EFFECTS OF WATER RECIRCULATION ON PILOT-SCALE MICROALGAE CULTIVATION USING FLUE GAS CO_2

By

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ABSTRACT

EFFECTS OF WATER RECIRCULATION ON PILOT-SCALE MICROALGAE CULTIVATION USING FLUE GAS CO_2

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This study investigates the effects of media recirculation on microalgal species *C. sorokiniana* growth in a pilot-scale algae photobioreactor (APB). Two culture conditions, freshwater and recirculated boiler water, were conducted on the APB to determine the effect of recirculation on algal growth. The results showed that microalgae cultivation under recirculation conditions was stable over a period of four months. Biomass productivities during the 1_{st} through 4_{th} months of recirculation (0.26, 0.23, 0.20, and 0.18 g L^{-1} d^{-1}, respectively) were not significantly different from the culture on freshwater (0.22 g L^{-1} d^{-1}). Furthermore, the relationship between eukaryotic and bacterial domains remained consistent throughout the four months of recirculation (80.7, 87.1, 83.1, and 82.1%, respectively and 19.2, 12.8, 16.9 and 17.8%, respectively). This was not significantly different from the abundance of each domain in freshwater cultivation (83.7% eukaryotic and 16.2% bacterial). A 1 m_3 photobioreactor was then envisioned for mass, energy and exergy analyses. The mass balance analysis concluded that a 98% reduction in freshwater usage and 25% reduction in nutrients could be achieved during cultivation operating under recirculation conditions for a year, while maintaining a biomass productivity of 1.2 kg wet algal biomass and 0.4 kg CO_2 sequestered per day. Both systems require an energy input of 219 kWh $unit^{-1}$ d^{-1}. The exergy balance analysis concluded that without considering solar irradiation, the rational exergy efficiency of the culture with water recirculation was more than double that of freshwater.

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CHAPTER 1: LITERATURE REVIEW

INTRODUCTION

Carbon dioxide (CO_2) is a major greenhouse gas and principal contributor of global climate change. Emissions of CO_2 are largely caused by anthropogenic activities such as burning fossil fuels, electricity and heat production, and deforestation. As the global population continues to increase, global energy consumption has also increased accordingly. Worldwide energy demand is driven by a growing global economy along with higher heating and cooling requirements in certain areas of the world [1]. Over half of the growth in energy needs in 2018 was due to a higher electricity demand [1]. Rising energy demands is a main reason for the continued increase of CO_2 levels in the atmosphere. While many methods for CO_2 mitigation exist, such as implementation of alternative energy sources, demand side management and climate engineering, CO_2 sequestration represents another possible solution to decrease atmospheric CO_2 and mitigate global warming.

CO_2 sequestration can be defined as the capture and long-term storage of carbon that would otherwise be emitted to or remain in the atmosphere [2]. Mitigation of CO_2 has been attempted through a number of sequestration methods, utilizing a variety of chemical, physical and biological processes. One such method is biological carbon capture through microalgae cultivation. Microalgae are photosynthetic organisms with the ability to convert atmospheric CO_2 into glucose for their growth using solar energy. Microalgae have high capabilities to fix CO_2 and generate biomass, offering attractive advantages over other terrestrial sequestration methods. They are able to duplicate cell biomass 100 times faster than terrestrial plants and are able to fix CO_2 10-50 times more efficiently [2]. It has been approximated that 1.83 kg of CO_2 can be fixed for every 1 kg of biomass produced [2]. Microalgae remain photosynthetically efficient even

under a range of CO_2 concentrations [3]. Furthermore, the cultivation of microalgae biomass does not require arable land [3]. In addition to CO_2 capture, microalgae biomass is characterized by high protein, lipid, and carbohydrate content, which provides a good source for value-added products including pharmaceuticals, biofuels, and nutritional supplements [3, 4]. As a result, algae cultivation offers an attractive solution for CO_2 capture from major carbon-emitting sources such as power plants.

Photoautotrophic microalgae cultivation for CO_2 sequestration requires several components for the successful accumulation of biomass. To maintain a high yield, systems require light for photosynthesis, CO_2 as a carbon source, a constant supply of several inorganic nutrients, and water [2, 5]. The major sources of CO_2 used for cultivation are from air or flue gas. CO_2 is available with atmospheric concentrations of 0.03–0.06% (v/v) or with power plant flue gas ranging from 6–15% (v/v) [2, 6]. Light requirements are provided through natural sunlight or illumination provided by artificial fixtures. Inorganic nutrients, such as nitrogen (N), phosphorous (P), and iron (Fe), can be provided directly to the culture or may be obtained through growth in nutrient-rich water sources, such as wastewater. Microalgae cultivations can be performed in a variety of water sources including freshwater, saltwater or brackish water, depending on the strain in question.

Large-scale microalgae cultivations are typically performed under controlled conditions in either open or closed algae photobioreactor (APB) systems. Open systems, such as raceway and open ponds, can be mixed or unmixed ponds used for the mass cultivation of algae with constant exposure to the environment [7, 8]. These systems are characterized by minimal capital and operating cost due to lower energy inputs, such as low mixing requirements and use of natural sunlight for illuminance [4, 7]. A major disadvantage of open ponds is the large area

requirement for scaling up and significant CO_2 losses to the atmosphere [5, 7]. Additionally, as open ponds are continuously exposed to the environment, these systems are more susceptible to contamination and adverse weather conditions [9]. Thus, location is an essential factor in the design of open ponds systems. Furthermore, they are more challenging in terms of controlling growth parameters including light exposure, temperature, and evaporation [5].

Microalgae cultivations performed in APB systems are typically grown in closed tubes or bags, which both reduces exposure to the environment and allows for greater control of growth parameters [7, 9]. CO_2 is also utilized more efficiently in these systems [5]. Artificial light sources, such as light emitting diodes (LEDs), are commonly used in APB systems, allowing for increased light intensity and exposure. Limited exposure to the environment decreases the probability of contamination, complications from adverse weather, and evaporation [5]. These characteristics correlate to a smaller area requirement and versatile location options. However, APBs tend to have issues with biofilm accumulation, overheating, and cleaning issues [7]. Most importantly, they incur very high capital and operating costs [7]. Despite these disadvantages, APBs are more efficient than open pond systems in terms of controlling growth parameters while also offering higher biomass productivity [5].

Several major technical challenges on large-scale algal cultivation hinder commercial algal production, including low algal biomass yield in outdoor conditions, lack of long-term stability, and high water and nutrient requirements. Additionally, the area required for CO_2 consumption to balance industrial CO_2 emissions is extremely large, leading to technical and economic limitations for large-scale microalgae cultivation [10]. Improvements in operational efficiency within APB operations is essential for technical and economic feasibility of this technology on a commercial scale.

One of the main operational limitations is the cost and environmental impacts related with the consumption of freshwater and nutrients [11]. These resources are key components in algae cultivation, as a constant supply of water and certain inorganic nutrients are required to achieve and maintain high biomass productivity. However, it is predicted that a significant cost reduction of at least 50% could be achieved if nutrients and water are obtained at a lower cost [11]. Both freshwater and nutrient usage in microalgae cultivation pose challenges to system sustainability. Freshwater is a natural resource that is becoming increasingly scarce, as freshwater aquifers are currently facing unsustainable rates of extraction [12]. Furthermore, life cycle assessments (LCAs) often find that the life cycle burden of microalgae cultivation comes from nutrient production occurring upstream of algae cultivation facilities [12]. Therefore, maximizing water and nutrient use efficiency is a significant factor in improving the overall feasibility of the technology. Current methods used to address water and nutrient challenges involve incorporating alternative water sources into microalgae cultivation systems. Potential alternative water sources include saltwater, brackish water, or recycled freshwater [3, 11, 12].

Certain strains of algae are known to tolerate high concentrations of salt and can be used in systems utilizing saltwater [4]. However, large requirements of saltwater may also be an issue [12]. The outcome of drawing from saltwater aquifers, a nearly untouched resource, is unknown and carries a high risk to coastal environments which are known to be highly productive ecosystems [12]. Additionally, saltwater would require commercial systems to be located near coastal regions to reduce the distance required for water transport [12]. The alternative option would require long distance pipelines that may drastically increase cost in addition to possible environmental and social impacts [11, 12]. Another option is to use wastewater to reduce reliance on freshwater while also providing nutrients to the algae [4, 5]. It would also provide

biological cleaning options for municipal wastewater and would lower environmental impacts and treatment costs [12]. Drawbacks associated with cultivation in wastewater include fluctuating nutrient level concentration, increased turbidity causing light penetration issues, and rigorous use of toxic chemicals [13]. The final practice involves the recirculation of freshwater and culture media. Recirculation can reduce freshwater requirements while also reducing nutrient usage. An LCA study performed by Yang et al. in 2011 found that when harvesting water was completely recycled, nutrient usage decreased by about 55% and freshwater usage decreased by 84% [12]. In 2020, Fret et al. demonstrated a 77% decrease in water footprint and 68% reduction in nutrients using media recirculation [14]. However, the full potential of media recirculation across many microalgal species has yet to be completely explored in the field of large-scale cultivation.

It is important to note that in order to maintain economic viability, large quantities of biomass and value-added products derived from algal biomass remain an essential component in microalgae cultivation systems. Although recirculation can reduce water and nutrient usage, the impact of recirculated media on microalgae growth and biomass, and thus the correlated economic value of biomass, is still widely unknown. To determine whether or not this approach is feasible, research must be completed to evaluate the effect of reused resources on algal growth and composition in pilot and large-scale operations.

EFFECTS OF MICROBIAL COMMUNITY

In order to fully exploit the industrial potential of algal biomass in CO_2 sequestration, operational efficiency must be achieved through reduction of nutrient and water usage. Although there is much research on APB design and biomass productivity optimization, minimal research was conducted on the integration of recycled water and nutrient sources into APBs. As the

overall system operation and effects on biomass composition is still largely unknown, performance indicators should be investigated to further determine feasibility.

Microbial community structure and function has the potential to indicate system operational status, including systematic stability. Although knowledge on complex microbial relationships between microalgae and bacteria is still limited, monitoring the presence and abundance of certain species can reveal potential pathogens that may be responsible for system failures [8, 15, 16]. Additionally, monitoring microbial community is useful in identifying helpful taxa that may correlate to improvements in system performance [8, 17, 18].

Microalgal strain selection and Chlorella sorokiniana

Microalgae are unicellular, photosynthetic organisms that live in a wide range of aquatic environments such as lakes, rivers, ponds, oceans, and are even known to be found in certain types of industrial effluents [4, 5, 7]. Microalgae can be prokaryotic, such as cyanobacteria, or eukaryotic like green algae [3]. In order to sustain growth, microalgae require light energy to convert water and CO_2 into biomass through photosynthesis. In addition to light, water, and CO_2, microalgal growth also requires both macro and micronutrients. Carbon is the most important element for microalgal nutrition, with dried algal biomass containing approximately 50% carbon, followed by nitrogen and phosphorous, which account for 10-20% of algae biomass [11, 19]. Other commonly required elements include macronutrients Na, Mg, Ca and K and micronutrients Mo, Mn, B, Co, Fe, Zn and other trace elements [4].

Although approximately 40,000 different species of microalgae have been reported, only a handful of those strains are considered feasible for mass cultivation [2, 4]. Mass cultivation of microalgae requires a strain that can tolerate a wide range of conditions such as temperature, pH,

salinity and light intensity. Robust strains that are commonly employed in APBs for CO_2 sequestration include, but are not limited to, *Nannochlorophsis sp., Dunaliella sp., Scendesmus sp.* and *Spirulina sp.* [5, 15]. Additionally, *Chlorella* is a species with industrial potential because it can grow both photoautotrophically and heterotrophically with high biomass concentration [20]. This microalgae is also commercially important, with global annual sales of greater than $38 billion USD [20]. *Chlorella* is known to produce value-added chemicals, such as ß1,3-glucan and carotenoids, and shows promise for biofuels production under heterotrophic conditions [20]. Although many species of *Chlorella* have been studied for large-scale algae cultivation, one recent strain of interest is the species *Chlorella sorokiniana*.

Originally isolated by Sorkin and Myers in 1953, *C. sorokiniana* is a type of green microalgae commonly used in large-scale APBs due to its high photosynthetic productivity and ability to grow at temperatures up to 38-42°C [21, 22]. *C. sorokiniana* is small in size (2-4.5 µm diameter) and often found in freshwater and soils [23, 24]. It is one of the only known species of *Chlorella* that tolerates high temperatures and light intensity, making it beneficial for many types of cultures [21, 22, 25]. It has also been shown to grow in wastewater, under conditions that other algal species may find unfavorable [26]. Furthermore, it has shown resistance to high concentrations of CO_x NO_x and SO_x, compounds that are typically found in power plant flue gas emissions and exhibit potential toxicity to some microalgal species [21]. Under photoautotrophic conditions, cell doubling times are found to be as low as 4-6 h [24]. On average, *C. sorokiniana* is composed of 40% protein, 30-38% carbohydrate and 18-22% lipid [24]. Prior research has shown that this robust species has industrial potential and is well suited for large-scale production in air- and liquid- mixed photobioreactors, while also producing compounds of commercial interest including antioxidants (i.e., carotenoids) [24].

Effect of bacterial community

Microalgae can either be cultivated as a pure culture, containing only the species of interest, or as a co-culture, containing microalgae and other micro-organisms simultaneously. Pure cultures are highly impractical because they are difficult to maintain and are characterized by high capital and operating costs, thus, co-cultures are becoming more common in the field of mass microalgae cultivation [16]. When considering a co-culture system, the analysis of both selected microalgal strain and the overall microbial community is of great importance. Although bacteria have often been considered contaminants that have the potential to inhibit or kill microalgae cultivations, algae-bacteria interactions have many possible effects [17, 18, 27].

In nature, many algae-bacteria interactions occur, with relationships ranging from mutualism to parasitism [16, 17]. Many relationships are still unexplored, especially under lesser-known conditions such as recirculated growth medium. In both natural and industrial processes, there is evidence of microalgae and bacteria living together in complex communities. Many of those described in engineered systems are also of the same genera found in natural environments [18]. Although studies are limited regarding microalgae-bacteria relationships in APB co-culture systems, the presence of several taxa have been documented. An analysis of several large-scale system studies showed that Proteobacteria, particularly Gammaproteobacteria, were associated in all of the microalgae cultivation communities studied [15]. The study also found the presence of several common bacterial orders, Cytophagales, Flavobacteriales, Pseudomonadales, Burkholderiales, Caulobacterales and Rhodobacterales, though these bacteria were not consistent over all studied systems [15].

The performance of microalgal species is highly affected by various factors such as pH, temperature, nutrient concentration, and light intensity [16]. It is thought that the presence of

bacteria in co-culture systems may lead to more robust communities that can better withstand environmental challenges through communication and division of labor [16]. In general, co-cultures have shown improvements in yields of biomass, lipids, and other value-added products in comparison to pure cultures [16]. This suggests a positive effect of algae-bacteria symbiosis on algal growth. For instance, one study showed that of 326 algal species studied, 171 species required an external supply of vitamin B_{12} [17]. It has also been shown that some bacterial species are known to supply vitamin B_{12} to algae in exchange for fixed carbon [8]. Similarly, other bacterial groups may help regulate available nutrients like iron, nitrogen, and phosphates or by releasing growth hormones [8].

Although limited, there have been several studies regarding positive relationships between *Chlorella* and several bacterial groups. A review performed by Lian *et al* discussed a number of bacteria that have been found beneficial to *C. vulgaris*, including members of the genera Bacillus, Flavobacterium, Rhizobium, Hyphomonas and Sphingomonas [15]. The review also discussed the species, *B. pumilus ES4,* which has been shown to promote *C. vulgaris* growth by providing fixed atmospheric nitrogen [15]. Amavizca *et al* found similar results in a *C. sorokiniana* co-culture, where *B. pumilus ES4* and *Azospirillum brasilense Cd* were shown to remotely induce increases in total lipids, carbohydrates, and chlorophyll *a* [28]. Another *Chlorella* species, *C. ellipsoidea,* showed increased cell density when accompanied by *Brevundimonas* sp., while *C. sorokiniana IAM C-212* was shown to have an increased growth rate when grown with *Microbacterium trichotecenolyticum* [15].

However, within their natural environment, microalgae are still at risk of viruses, parasites, and bacterial pathogens, though many have not been identified [8, 15]. Additionally, there is a greater risk of infection and inhibition from bacteria, fungi, and viruses found in greater

concentrations in recycled waters [11]. Co-inhabiting species may compete for existing nutrients, resulting in decreased growth of the algae [8, 17]. Another potential threat is through bacterial parasitism of algae, where algae cells are lysed by enzymes, allowing bacteria to use intracellular compounds of algae as nutrients [17]. Lian *et al* found that rot symptoms are commonly due to gram-negative members belonging to the genera Alteromonas, Cytophaga, Flavobacterium, Pseudomonas, Saprospira, Vibrio and Pseudoalteromonas [15]. In addition to bacterial pathogens, some types of algae can also be parasitic [8, 17]. An algaelytic protist, Pseudobodo sp. KD51 s, caused more than a 50% decrease in chlorophyll content of *C. vulgaris* within three days of inoculation [15].

EFFECT OF CULTURE MEDIA

As freshwater and nutrients are limited resources, the integration of recycled media into APB systems is a potential solution [11]. However, the recirculation of freshwater may result in the accumulation of numerous compounds at a level not normally exhibited in cultures frequently replenished with freshwater. Many studies have investigated the use of algae in treating various water sources, suggesting microalgae are tolerant to a wide variety of water sources and compounds. Research has also been conducted on the use of recirculated freshwater on a variety of commonly cultivated microalgal species, but specific knowledge of the effects of *C. sorokiniana* on recycled media is limited. As tolerances and thresholds to various compounds differ between microalgae species, it is still widely unknown how cultures of *C. sorokiniana* respond to recycled media.

Effect of accumulation and inhibitory compounds

While algal cells require a certain amount of nutrients and minerals, the overabundance of any one compound may negatively impact growth. For instance, C, N and P are the most

important nutrients required for microalgae cultivation, but an oversupply can result in increased stress and reduced growth [29]. Similarly, micronutrients, such as Fe, also have a supply threshold, however, it is much narrower than that of macronutrients [29]. For example, in a study performed by Wan *et al*, Fe was found to be beneficial to *C. sorokiniana* at concentrations up to 10^{-5} mol L^{-1}, but was toxic at 10^{-3} mol L^{-1} [30]. An investigation on NaCl concentration found that *C. sorokiniana* tolerated levels up to 0.3 M, but also showed a decreased growth rate [31]. Heavy metals such as cadmium (Cd), lead (Pb), and mercury (Hg) are unnecessary for algae growth and have been shown to negatively impact cells at very low concentrations [15, 32]. For example, a study performed by Carfagna *et al* found that the algal cell structure and physiological characteristics such as growth, photosynthesis, respiration and enzyme activities were affected in a strain of *C. sorokiniana* when exposed to certain levels of Pb and Cd [23]. Additionally, the study found that Pb and Cd induced a reduction in total chlorophyll content and decreased soluble protein. Similarly, Liang *et al* found that *C. sorokiniana* was able to tolerate levels of Pb (total), Copper (Cu) and Cd at levels of 0.249 mg/L, 0.485 mg/L, and 46.108 mg/L, respectively [33]. Both studies found that *C. sorokiniana* showed a high tolerance to Pb over the other studied metals, which is likely due to the intra- and extracellular mechanisms possessed by microalgae that prevents metal toxicity [23]. Other heavy metals like zinc, which is beneficial to algae growth in small amounts, may inhibit productivity after a certain concentration [34]. Spence observed inhibition in *C. sorokiniana* under recycled media conditions but was unable to identify if inhibition was due to the accumulation of zinc or inhibitory secondary metabolites [34]. Accumulation is noteworthy for systems using recycled media, since even a minor accumulation of nonessential compounds can be toxic to algae [35].

Auto-inhibitory compounds are another potential challenge for systems implementing recycled growth media. Auto-inhibitory compounds are naturally occurring substances released by a cell to inhibit or provide feedback on the organism's own growth or to inhibit or check the growth of other species [32, 36]. Auto-inhibitors are typically present in ultrahigh density microalgae cultures, typically characterized by concentrations of at least 10 g cell mass L^{-1}, though concentrations at this level are rarely reported in photoautotrophic cultivations [37, 38]. Several species of microalgae have been shown to release extracellular compounds with inhibitory or algicidal properties. *C. pyrenoidosa*, for example, was found to produce polyunsaturated fatty acids, linoleic and linolenic acid, which resulted in inhibitory effects on growth [39, 40]. A genus of algae commonly used as aquaculture feed, Nannochloropsis, is known to release a thick and multilayered parent cell wall during cell division, which may potentially reduce culture growth and productivity using recycled media [29]. Cell wall remains caused the formation of aggregates in the culture, which are thought to entrap cells, bacteria and debris, leading to unsuitable growth conditions [41]. Additionally, the accumulation of dissolved organic matter can be conducive to algae contamination and can also inhibit algae growth at certain thresholds [38]. In general, the manner in which these substances inhibit growth is widely unknown and many of the substances involved have not been fully characterized [32, 40].

As previously discussed, co-culture systems have been proven to have symbiotic effects on the growth of algae and are often easier to maintain than pure cultures, which explains why many systems today employ co-cultured microbial communities [42]. However, mixed cultures also present the issue of allelopathy, in which co-inhabiting organisms produce biochemicals, known as allelochemicals, capable of influencing the growth and survival of other organisms [43] . Similar to auto-inhibitory compounds, very few algicidal metabolites have been

characterized to date [44]. Allelopathy has frequently been studied, specifically for the role of algicidal bacteria in algal blooms and can provide insight on compounds that may also be present in mixed cultures. An example of this is seen in the relationship between *C. vulgaris* and bacteria Pseudomonas. When allowed to grow at high cell concentrations, the bacteria is found to express self-regulation and inhibitory effects on *C. vulgaris* through excreting chemical substances [35]. Algicidal pigments produced by marine bacteria have also been isolated and identified [45]. This is not an uncommon occurrence among co-cultures, as many bacteria are able to produce allelochemicals. Specifically, many gram-negative bacteria produce chemicals, such as acetylated homoserine lactones, that are used to regulate the production of secondary metabolites and facilitate quorum sensing [45]. Although, the interaction between bacteria and algae is highly dynamic and the threshold for inhibition is often dependent upon culturing conditions, dominating microalgae strain, and resource availability [39]. The ability of a bacterial species to dominate over an algae species is also contingent with nutrient concentration and stability. The imbalance of nutrients may cause a normally symbiotic culture to transition into the collapse of an algal species through bacterial domination [38]. Additionally, the accumulation of toxins in recycled media may amplify these effects [34].

Another potential factor that should be considered while implementing recycled media is the use of anti-foaming agents. Anti-foaming agents are commonly employed in large-scale microalgae cultivations in order to reduce foams occurring due to the introduction of gases into the culture medium [46]. Foaming is a potentially serious problem in bioreactors and can result in overflows, loss of culture and products, along with operational problems with machinery such as pumps [47]. Antifoams may be composed of a variety of different materials, such as silicone or polypropylene glycol. It is known that certain antifoams can affect the growth rates and

surface properties of prokaryotic and eukaryotic organisms [46]. Both negative and positive effects have been shown, however, there is relatively little information on how antifoams affect biological processes [46]. One way in which antifoams may affect biological processes is through their effect on dissolved oxygen (DO) content and volumetric mass oxygen transfer coefficients within a system [46]. For example, Al-Masry showed that a silicone-based antifoam had negative impacts on the mass transfer coefficient and gas velocity within the culture media of 55 and 700 L airlift reactors [47]. In contrast, Koch *et al* showed that an antifoam containing silicone oil only had a significant effect at the beginning of a process but decreased over time, and had varying effects over the growth of the microbial strains tested [48]. It has been suggested that some organisms may possess the ability to utilize antifoams as a way to increase their growth rate and improve protein production [46]. However, the impact of various antifoaming agents on differing prokaryotic and eukaryotic organisms still remains unknown.

Effect on growth and composition

The replenishment of freshwater in microalgae cultivation after harvesting is a common practice used to frequently purge unwanted contaminants, such as pathogenic bacteria and toxic compounds, that are thought to be detrimental to algae growth [11, 38]. The introduction of recycled media into cultivation systems may contain microalgal cell debris and organic compounds, which may have negative impacts on biomass growth and composition [13]. However, certain strains of microalgae, such as *C. sorokiniana*, are known to be highly robust and tolerant to a wide range of conditions, allowing them to thrive even in harsh conditions that may develop from recycled media.

Several studies have investigated the impact of recycled media on microalgae growth and composition over a variety of strains, culture conditions, and experimental scales. Studies show a

wide range of results from increasing to decreasing growth rate and differences in composition. A review performed by A. Shahid *et al* found that over a range of studies, *Desmodesmus, Tetraselmis, Arthrospira,* and *Hormotila sp.* generally showed the most promising growth on recycled media [13]. Some species, such as *Scenedesmus sp.* and *Nannochloropsis salina,* showed no negative impact on cell growth but had reduced protein and/or lipid composition [13]. Other strains, including *Teradesmus obliquus* and *C. zofingiensis,* showed increased lipid and carbohydrate content and improved biomass growth, respectively [13]. The review also discussed results based on whether or not recycled media was replenished with additional nutrients. For example, *C. vulgaris* was found to grow successfully in nutrient replenished media. Other species, including *Scenedesmus sp.* and *C. kessleri,* were found to grow for a finite number of cycles on recycled, but not replenished, media before negative impacts occurred [13]. Some of the reviewed studies found that the accumulation of organic matter, such as with *Arthrospira platensis,* were at fault for reduced growth [13, 38]. Fret *et al* found that media recirculation had no impact on *Nannochloropsis* sp. and *Tisochrysis utea* productivity when cultivated on microfiltered replenished media [14]. Limited research is available regarding cultivation of *C. sorokiniana* on recycled media. However, a research study conducted by Spence found a 3 - 18% reduction in growth rate in *C. sorokiniana* cultured at lab scale when media was recycled and replenished 1 - 4 times, respectively [34].

SUMMARY OF KNOWLEDGE GAPS

Through this literature review it can be shown that media recirculation in microalgae cultivation systems is a potential solution to minimize water and nutrient usage, thus minimizing cost and environmental impact. However, due to a lack of commercial systems, large-scale microalgae cultivation is still an immature technology and information on the effects of

recirculation is still limited [12]. Further research is required to determine the feasibility of large-scale media recirculation in photoautotrophic microalgae cultivation. Specific research on microalgal strain *C. sorokiniana* within large-scale photobioreactor systems is also limited. This includes effects of recirculation on microbial community and *C. sorokiniana*, effects of recirculation on growth and biomass composition, and the overall effect on system performance. Taking into account the reviewed literature, it is expected that accumulated compounds, both mineral and microbial, will pose a challenge to system stability of large-scale and long-term algae cultivation under recirculation conditions.

OBJECTIVES AND HYPOTHESIS

The overall hypothesis is that freshwater and nutrient utilization can be reduced through the integration and recirculation of alternative water sources and that recirculation would have no effect on system stability over a finite period. The objectives of this research were to: 1) study system stability under recirculation conditions; 2) minimize freshwater usage through media recirculation; and 3) minimize nutrient usage through media recirculation.

CHAPTER 2: CONTINUOUS MICROALGAE CULTIVATION ON FLUE GAS IN A WATER RECIRCULATING PHOTO-BIOREACTOR SYSTEM

ABSTRACT

Growth media recirculation is a potential solution to address water and nutrient challenges in large-scale microalgae cultivation. A pilot-scale algae photobioreactor (APB) was used to culture *C. sorokiniana* on flue gas from the T.B. Simon Power Plant at Michigan State University. Two culture conditions, freshwater and recirculated boiler water, were conducted on the APB to determine the effect of recirculation on algal growth. The results showed that microalgae cultivation under recirculation conditions was stable over a period of four months. Biomass productivities during the 1st through 4th months of recirculation (0.26, 0.23, 0.20, and 0.18 g L^{-1} d^{-1}, respectively) were not significantly different than freshwater (0.22 g L^{-1} d^{-1}). Furthermore, the relationship between eukaryotic and bacterial domains remained consistent throughout the four months of recirculation (80.7, 87.1, 83.1, and 82.1%, respectively and 19.2, 12.8, 16.9 and 17.8%, respectively). This was not significantly different than the abundance of each domain in freshwater cultivation (83.7% eukaryotic and 16.2% bacterial). A 1 m^3 photobioreactor was then envisioned for a mass, energy and exergy analysis. The mass balance analysis concluded that a 98% reduction in freshwater usage and 25% reduction in nutrients could be achieved during cultivation operating under recirculation conditions for 1 year, while maintaining a biomass productivity of 1.2 kg wet algal biomass and 0.4 kg CO_2 sequestered per day. The exergy balance analysis concluded that without considering solar irradiation, the culture with water recirculation more than doubled the rational exergy efficiency of the freshwater culture.

INTRODUCTION

Carbon dioxide (CO_2) is a major greenhouse gas and principal contributor of global climate change. A large source of emissions is derived from power generation, such as power plants, which accounted for approximately 38% of total energy-related CO_2 emissions in 2018 [1]. While many methods for CO_2 mitigation exist, biological CO_2 sequestration through microalgae cultivation represents a possible solution to decrease atmospheric CO_2 and mitigate global warming.

Microalgae have high capabilities to capture CO_2 and generate biomass, offering attractive advantages over other terrestrial sequestration methods. They are able to duplicate cell biomass 100 times faster than terrestrial plants, capture CO_2 10-50 times more efficiently, and do not require arable land [2]. Certain strains of microalgae remain photosynthetically efficient under a large range of CO_2 concentrations [3]. In addition to CO_2 capture, microalgae biomass is characterized by high protein, lipid, and carbohydrate content, which provides a good source for value-added products including pharmaceuticals, biofuels, and nutritional supplements [3, 4]. As a result, microalgae cultivation offers an attractive solution for CO_2 capture from major carbon-emitting sources such as power plants.

Large-scale microalgae cultivations are typically performed under controlled conditions in either open or closed algae photobioreactor (APB) systems. Although open systems are characterized by lower capital and operating costs, closed APBs utilize CO_2 more efficiently while also offering higher biomass productivity [5, 7]. However, several major technical challenges on large-scale algal cultivation hinder commercial algal production, including lack of long-term stability, high water and nutrient requirements, and an extremely large area requirement to balance industrial CO_2 emissions [4, 5, 10]. Improvements in operational

efficiency within APB operations is essential for technical and economic feasibility of this technology on a commercial scale.

One of the main operational limitations is cost and environmental impacts due to the consumption of freshwater and nutrients [11, 12, 14]. A constant supply of water and certain inorganic nutrients are required to achieve and maintain high biomass productivity [12]. However, freshwater and nutrient usage in microalgae cultivation pose challenges to system sustainability. Freshwater is a natural resource that is becoming increasingly scarce, as freshwater aquifers are currently facing unsustainable rates of extraction [12]. Life cycle assessments (LCAs) find that the life cycle burden of microalgae cultivation comes from nutrient production occurring upstream of algae cultivation facilities [12]. Therefore, maximizing the efficiency of water and nutrient use is a critical factor in improving the feasibility of the technology.

Growth media recycling is a potential solution to address water and nutrient challenges in large-scale microalgae cultivation [3, 11-14]. Recirculation can reduce the freshwater requirement while also reducing nutrient usage. An LCA study performed by Yang *et al* found that when harvesting water was completely recycled, nutrient usage decreased by about 55% and freshwater usage decreased by 84% [12]. Fret *et al* achieved a 77% decrease in water and 68% reduction in nutrients using media recirculation [14]. However, the full potential of media recirculation across many microalgal species has yet to be completely explored in the field of large-scale cultivation.

This research studied a long-term media recirculation operation in a pilot-scale APB using the flue gas from a power plant. The objectives of the study were to minimize freshwater

and nutrient usage, evaluate biomass production and algal assemblage stability of the cultivation under recirculation conditions, and compare the culture under media recirculation and freshwater conditions using mass, energy, and exergy analyses.

MATERIALS AND METHODS

Algal Assemblage

The microalgae strain *Chlorella sorokiniana* MSU was isolated from the Great Lakes region for use in seeding the APB. *C. sorokiniana* isolates were stored on Tris-Acetate-Phosphate (TAP) agar medium [49] at room temperature and exposed under constant fluorescent light. Modified liquid TAP medium was used for all photoautotrophic cultures. The modified TAP medium is based on a reference study [50] and contained the following substances: 3.75 mmol L^{-1} of NH_4NO_3, 0.34 mmol L^{-1} of $CaCl_2 \cdot 2H_2O$, 0.4 mmol L^{-1} of $MgSO_4 \cdot 7H_2O$, 0.68 mmol L^{-1} of K_2HPO_4 (anhydrous), 0.45 mmol L^{-1} of KH_2PO_4 (anhydrous), and 0.09 mmol L^{-1} $FeCl_3 \cdot 6H_2O$. Nutrient stock solutions were prepared using deionized water. Microalgae cultivations were performed in the closed, but not aseptic, APB.

Pilot photobioreactor system and operations

A $PHYCO_2$ APB unit previously installed in the T.B. Simon Power Plant was used in this study [51]. All cultures were approximately 100 L. The culture was exposed to 24 h lighting conditions provided by twelve red and blue LED light bars (Independence LED Lighting LLC, USA). The LED light bars delivered a continuous photosynthetic photon flux density (PPFD) of approximately 407 μmol m^{-2} s^{-1} to support algae growth. PPFD was measured using a LI-190R Quantum Sensor and LI-250A light meter (LI-COR, Lincoln, Nebraska). Additional system specifics of the APB are provided elsewhere [50]. The natural gas fired flue gas, containing 7.5 ± 1.15% v/v of CO_2, was directly pumped from the stack into the APB at a flow rate of 120

L/m^3/min. The unit ran for approximately 7 months. Data used in this study were collected from May 2nd 2019 to November 15th 2019.

Two semi-continuous cultures were cultivated under two water sources (freshwater and recirculated boiler water). Initial harvesting began once biomass productivity reached 0.22 g L^{-1} day^{-1}. A 50% harvest ratio was previously optimized for maximizing biomass concentration and was thus the only harvesting ratio used in this study [50]. The specified water source (freshwater or recirculated boiler water) was used to refill the APB reactor after harvesting. Water was stored in a 380 L storage tank for up to 5 days before being fed into the APB. Boiler water was obtained directly from power plant boilers. Freshwater cultivation was performed using the tap water from Michigan State University. Under recirculation conditions, biomass was removed through centrifugation and remaining broth was recirculated back to the APB. Nutrients were replenished to that of modified TAP media, with the exception of total phosphorus, which was allowed to accumulate within the reactor to simplify daily nutrient additions. The pH was maintained at 6.6 ± 0.09 for the freshwater treatment and 6.2 ± 0.27 for the recycle treatment.

Chemical analysis

Samples were analyzed daily for dry biomass weight, pH and nutrient concentrations (total nitrogen (TN), total phosphorus (TP), nitrate (NO_3-N) and ammonia (NH_3-N)). Algal biomass was concentrated for biomass productivity measurements using a Dolphin Alfa Laval MAB204 Centrifuge. Wet biomass was weighed and then dried at 105°C for 24 h for dry weight determination. Sample pH was measured using a pH meter (Fisherbrand™ accumet™ AB15 + Basic, Fisher Scientific Co., Pittsburgh, PA). Nutrient concentrations were tested in the liquid supernatant using nutrient test kits (HACH Company, Loveland, Colorado) equivalent to EPA methods [52]. Trace element analysis of the liquid supernatant, elemental analysis, and biomass

composition of select samples was conducted by Dairy One (Ithaca, NY). Additional elemental (CHNS) analysis was conducted by Atlantic Microlab (Norcross, GA).

Microbial analysis

DNA Extraction

Microbial community samples (1.5 mL) collected for DNA analysis were taken once per week throughout the study and stored at -20°C until extraction. To remove nutrient media, algae samples were centrifuged using an Eppendorf 5416R centrifuge at 10,000 rpm for 5 min and the supernatant was discarded. The remaining pellet was washed and resuspended once with deionized water, and the supernatant was discarded. The final remaining pellet was used for DNA extraction using a DNeasy® PowerSoil® DNA Isolation Kit (Qiagen, Germany). DNA extracts were eluted with 100 μL of 10 mM Tris-HCl (pH 8.5) and the concentration and purity were determined using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, USA). Extracted DNA samples were stored at -80°C for several weeks before use in real-time PCR quantification and high-throughput sequencing (Illumina MiSeq flow cell).

Illumina preparation and sequencing

Illumina sequencing was performed for the 16S rRNA gene region to assess the bacterial community. The PCR conditions were as follows: 1.0 μL DNA template (10x diluted of microbial community DNA), 0.5 μL of 100 μM forward primer (IDT, Pro341F 5′-CCTACGGGNBGCASCAG-3′), 0.5 μL of 100 μM reverse primer IDT, Pro805R 3′-GACTACNVGGGTATCTAATCC-5′), 12.5 μL 2x Supermix (Invitrogen, USA), and 10.5 μL PCR grade water. The PCR program used for all assays were as follows: 96°C for 2 min, followed by 30 cycles of 95°C for 20 s, 52°C for 30 s, and 72°C for 1 min, and a final elongation

period of 72°C for 10 min. Amplicons were quality-tested and size-selected using gel electrophoresis (1.0% (w/v) agarose concentration and 1× TAE run buffer). Samples were then diluted to normalize DNA concentrations within 5-10 ng μL^{-1} by measuring the DNA concentration with the PicoGreen® dsDNA quantitation assay (Invitrogen, USA) and Fluostar Optima microplate reader (BMG Labtech, Germany). The normalized PCR products were then sequenced at the Michigan State University (MSU) Research Technology Support Facility (RTSF). Illumina MiSeq (pair-end 250 bp) targeting on V3_V4 hypervariable regions was used to carry out the sequencing. Fastq files from the high-throughput sequencing were analyzed using the QIIME2 database to generate taxonomic/phylogenetic data for statistical analysis [53].

Statistical analysis

All statistical analyses were performed using R statistical software (Version 3.6.3). The data with normal distribution and equal variance were analyzed using one-way analysis of variance (ANOVA). When data violated the normality assumption and equal variance, the Kruskal-Wallis test was used. Tukey and Conover's pair-wise rank comparison post-hoc tests were used following ANOVA and Kruskal-Wallis tests, respectively. A significance value of $\alpha = 0.05$ was used for all tests.

Microbial analysis was performed using the R libraries Vegan, ggplot2, phyloseq, and MASS on taxonomic/phylogenetic data to graph relative abundances of samples. Non-metric multi-dimensional scaling analysis (NMDS) was then used to correlate the dissimilarities between culture conditions, reactor performance, and phenotype abundance.

Mass balance analysis

A mass balance analysis was conducted on a 1 m^3 APB unit to compare freshwater and recirculation cultivation. The envisioned APB unit has a volume 10 times greater than the experimental testing unit. The gas transfer in the APB is operated through airlift, which requires a high inlet flue gas flow rate of 212.5 kg per day. A 50% harvesting ratio is used for both scenarios. Flue gas CO_2 removal rate is based on biomass productivity and carbon content of algal biomass under each condition. After separation of biomass from medium using a centrifuge, medium is discarded under freshwater conditions or reintroduced to the APB under recirculation conditions. Slightly overdosing nutrients is a common practice to prevent nutrient limitation [14]. Using this practice, any discharged media contains a fraction of the initial nutrient content. All performance parameters are based on pilot-scale experimental data.

Energy balance analysis

An energy balance analysis was conducted for the envisioned 1 m^3 APB unit based on the pilot operational data. The electricity input for the LEDs was based on an average photosynthetic photon flux density (PPFD) of 407 μmol m^{-2} s^{-1} and reactor surface area of 46.2 m^2. PPFD was converted to electrical input using the conversion factor of 1 w m^{-2} to 2.1 μmol m^{-2} s^{-1} [54]. The centrifuge is a 5 kW unit and the average running time is 3 min d^{-1}. The harvesting water pump is a 0.5 kW unit with an average running time of 8 min d^{-1}. The water refill pump is a 0.5 kW unit with an average running time of 8 min d^{-1}. The inlet centrifuge pump is a 0.5 kW unit and runs for 3 min d^{-1}. The outlet centrifuge pump is a 0.5 kW unit and runs for 3 min d^{-1}. The flue gas pump is a 0.146 kW unit with an average running time of 24 h d^{-1}. The energy content of algal biomass as biofuel was not considered in the energy balance calculation.

Exergy analysis

An exergy analysis was conducted on microalgae biomass accumulation under recirculation and freshwater conditions, based on the reference approach [55]. The mass and energy balance data were used to carry out the exergy analysis. The exergy flow rates of individual compounds were calculated as the sum of their physical and chemical exergy flow rates:

$$B_{x\,k} = B_{j\,k}^{ch} + B_{j\,k}^{ph}$$

where k is the kth component in the process, $B_{j\,k}^{ch}$ is the chemical exergy rate (kW) of the kth component, $B_{j\,k}^{ph}$ is the physical exergy rate (kW) of the kth component, and $B_{x\,k}$ is the process exergy rate (kW) of the kth component. $B_{j\,k}^{ch}$ and $B_{j\,k}^{ph}$ are defined as follows:

$$B_{j\,k}^{ch} = \frac{m_k \cdot b_k^{ch}}{86400}$$

$$B_{j\,k}^{ph} = \frac{m_k}{86400}(h - h_0 - T_0(s - s_0))$$

where m_k is the mass flow rate (kg/day) of the kth component, b_k^{ch} is the specific chemical exergy (kJ/kg) of the kth component; h and s are the specific enthalpy (kJ/kg) and specific entropy (kJ/kg•K), respectively, at the given conditions, h_0 and s_0 are the specific enthalpy and specific entropy of the reference environment, respectively, and T_0 is the reference temperature (298.15 K). The physical exergies of components with similar temperature to the reference environment were negligible in comparison with chemical exergy rates. The rational exergy efficiency (ψ) was calculated as the desired exergy output divided by the used exergy input:

$$\psi = \frac{B_{desired}^{out}}{B_{used}^{in}} \times 100\%$$

where $B_{desired}^{out}$ is the exergy rate (kW) of the desired product in the output stream and B_{used}^{in} is the exergy (kW) used deduced from the exergies of wasted (B_{waste}^{out}) and desired ($B_{desired}^{out}$) products and irreversibility (I) of the system. $B_{desired}^{out}$ and B_{used}^{in} are defined as followed:

$$B_{desired}^{out} = B_{Algal\ biomass}^{out} + B_{Water\ in\ algal\ biomass}^{out}$$

$$B_{used}^{in} = B_{desired}^{out} + I + B_{waste}^{out}$$

where $B_{Algal\ biomass}^{out}$ is the exergy rate (kW) of the desired algal biomass product and $B_{Water\ in\ algal\ biomass}^{out}$ is the exergy rate (kW) of the water in the desired product. I is the irreversibility or exergy destruction (kW) of the process and B_{waste}^{out} is the sum of the exergy rates (kW) of the undesirable products in the output stream. I and B_{waste}^{out} are defined as followed:

$$I = B_{total}^{in} - B_{total}^{out}$$

$$B_{waste}^{out} = B_{Water}^{out} + B_{NH4NO3}^{out} + B_{KH2PO4}^{out} + B_{K2HPO4}^{out} + B_{MgSO4}^{out} + B_{CaCl2}^{out} + B_{FeCl3}^{out} + B_{Antifoam}^{out} + B_{CO2}^{out}$$

where B_{total}^{out} is the total exergy in the output stream (kW) and B_{total}^{in} is the total exergy in the input stream (kW). The component calculation of B_{total}^{in} and B_{total}^{out} is expressed as followed:

$$B^{in}_{total} = B^{in}_{Water} + B^{in}_{NH4NO3} + B^{in}_{KH2PO4} + B^{in}_{K2HPO4} + B^{in}_{MgSO4} + B^{in}_{CaCl2} + B^{in}_{FeCl3} + B^{in}_{Antifoam} + B^{in}_{CO2} + B^{in}_{Solar\ irradiation} + B^{in}_{Exergy\ for\ flue\ gas\ pump} + B^{in}_{Exergy\ for\ water\ refilling\ pump} + B^{in}_{Exergy\ for\ harvesting\ pump} + B^{in}_{Exergy\ for\ centrifuge} + B^{in}_{Exergy\ for\ storage\ pump} + B^{in}_{Exergy\ for\ centrifuge\ pump}$$

$$B^{out}_{total} = B^{out}_{Water} + B^{out}_{NH4NO3} + B^{out}_{KH2PO4} + B^{out}_{K2HPO4} + B^{out}_{MgSO4} + B^{out}_{CaCl2} + B^{out}_{FeCl3} + B^{out}_{Antifoam} + B^{out}_{CO2} + B^{out}_{Algal\ biomass} + B^{out}_{Water\ in\ algal\ biomass}$$

The most significant exergy demand is from solar or illumination energy, which accounts for more than 94% of the total daily exergy demand. As solar energy remains constant under water recirculation and freshwater conditions, rational exergy without solar energy was considered in order to delineate differences of lesser magnitude between two conditions. Rational exergy efficiency without considering solar energy (ψ without solar irradiation) was calculated as the desired exergy output divided by the used exergy input without solar energy:

$$\psi_{without\ solar\ irradiation} = \frac{B^{out}_{desired}}{B^{in}_{used\ without\ solar\ irradiation}} \times 100\%$$

where $B^{in}_{used\ without\ solar\ irradiation}$ is the exergy (kW) used deduced from the exergies of wasted (B^{out}_{waste}) and desired ($B^{out}_{desired}$) products and irreversibility ($I_{without\ solar\ irradiation}$) of the system, without solar energy. $B^{in}_{used\ without\ solar\ irradiation}$ is defined as:

$$B^{in}_{used\ without\ solar\ irradiation} = B^{out}_{desired} + I_{without\ solar\ irradiation} + B^{out}_{waste}$$

where $I_{without\ solar\ irradiation}$ is the irreversibility (kW) of the process without solar energy, $I_{without\ solar\ irradiation}$ is defined as:

$$I_{without\ solar\ irradiation} = B^{in}_{total\ without\ solar\ irradiation} - B^{out}_{total}$$

RESULTS AND DISCUSSION

Effects of water recirculation on algae growth, nutrient consumption, and biomass composition

The continuous cultures were conducted using the APB to investigate the effects of water recirculation on algal biomass production, composition, and nutrient consumption. The data demonstrate that water recirculation does not impact biomass growth in terms of biomass productivity compared to freshwater condition. The data also suggest that while there were significant differences in nutrient consumption, these trends do not significantly impact total biomass production or composition between conditions.

Monthly biomass productivity (Fig. 1a) and nutrient consumption (Fig. 1b) were analyzed to determine the optimal recirculation period until recirculated water should be replaced with freshwater. Although biomass productivity showed a decreasing trend over a period of four months of water recirculation, the 1st through 4th months of recirculation showed no significant differences ($P > 0.05$) in biomass productivity (0.26, 0.23, 0.20, and 0.18 g L^{-1} d^{-1}, respectively) in comparison to freshwater (0.22 g L^{-1} d^{-1}).

TN consumption during the 1st and 2nd months of recirculation (28.3 and 30.0 mg TN L^{-1} d^{-1}, respectively) was significantly different ($P < 0.5$) than freshwater (22.0 mg TN L^{-1} d^{-1}), but was not significantly different ($P > 0.05$) during the 3rd and 4th months (20.6 and 22.9 mg TN L^{-1} d^{-1}, respectively) than freshwater. NH_3-N consumption was significantly different ($P < 0.05$) than freshwater during the 2nd, 3rd and 4th months of recirculation (9.1, 7.0 and 6.9 mg NH_3-N L^{-1} d^{-1}, respectively), but was not significantly different ($P > 0.05$) than freshwater (11.9 mg NH_3-N L^{-1}

d-1) during the 1st month of recirculation (11.1 mg NH_3-N L^{-1} d^{-1}). NO_3-N consumption also showed significant differences ($P > 0.05$) during the 1st, 2nd and 4th months (12.9, 11.7 and 11.3 mg NO_3-N L^{-1} d^{-1}, respectively), but was not significantly different ($P > 0.05$) than freshwater (6.1 mg NO_3-N L^{-1} d-1) during the 3rd month (7.41 mg NO_3-N L^{-1} d^{-1}). TP consumption was significantly different ($P < 0.05$) during the 1st and 3rd months of recirculation (3.3 and 1.9 mg TP L^{-1} d^{-1}, respectively) but was not significantly different ($P > 0.05$) than freshwater (2.6 mg TP L^{-1} d-1) during the 2nd and 4th months of recirculation (2.8 and 2.2 mg TP L^{-1} d^{-1}, respectively).

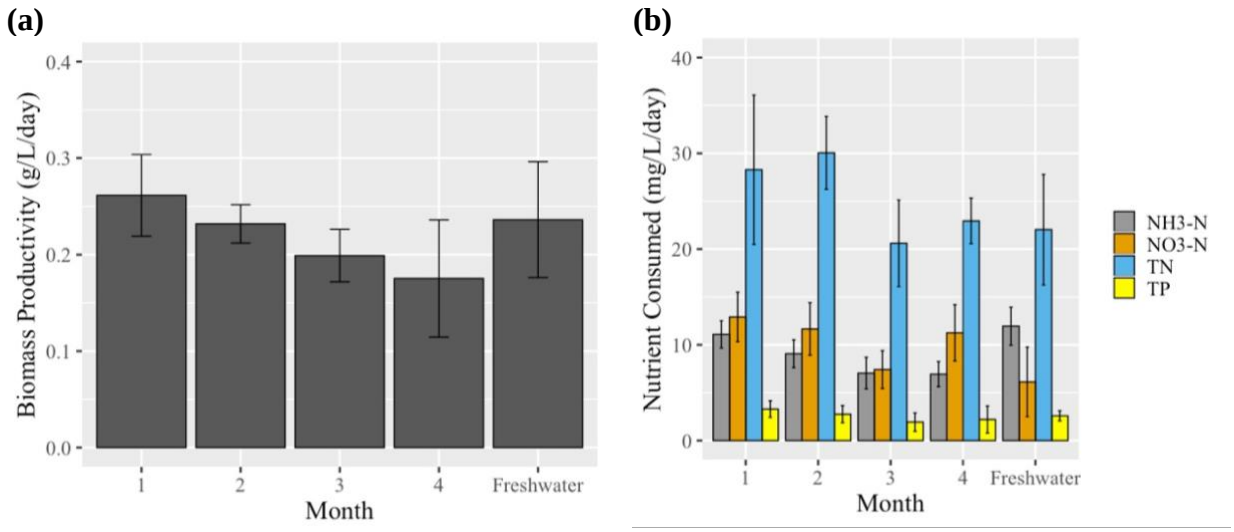

Figure 1: Biomass production and nutrient consumption. **a.)** Biomass productivity over 4-month recirculation period compared with freshwater. **b.)** Nutrient consumption over 4-month recirculation period compared with freshwater.

The biomass productivity data suggest that a four-month time period is an acceptable duration for water recirculation before recirculated water should be replaced with freshwater. Biomass productivity was not significantly different than freshwater during any month, regardless of differences in monthly nutrient consumption. Furthermore, biomass components (both elements and macromolecules) between recirculation and freshwater conditions showed similar content (Table 1), suggesting that recirculation did not greatly affect biomass composition. However, four months of recirculation is a conservative recirculation estimate, as

the 4th month did not show significant differences in biomass productivity compared to the freshwater condition. Thus, the actual maximum recirculation period under the recirculation conditions cannot be concluded.

Table 1: Biomass composition under recirculation and freshwater conditions

Component (% of biomass)	Recycle	Freshwater
C [a]	46.2 ± 0.89	46.4 ± 0.44
H [a]	7.1 ± 0.05	7.1 ± 0.08
O [c]	32.3 ± 1.08	30.5 ± 0.09
N [a]	8.5 ± 0.35	8.7 ± 0.27
P [b]	1.9 ± 0.10	2.4 ± 0.25
S [a,b]	0.6 ± 0.09	0.6 ± 0.09
Carbohydrate [b, d]	19.2 ± 2.61	19.5 ± 2.44
Lipid [b]	12.2 ± 0.47	9.7 ± 1.68
Protein [b]	59.1 ± 2.47	58.6 ± 2.41
Ash [b]	9.6 ± 0.36	12.2 ± 1.52

a: Value obtained from Atlantic Microlabs
b: Value obtained from DairyOne Inc.
c: Value obtained through calculating 100 - SUM (macro and micronutrient composition). No ash included. See appendix for details on detailed mineral content of biomass.
d: Carbohydrates = NFC + NDF. See Table 4 in the appendix for composition values.

Effects of water recirculation on algal assemblage

Microbial community composition is a useful indicator for APB system operational status. The unique co-culture assemblage was monitored to further assess APB stability under recirculation conditions. Illumina library preparation and sequencing were performed at the MSU RTSF and QIIME 2™ was used for all sequence analyses. Sequence analysis showed a high abundance of cyanobacteria, which is known to have 85-93% 16s rRNA gene sequence similarity with microalgal strain *C. sorokiniana* [56]. Further analysis through microscopic imaging did not indicate the presence of cyanobacteria, which are frequently characterized by

blue-green color and various shape, size and form, unlike *C. sorokiniana* [57]. Thus, the sequence was interpreted as microalga *C. sorokiniana* for all further analyses.

The unique algae-bacteria assemblage was monitored to further assess APB stability under recirculation conditions. Microbes with less than 0.5% of relative abundance are not discussed. The relative abundances of the dominant microbial communities at three taxonomic levels (Domain, Phylum and Class) are presented in Fig. 2. Communities are shown over a four-month recirculation time period and under freshwater conditions. These communities belong to a total of 2 domains, 8 phyla and 9 classes. The domains present are eukarya (containing microalgal species of interest, *C. sorokiniana*) and bacteria (Fig. 2a). The dynamic data over the recirculation period show that the relationship between bacteria and algae abundance remained relatively stable. No significant differences ($P > 0.05$) were observed for eukarya abundance in the 1st through 4th month of recirculation (80.7, 87.1, 83.1, and 82.1%, respectively) in comparison to freshwater conditions (83.7%). Similarly, no differences ($P > 0.05$) in abundance were observed in the bacterial domain for the 1st through 4th month (19.2, 12.8, 16.9 and 17.8%, respectively) compared to freshwater conditions (16.2%).

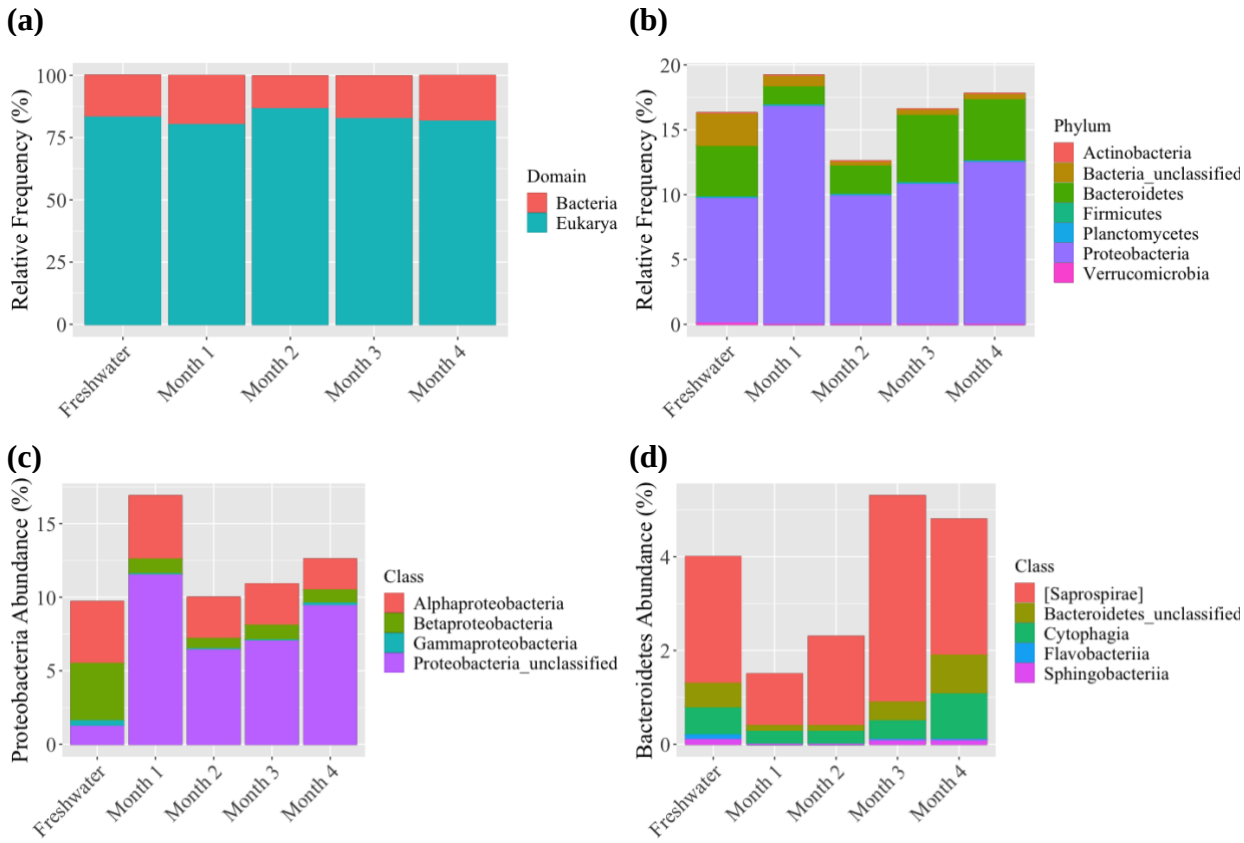

Figure 2: Relative abundance of bacterial communities. **a)** at domain level. **b)** at phylum level. **c)** at Proteobacteria class level. **d)** at Bacteroidetes class level.

At the bacterial phylum level, Proteobacteria (9.5-16.8%), Bacteroidetes (1.5-5.3%) and Bacteria unclassified (0.3-2.5%) abundance dominated both freshwater and recirculation communities (Fig. 2b). These findings are consistent with other environmental and large-scale microalgae cultivation studies [8, 58].

The phylum Proteobacteria (Fig. 2c) was the most abundant group under both freshwater and recirculation conditions. Proteobacteria abundance in the 1st month of recirculation (16.8%) was significantly higher ($P < 0.05$) than during freshwater conditions (9.5%) while the 2nd, 3rd and 4th months of recirculation (10.1, 11.1 and 12.5%, respectively) showed no significant difference ($P > 0.05$). Under recirculation conditions, Proteobacteria unclassified was the most abundant (6.5-11.7%) class, followed by Alphaproteobacteria and Betaproteobacteria (2.0-4.2%

and 0.7-1.1%, respectively). Under freshwater conditions, Alphaproteobacteria and Betaproteobacteria were the most abundant classes (4.1 and 3.9%, respectively), followed by Proteobacteria unclassified (1.3%). No significant differences ($P > 0.05$) were observed for Alphaproteobacteria abundance during the 1st through 4th months of recirculation (4.2, 2.9, 2.9 and 2.0%, respectively) when compared with freshwater conditions (4.1%). However, significant differences were identified in the abundance of the Betaproteobacteria and Proteobacteria unclassified classes. Betaproteobacteria abundance was significantly less ($P < 0.05$) during the 1st through 3rd months of recirculation (1.0, 0.7 and 1.1, respectively) compared to freshwater conditions (3.9%), although the 4th month showed no difference (0.9%). Proteobacteria unclassified abundance was significantly higher ($P < 0.05$) during all four months of recirculation (11.7, 6.5, 7.1 and 9.5%, respectively) compared to freshwater conditions (1.3%).

The Bacteroidetes phylum (Fig. 2d) was another dominant bacterial group among recirculation and freshwater. No significant differences ($P > 0.05$) were observed between any months of recirculation (1.5, 2.3, 5.3 and 4.9%, respectively) when compared to freshwater conditions (4.1%). However, significant differences ($P < 0.05$) were observed between the months of recirculation. Under both freshwater and recirculation conditions, the most abundant class within Bacteroidetes was [Saprospirae] (phylum is disputed) (1.1-4.4%). No significant differences ($P < 0.05$) in [Saprospirae] were observed between the 1st through 4th months of recirculation (1.1, 1.9, 4.4 and 2.9%, respectively) when compared with freshwater abundance (2.8%). Although, significant differences ($P < 0.05$) were observed between months of recirculation.

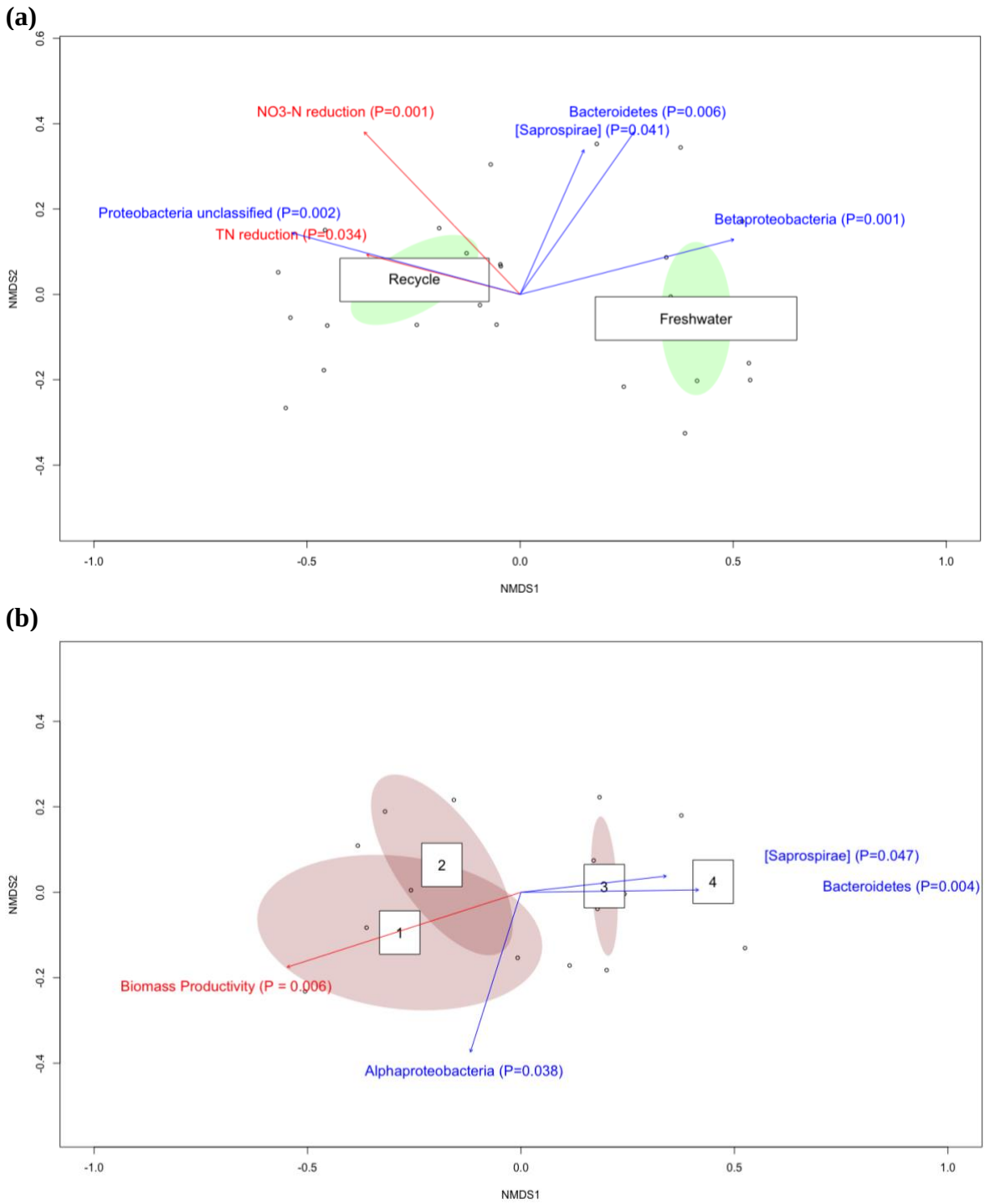

Figure 3: Non-metric multidimensional scaling (NMDS) analysis of microbial community relative abundance for the APB system*. **a.)** Culture condition. **b.)** Water recirculation time. *: Green ellipses indicate culture condition and red indicate recirculation time. The red arrows indicate significant operational parameters ($P < 0.05$) of the APB. Blue arrows indicate significant microbial groups ($P < 0.05$).

Non-metric multidimensional scaling (NMDS) analysis was conducted to determine the linkage of 16S rRNA gene sequences to environmental condition (Fig. 3a) and recirculation time (Fig. 3b). Experimental condition, significant operational parameters and key bacterial groups were compared with microbial community distances to determine the interaction between microbial community, environmental condition, and water recirculation time. Recirculation conditions significantly (P=0.001) shifted the microbial communities (green ellipses in Fig. 3a), as did time (P=0.001) of recirculation (red ellipses in Fig. 3b). One bacterial phyla (Bacteroidetes) and three bacterial classes (Betaproteobacteria, [Saprospirae] and Proteobacteria unclassified) were significantly ($P < 0.05$) influenced under recirculation and freshwater conditions. Additionally, one bacterial phyla (Bacteroidetes) and two bacterial classes (Alphaproteobacteria and [Saprospirae]) were significantly ($P < 0.05$) influenced by time of recirculation.

Bacteroidetes was affected by both culture condition and recirculation time. Most notably, Fig. 3b shows the increased abundance of Bacteroidetes over increasing time of recirculation. Members of the Bacteroidetes phylum are highly diverse, but are frequently recognized as specialists for degrading complex organic matter, such as proteins and carbohydrates [58]. The accumulation of organic matter over the recirculation period, such as algal cell components, is a potential carbon and energy source for Bacteroidetes. Unlike freshwater conditions, the accumulation of unassimilated compounds is a critical consideration for recirculated media.

The class [Saprospirae], was found to be the most abundant class within Bacteroidetes and was also significantly affected by culture condition and time. Recirculation time (Fig. 3b) showed a clear relationship with [Saprospirae], with relative abundances increasing over time.

Further analysis showed that Chitinophagaceae was the dominating bacterial family from the [Saprospirae] class. Previous studies suggest that Chitinophagaceae may provide molecules and enzymes which act as potential stimulants for plant growth [59]. Another study [60] on algal-bacteria symbiosis also reported the presence of Chitinophagaceae in association with algal growth in wastewater, and suggests that the growth of this bacterial group was favored due to its co-existence with algae.

Fig. 3 suggests that significant changes in TN and NO_3-N consumption (P=0.034 and P=0.001, respectively) occurred under recirculation conditions. This trend may indicate that the bacterial consortia utilized nitrogen compounds differently between freshwater and recirculation culture conditions. Betaproteobacteria, for example, was found to be significantly influenced (P=0.001) by culture condition, showing a higher relative abundance under freshwater conditions than during recirculation (Fig. 3a). The Betaproteobacteria class is known to contain many species of ammonia oxidation bacteria [61], which provides a possible explanation for differences in nitrogen consumption patterns among culture conditions.

A particularly interesting study conducted by Sambles *et al* discusses microbial community changes in an algae-bacteria co-culture due to repeated rinsing. Certain groups, such as Alphaproteobacteria, increased after rinsing [62]. Under recirculation conditions, Alphaproteobacteria was shown to decrease over time. It is possible that rinsing with freshwater may provide a favorable environment for this group of bacteria. Sambles *et al* also identified certain bacterial orders that were not removed from the culture by rinsing, while others were removed after rinsing [62]. This suggests that microbes not removed by rinsing were likely very closely associated or attached to algal colonies [62]. Bacteria unclassified and Proteobacteria unclassified were shown to be affected by culture condition and/or recirculation time. Although

these bacterial groups could not be further classified beyond the domain and phylum levels, respectively, speculations can be made as to why differences in abundance exist between conditions. It is possible that daily harvesting disrupts algae-bacteria relationships. While this disruption remains permanent under freshwater conditions, media recirculation may reinforce such relationships, as previously rinsed microbes are eventually re-introduced into the system. It is likely that more detailed relationships such as this are masked under currently unclassified groups.

Furthermore, although biomass productivity under recirculation was not found to be significantly different than freshwater, it is clear in Fig. 3b that biomass productivity showed an overall decreasing trend over the recirculation period. These results are consistent with similar small-scale algae cultivation studies on media recirculation in microalgae cultivation [14]. It remains uncertain why biomass decreased over recirculation. One potential cause for decrease may include unidentified pathogenic bacteria that were allowed to grow under prolonged residence time within the system and harm algal cells. Some species in the order of Cytophagia, for example, are capable of lysing a variety of algae cells [8]. Another possible factor is the gradual accumulation of substances that may be detrimental to algal health. Potential accumulating substances could be inhibitory compounds from bacterial or algal cells, nutrients, minerals, or antifoaming agent used to control foaming during algae cultivation. Compounds such as inhibitory substances and antifoaming agent are not assimilated into the microalgae biomass, allowing concentration to rise as media is recirculated. Select nutrients and minerals, such as potassium (K) and P were also found to accumulate within the reactor, as algal biomass consumption rate was likely less than the amount added each day. Table 8 in the appendix

provides more detailed information on micronutrient accumulation in the culture media over the recirculation period and under freshwater conditions.

Microbial analysis demonstrated that the relationship between microalgae and bacteria domains was relatively stable for the recirculation period and did not display and major differences between conditions. However, it is apparent that the intricacies and complexities of microbial community on biomass growth cannot be fully characterized by the obtained results. Understanding microbial community structure and function within microalgae cultivation is essential for large-scale microalgae cultivation. In order to determine the true stability and functional relationships between microalgae and bacteria, more detailed analysis on community structure and function at genus or species level is required.

Mass and energy balance of the water recirculating photobioreactor system

Using experimental data, a mass and energy balance analysis was conducted on a 1 m^3 APB unit (Fig. 4 and Table 3). Under freshwater conditions, the 1 m^3 unit produces 1.1 kg of wet algal biomass per day (Fig. 4a). The daily nutrient requirements are 0.036 kg nitrogen and 0.008 kg of phosphorus. Additionally, 500 kg of water is required each day to replenish 498.9 kg of water discharged from centrifuging and to replace water contained in algal biomass. The discharged water contains approximately 0.011 kg nitrogen and 0.003 phosphorus. The culture sequesters approximately 0.4 kg of CO_2 per day.

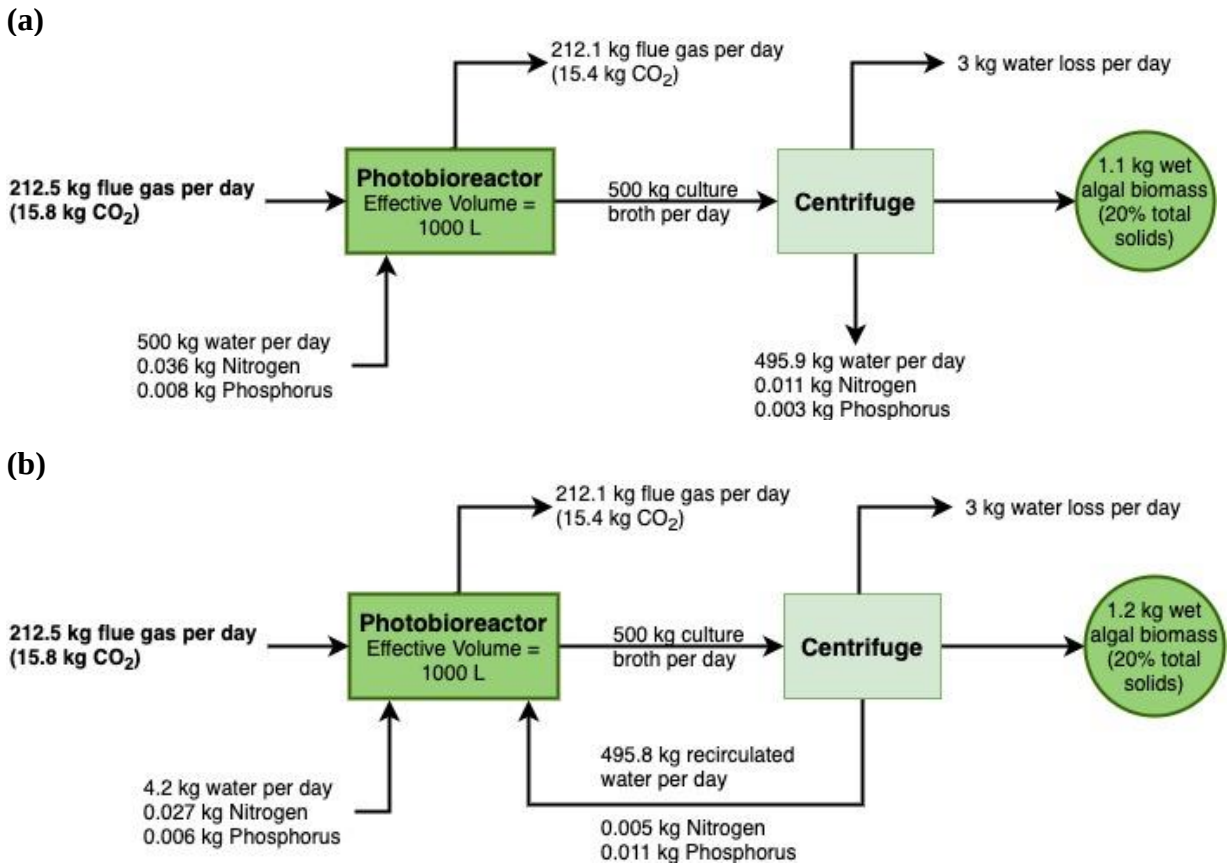

Figure 4: Mass balance of the pilot APB unit*. **a.)** Mass balance under freshwater conditions**. b.)** Mass balance under recirculation conditions.

*: Calculation of CO_2 fixation under freshwater conditions is based on microalgal cell formula: $CH_{1.84}O_{0.49}N_{0.16}P_{0.020}S_{0.005}Fe_{0.009}K_{0.008}Ca_{0.005}Mg_{0.003}$

*: Calculation of CO_2 fixation under recirculation is based on microalgal cell formula: $CH_{1.84}O_{0.52}N_{0.16}P_{0.016}S_{0.005}Fe_{0.006}K_{0.009}Ca_{0.003}Mg_{0.003}$

Under recirculation conditions, the photobioreactor produces 1.2 kg of wet algal biomass per day (Fig. 4b). The nutrients required are 0.027 kg nitrogen and 0.006 kg of phosphorus per day, and 495.8 kg of water is able to recycle after separation of algal biomass from media by centrifugation. Recirculated water contains 0.005 kg of nitrogen and 0.011 kg of phosphorus. Thus, only 4.2 kg of freshwater is required each day to replenish 3 kg of water discharged from centrifuging and to replace water contained in algal biomass. The culture sequesters approximately 0.4 kg of CO_2 per day.

The mass balance analysis shows that for a 1-year cultivation operating under recirculation conditions, approximately 179,000 kg of freshwater, 3.2 kg of nitrogen, and 0.7 kg of phosphorus can be conserved (Table 2). This represents a 98% reduction in freshwater, and 25% reduction of both nitrogen and phosphorus over freshwater cultivation. However, it should be noted that because phosphorus was allowed to accumulate to higher concentrations under recirculation than in freshwater conditions, the theoretical P requirement to maintain the same biomass productivity and CO_2 sequestration rate may be lower than reported.

Table 2: Resources saved of the envisioned 1000 L algae photobioreactor for 1-year operation under recirculation conditions*.

Resource	Amount Saved (kg)	Reduction
Freshwater	179,000	98 %
Nitrogen	3.2	25 %
Phosphorus	0.7	25 %

*4-month water usage before replacing with new freshwater
*Based on mass balance and pilot operational data
*In comparison with freshwater treatment conditions

The energy balance analysis shows that a net energy input of 219 kWh-e unit $^{-1}$ day $^{-1}$ is required to power the APB unit under both freshwater and recirculation conditions (Table 3). The electricity demands for the LED lights and centrifuge are 215 and 0.25 kWh unit $^{-1}$ day $^{-1}$, respectively. The energy required for the APB water addition and culture harvesting pumps are 0.07 and 0.07 kWh unit $^{-1}$ day $^{-1}$, respectively. The energy required for centrifugation pumps is 0.03 kWh unit $^{-1}$ day $^{-1}$, each. The energy requirement for the flue gas pump is 3.50 kWh unit $^{-1}$ day $^{-1}$. The most significant energy demand comes from the electrical input to the LED lights, which accounts for 98% of the total daily energy demand.

Table 3: Energy balance of the envisioned 1000 L algae photobioreactor *

Component	Energy Required
Electrical input to LED lights (kWh/unit/day)	-215
Electrical input to centrifuge (kWh/unit/day)	-0.25
Electrical input to water addition (kWh/unit/day)	-0.07
Electrical input to culture harvesting (kWh/unit/day)	-0.07
Electrical input to centrifuge pump (tank to centrifuge) (kWh/unit/day)	-0.03
Energy input to centrifuge pump (centrifuge to sink) (kWh/unit/day)	-0.03
Energy input to flue gas pump (kWh/unit/day)	-3.50
Net energy (kWh-e/unit/day)	**-219**

*: The energy balance analysis was based on the mass balance and pilot operational data. Energy input is negative and energy output is positive.

Exergy analysis of the water recirculating photobioreactor system

An exergy analysis was conducted to calculate the rational exergy efficiencies and compare process effectiveness between water recirculation and freshwater conditions (Fig. 5). The rational exergy efficiency under recirculation and freshwater conditions were 0.64 and 0.59%, respectively. The rational exergy efficiencies without considering solar energy were 23 and 10%, for recirculation and freshwater, respectively. The data indicate that microalgae cultivation under recirculation conditions had a better exergetic performance than cultivation under freshwater conditions.

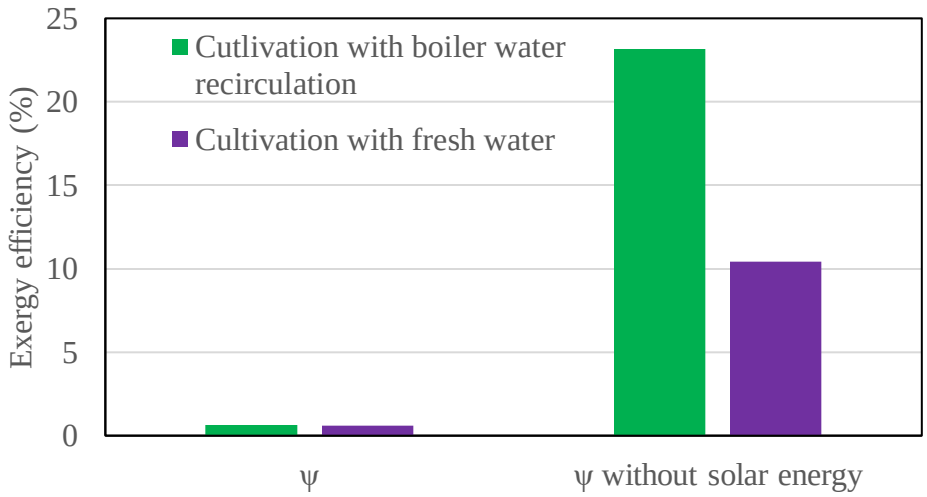

Figure 5: Rational exergy efficiencies of freshwater and water recirculation cultivations.

The freshwater control used a greater amount of chemicals and freshwater in the input stream and wasted a greater amount of product in the output stream, while maintaining a similar output in the desired product compared to recirculation. In contrast, recirculation minimized water and nutrient usage in the input and output streams, as water loss from centrifugation and flue gas were the only output flows with undesirable products. Furthermore, the input stream required less water and nutrient input per day due to cross-over products from recirculation. Microalgae cultivation under recirculation conditions clearly exhibits an improved exergetic performance over freshwater conditions. The detailed exergy analysis is provided in the appendix (Tables 16 and 17).

CONCLUSION

A pilot-scale APB system for flue gas CO_2 sequestration and algal biomass production for microalgae species *C. sorokiniana* was studied under boiler water recirculation and freshwater conditions. The results indicate that water recirculation does not affect *C. sorokiniana* cultivation over the studied recirculation period. Biomass productivity was not significantly different between recirculation and freshwater conditions (0.23 and 0.22 g L^{-1} d^{-1}, respectively) and biomass content between conditions was comparable. Furthermore, the relationship between eukaryotic and bacterial domains remained stable between recirculation (81%, 87%, 83%, and 82%, respectively and 19%, 13%, 17% and 18%, respectively) and freshwater (84% eukaryotic and 16% bacterial). The water footprint was substantially reduced by 98% and nitrogen and phosphorus were each reduced by 25%. Recirculation conditions exhibited an increase in rational exergy efficiency more than double that of freshwater conditions.

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CHAPTER 3. CONCLUSIONS AND FUTURE WORK

CONCLUSIONS

C. sorokiniana is a species of green microalgae that is commonly employed in large-scale cultivations for CO_2 sequestration. In order to improve the economic efficiency and sustainability of microalgae cultivation operations, freshwater and nutrient usage need to be reduced. Thus, *C. sorokiniana* was cultivated on flue gas CO_2 under boiler water recirculation and freshwater conditions to determine the feasibility of media recirculation in a pilot-scale operation.

This research shows that the cultivation of *C. sorokiniana* on recycled media did not affect the growth in comparison to freshwater conditions in the pilot-scale unit over the recirculation period. No significant differences were observed in biomass productivity, composition, CO_2 sequestration and dominating microbial domains. Meanwhile, mass and exergy balances found that substantial amounts of nutrients and water could be conserved, therefore improving the overall efficiency of the system. It is important to note that a decreasing trend in biomass productivity was observed during the recirculation period and that significant differences were observed in parameters including nutrient consumption and microbial community at phylum and class level. The accumulation of unassimilated compounds was also observed. However, the underlying cause behind microalgal biomass productivity decrease was not identified in this study and therefore remains uncertain. Potential contributors to this trend are increased abundance of competing microorganisms, pathogenic bacteria, accumulation of unnecessary compounds, and likely, a combination of these.

Overall, this study concluded that media recirculation is a feasible approach in pilot and large-scale microalgae cultivation systems over the finite period of four months. After this finite

period, it is recommended that recirculated media should be replaced with freshwater to avoid significant decrease in biomass productivity.

FUTURE WORK

Future work in the area of media recirculation should be conducted to improve large-scale microalgae cultivation. The effect of water recirculation on additional economically relevant microalgal strains should be investigated, as various strains and microbial communities differ in response to cultivation conditions. Furthermore, a dynamic assessment of microbial community over the recirculation period should be performed. Closely monitoring changes in microbial community over time is crucial for identifying fluctuations in nutrient consumption and algal biomass growth. It also highlights areas for potential optimization, such as reducing bacterial communities that compete with microalgae for resources or enhancing communities that are beneficial to microalgal growth. Additionally, accumulating and inhibitory compounds under recirculation conditions should be identified and further characterized for their relationship with biomass productivity. The characterization of these compounds in combination with identification of harmful microbial groups can be used to recognize additional measures that should be taken to maximize recirculation period, such as media filtration or sterilization. The listed studies will contribute broadly to the area of microalgae cultivation by providing insight on areas that require optimization, thus further reducing natural resource consumption and increasing economic and environmental viability.

APPENDICES

APPENDIX A: TABLES AND FIGURES

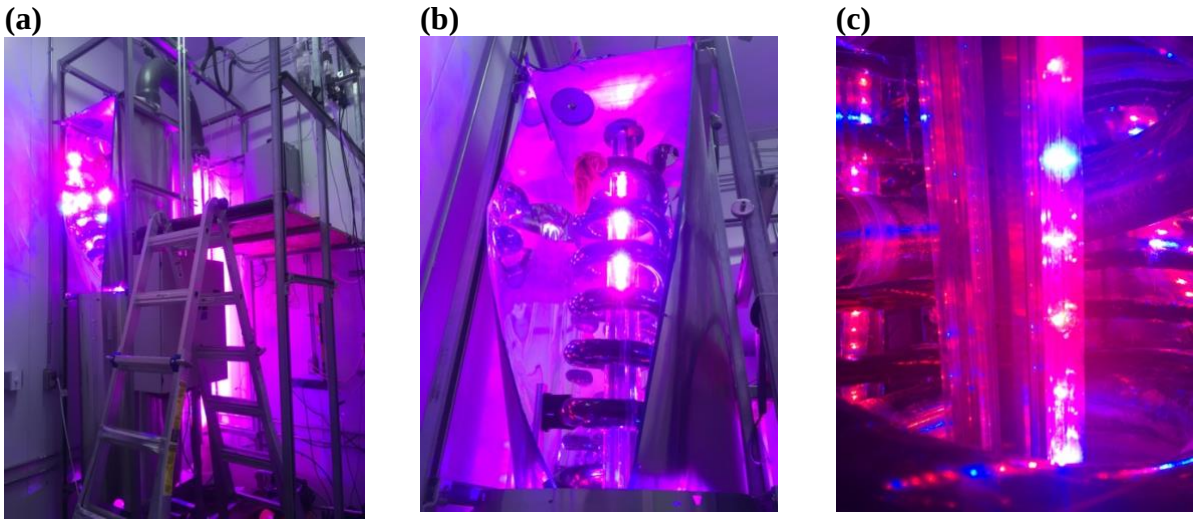

Figure 6: 100 L photobioreactor set up. **a.)** Helical coil (front) and up-tube (back). **b.)** Helical coil. **c.)** Red and blue LED light strip.

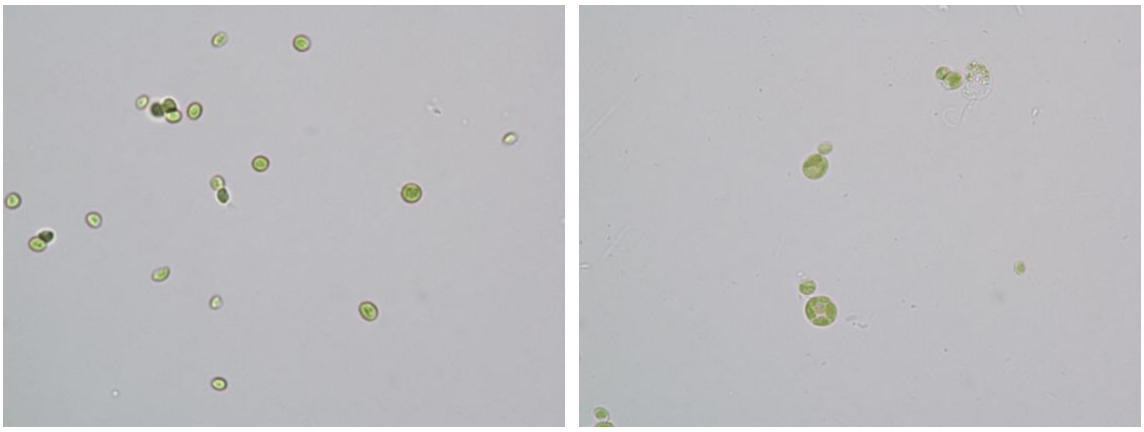

Figure 7: *C. sorokiniana* and microbial community.

Table 4: *C. sorokiniana* biomass composition. *

Treatment	Recycle				Freshwater		
Component	5/19-5/21	6/20-6/23	7/18-7/21	8/8-8/10	9/11-9/13	10/10-10/12	11/7-11/9
Moisture (%)	11.2	8	11.9	12.1	7.6	9.7	8.4
Dry Matter (%)	88.8	92	88.1	87.9	92.4	90.3	91.6
Crude Protein (%)	61.4	60	59.2	55.6	56.6	61.3	58
Available Protein (%)	59.8	57	55.6	55.1	55.2	59.9	57.3
ADICP (%)	1.5	3	3.5	0.5	1.4	1.4	0.7
Adjusted Crude Protein (%)	60.8	58	56.6	55.6	56.6	61.3	58
ADF (%)	15.7	20.1	16.9	9.4	7.1	1.4	0.7
Crude Fat (%)	12.4	12.1	12.7	11.6	8.8	11.6	8.6
Total Fatty Acids (%)	10.46	10.19	10.83	10.29	8.42	10.43	8.2
RUFAL (%)	5.06	4.65	4.97	4.93	3.7	5.04	3.86
TDN (%)	90	88	92	93	81	93	87
NEL (Mcal/Lb)	1.01	0.99	1.04	1.04	0.9	1.04	0.96
NEM (Mcal/Lb)	1.08	1.06	1.11	1.12	0.94	1.12	1.02
NEG (Mcal/Lb)	0.75	0.74	0.78	0.79	0.64	0.79	0.7
Calcium (%)	0.39	0.38	0.4	0.31	0.96	0.63	0.8
Phosphorus (%)	1.82	1.92	2.02	2.03	2.57	2.1	2.46
Magnesium (%)	0.3	0.3	0.31	0.28	0.31	0.3	0.28
Potassium (%)	1.3	1.33	1.39	1.35	1.09	1.22	1.19
Sodium (%)	0.012	0.01	0.012	0.008	0.009	0	0
Iron (ppm)	10900	14400	15000	16600	26100	14700	20500
Zinc (ppm)	36	40	43	55	486	313	477
Copper (ppm)	20	22	18	20	123	159	244
Manganese (ppm)	55	46	50	30	69	57	59
Molybdenum (ppm)	0.4	0.2	0.5	0.1	0.7	0.4	0.6
Sulfur (%)	0.68	0.64	0.65	0.63	0.66	0.72	0.71
Ash (%)	9.08	9.83	9.81	9.77	13.4	10.5	12.76
Chloride Ion (%)	0.1	0.12	0.16	0.14	0.05	0.01	0.01
Soluble Protein (% CP)	21	15	18	20	15	19	22
Lignin (%)	11.5	11.4	13.6	6.4	5.6	1.3	0.6
NDICP (%)	3.7	3.3	2.8	3.6	4.9	0.2	0.1
Starch (%)	0.9	0.6	0.2	1.3	1.5	0.9	0.7
NFC (%)	6.9	8.3	12.8	17.8	10.2	16	19.1
ESC (Simple Sugars) (%)	5.1	5.1	7.6	8.5	4.3	7.2	7.1
aNDFom (%)	10.3	9.8	5.5	5.2	11	0.7	1.5

*: Biomass processed by DairyOne Inc.

Table 5: *C. sorokiniana* CHNS composition. *

Component	Concentration (ppm)							
	Recycle						Freshwater	
	3/17-3/19	4/18-4/21	5/19-5/22	6/20-6/23	7/18-7/21	8/11-8/14	9/2-9/3	10/3-10/6
C	46.3	46.55	46.4	47.43	45.55	45.55	46.13	46.75
H	6.95	7.16	7.19	7.13	7.09	7.09	7.06	7.17
N	8.97	8.76	8.59	8.67	8.12	7.96	8.46	8.84
S	0.56	0.54	0.51	0.48	0.49	0.48	0.54	0.54

*: Data from Atlantic Microlab.

Table 6: *C. sorokiniana* fatty acid composition as % of dry matter. *

Fatty Acid	% of Dry Matter							
	Recycle					Freshwater		
	4/18-4/21	5/19-5/22	6/20-6/23	7/18-7/21	8/8-8/10	9/11-9/13	10/10-10/12	11/7-11/9
C12:0 Lauric	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C14:0 Myristic	0.03	0.03	0.03	0.04	0.03	0.03	0.03	0.02
C16:0 Palmitic	1.70	1.82	1.79	1.89	1.88	1.57	1.76	1.53
C16:1 Palmitoleic	0.10	0.08	0.09	0.09	0.07	0.09	0.07	0.09
C18:0 Stearic	0.07	0.07	0.06	0.07	0.08	0.05	0.06	0.05
C18:1 Oleic	1.15	1.37	1.21	1.25	1.34	0.98	1.32	1.09
C18:2 Linoleic	2.31	2.3	2.28	2.55	2.46	1.75	2.11	1.55
C18:3 Linolenic	1.16	1.38	1.16	1.17	1.14	0.97	1.61	1.22
C20:0 Arachidic	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
C20:1 Gadoleic	0.00	0.00	0.00	0.01	0.00	0.00	0.01	0.01
C20:5 Eicosapentaenoic (EPA)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C22:0 Behenic	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C22:6 Docosahexanoic (DHA)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C24:0 Lignoceric	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
Other	2.82	3.38	3.55	3.75	3.28	2.95	3.44	2.61
Total Fatty Acids	4.61	5.06	4.65	4.97	4.93	3.7	5.04	3.86
Saturated	9.36	10.46	10.19	10.83	10.29	8.42	10.43	8.2
MUFA	2.82	3.38	3.55	3.75	3.28	2.95	3.44	2.61
PUFA	4.61	5.06	4.65	4.97	4.93	3.7	5.04	3.86
RUFAL	9.36	10.46	10.19	10.83	10.29	8.42	10.43	8.2

*: Results from DairyOne, Inc.

Table 7: *C. sorokiniana* fatty acid composition as % of total fatty acids. *

Fatty Acid	% of Total Fatty Acids							
	Recycle					Freshwater		
	4/18-4/21	5/19-5/22	6/20-6/23	7/18-7/21	8/8-8/10	9/11-9/13	10/10-10/12	11/7-11/9
C12:0 Lauric	0.03	0.03	0.03	0.03	0.04	0.04	0.03	0.04
C14:0 Myristic	0.31	0.31	0.31	0.33	0.33	0.34	0.28	0.28
C16:0 Palmitic	17.73	16.99	17.18	17.03	17.83	18.28	16.5	18.27
C16:1 Palmitoleic	1.07	0.72	0.85	0.85	0.65	1.05	0.69	1.13
C18:0 Stearic	0.71	0.63	0.57	0.64	0.73	0.65	0.54	0.57
C18:1 Oleic	12.35	13.24	11.95	11.59	13.1	11.68	12.74	13.43
C18:2 Linoleic	24.87	22.18	22.56	23.75	24.05	21	20.41	19.07
C18:3 Linolenic	12.6	13.45	11.63	11	11.25	11.75	15.69	15.15
C20:0 Arachidic	0.06	0.05	0.05	0.05	0.06	0.06	0.06	0.07
C20:1 Gadoleic	0.05	0.03	0.01	0.05	0.03	0.05	0.05	0.09
C20:5 Eicosapentaenoic (EPA)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C22:0 Behenic	0.03	0.03	0.03	0.03	0.03	0.04	0.03	0.04
C22:6 Docosahexanoic (DHA)	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
C24:0 Lignoceric	0.11	0.10	0.09	0.08	0.09	0.09	0.08	0.10
Other	30.08	32.26	34.74	34.56	31.83	34.96	32.92	31.75
Total Fatty Acids	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Saturated	18.98	18.13	18.26	18.19	19.1	19.51	17.51	19.38
MUFA	13.46	13.98	12.81	12.5	13.78	12.78	13.48	14.65
PUFA	37.47	35.63	34.19	34.75	35.29	32.75	36.09	34.22
RUFAL	-	-	-	-	-	-	-	-

*: Results from DairyOne, Inc.

Table 8: Algal supernatant and boiler water composition. *

Component (ppm)	Recycle									Freshwater			
	Before Mixing Storage Tank Water	After Mixing Storage Tank Water	Boiler Water	5/7/19	5/21/19	6/4/19	6/18/19	7/16/19	7/26/19	8/10/19	9/4/19	9/17/19	10/2/19
Calcium (Ca)	96.33	103.50	2.71	114.56	115.58	120.84	107.48	103.66	100.70	98.46	108.60	118.22	115.88
Phosphorus (P)	19.00	25.17	13.29	9.12	12.06	22.78	25.22	28.92	31.10	17.90	5.44	11.32	4.98
Magnesium (Mg)	48.31	49.95	0.65	49.22	52.02	53.74	49.16	51.56	51.14	49.08	37.52	39.64	38.28
Potassium (K)	291.50	306.10	0.00	119.16	165.82	209.60	219.60	275.00	287.00	289.60	8.80	8.98	10.42
Sodium (Na)	31.10	38.41	30.80	33.28	28.14	36.20	30.68	26.92	40.34	27.62	25.90	17.82	23.82
Iron (Fe)	1.05	9.63	0.03	0.00	0.00	0.08	0.30	0.14	0.12	0.26	0.08	0.00	0.02
Zinc (Zn)	0.00	0.31	0.02	0.00	0.00	0.00	0.00	0.00	0.02	0.00	0.00	0.00	0.00
Copper (Cu)	0.03	0.09	0.04	0.06	0.06	0.08	0.08	0.06	0.10	0.06	0.08	0.08	0.08
Manganese (Mn)	0.00	0.06	0.01	0.04	0.00	0.04	0.00	0.00	0.02	0.00	0.02	0.08	0.00
Molybdenum (Mo)	0.00	0.00	0.00	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.04

*Result from DairyOne, Inc.

Table 9: Biomass data under recirculation conditions.

Date	Biomass Harvested (g)	Moisture Content (%)	Biomass Productivity (g dry wt. L^{-1} day^{-1})
5/2-5/4	410.5	22.7%	0.31
5/5-5/9	619.4	22.9%	0.28
5/10-5/12	372.4	18.5%	0.23
5/13-5/15	465.5	21.6%	0.34
5/16-5/18	397.8	19.3%	0.26
5/19-5/22	477.6	21.5%	0.26
5/23-5/26	443.2	18.6%	0.21
5/27-5/29	372.7	20.6%	0.26
5/30-6/2	451.2	19.4%	0.22
6/3-6/5	372.6	30.0%	0.37
6/6-6/9	417.2	24.9%	0.26
6/10-6/12	337.1	21.2%	0.24
6/13-6/16	422.5	20.8%	0.22
6/17-6/19	320	21.2%	0.23
6/20-6/23	404.3	21.8%	0.22
6/24-6/26	361.7	21.7%	0.26
6/27-6/29	298.4	20.7%	0.21
6/30-7/3	420.3	21.3%	0.22
7/4-7/6	337.9	20.7%	0.23
7/7-7/10	412	19.1%	0.20
7/11-7/14	378.1	18.5%	0.17
7/15-7/17	321.1	20.5%	0.22
7/18-7/21	366.5	20.4%	0.19
7/22-7/24	277.2	21.4%	0.20
7/25-7/28	351.3	17.6%	0.15
7/29-7/31	200.8	22.8%	0.23
8/1-8/4	373.4	20.0%	0.19
8/5-8/7	329.7	18.1%	0.20
8/8-8/10	305	22.4%	0.23
8/11-8/14	186.7	18.9%	0.09

Table 10: Biomass data under freshwater conditions.

Date	Biomass Harvested (g)	Moisture Content (%)	Biomass Productivity (g dry wt. L^{-1} day^{-1})
8/29-9/1	329	18.1%	0.15
9/2-9/3	268	19.8%	0.27
9/4-9/5	213	17.2%	0.18
9/6-9/10	440	15.8%	0.14
9/11-9/13	173.5	15.8%	0.09
9/14-9/18	708.8	16.3%	0.23
9/19-9/22	955.9	14.7%	0.35
9/23-9/25	663.2	14.3%	0.32
9/26-9/28	452.2	15.7%	0.24
9/29-10/2	487.1	19.0%	0.23
10/3-10/6	539.9	20.7%	0.28
10/7-10/9	421.8	18.3%	0.26
10/10-10/12	359.9	18.6%	0.22
10/13-10/16	365.4	18.8%	0.17
10/17-10/18	182.2	20.8%	0.38
10/19-10/23	380.4	20.1%	0.25
10/24-10/27	496.3	17.9%	0.22
10/28-10/30	322.1	19.5%	0.21
10/31-11/3	390.1	19.7%	0.19
11/4-11/6	312.7	17.2%	0.18
11/7-11/9	243.7	16.2%	0.13
11/10-11/14	491.9	18.6%	0.18

Table 11: Operational data for recirculation cultivation.

Date	NO3-N (mg/L)		NH3-N (mg/L)		TN (mg/L)		TP-P (mg/L)		pH	
	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest
5/2/19	0.35	16.45	2.80	14.60	26.70	54.50	15.18	19.17	6.72	6.55
5/3/19	2.40	17.40	2.65	14.40	24.38	51.50	15.24	19.33	6.55	6.43
5/4/19	0.73	14.90	6.65	16.60	14.18	40.88	15.26	19.33	6.53	6.38
5/5/19	2.23	16.40	6.70	19.35	16.50	45.53	16.10	19.74	6.57	6.47
5/6/19	4.42	16.95	8.35	15.50	18.83	45.63	16.64	20.07	6.50	6.48
5/7/19	4.36	17.85	3.50	13.85	17.45	40.23	8.73	10.82	6.46	6.45
5/8/19	4.10	17.05	2.75	15.55	15.55	46.30	8.30	12.04	6.68	6.64
5/9/19	6.14	17.85	2.95	13.70	14.53	42.45	8.27	21.13	6.61	6.47
5/10/19	0.56	12.60	3.30	12.70	9.75	33.40	17.87	21.70	6.68	6.53
5/11/19	3.66	14.30	3.35	13.05	21.63	40.28	19.01	21.62	6.49	6.50
5/12/19	3.67	16.15	3.05	13.25	12.78	41.73	18.52	21.37	6.54	6.38
5/13/19	2.63	9.60	0.80	13.20	12.93	38.73	18.52	22.60	6.44	6.56
5/14/19	0.53	11.50	3.45	10.40	9.50	27.95	18.76	21.05	6.66	6.53
5/15/19	0.47	13.90	2.80	13.25	16.58	33.63	10.00	14.23	6.57	6.48
5/16/19	0.48	15.70	3.05	12.05	12.93	41.68	10.95	14.07	6.54	6.61
5/17/19	2.85	16.50	2.80	14.60	9.84	40.90	10.70	13.91	6.29	6.46
5/18/19	3.01	17.85	2.65	12.90	8.64	41.05	11.40	14.83	6.47	6.48
5/19/19	1.95	16.65	2.70	14.45	7.11	41.00	11.40	14.90	6.67	6.52
5/20/19	1.45	17.50	2.40	16.20	10.94	51.15	11.29	15.71	6.64	6.73
5/21/19	5.02	17.50	2.90	15.70	9.55	40.40	12.51	15.82	6.54	6.41
5/22/19	8.65	19.20	2.60	14.15	12.38	40.50	12.59	16.46	6.36	6.35
5/23/19	19.05	22.40	3.25	15.80	24.30	51.75	10.21	15.93	6.92	6.88
5/24/19	20.95	26.35	0.30	12.90	31.50	52.25	18.19	19.50	6.03	6.21
5/25/19	11.15	22.50	0.10	9.55	25.65	61.25	17.38	19.58	6.01	6.16
5/26/19	9.14	25.15	0.40	9.90	23.30	74.00	16.72	21.05	6.11	5.96
5/27/19	6.45	20.20	0.20	12.70	17.40	56.75	19.74	20.39	6.10	6.07
5/28/19	7.35	19.40	0.30	11.95	18.40	55.25	16.22	19.82	6.08	5.99
5/29/19	10.28	22.25	0.30	11.30	19.50	54.00	16.89	20.07	6.23	6.27
5/30/19	12.80	28.30	0.20	13.20	19.05	53.50	17.05	21.70	6.27	6.34
5/31/19	15.75	26.15	0.30	12.10	21.50	54.75	18.68	21.94	6.17	6.16
6/1/19	17.50	22.20	0.65	10.60	25.85	52.90	19.09	21.13	6.25	6.08
6/2/19	21.65	26.25	0.95	8.35	27.55	46.70	19.25	21.94	6.05	5.96
6/3/19	13.25	25.45	0.60	8.85	19.85	50.33	19.09	22.60	6.09	6.15
6/4/19	10.17	22.40	0.45	10.70	18.85	49.00	20.64	24.23	6.18	6.14
6/5/19	10.17	20.05	1.50	10.60	14.05	49.55	21.05	23.41	6.15	6.07
6/6/19	5.32	18.50	1.05	11.10	8.79	41.18	20.39	23.01	6.27	6.22
6/7/19	7.22	18.35	1.20	11.55	12.20	41.08	21.29	25.04	6.15	6.11
6/8/19	6.51	19.75	2.05	9.15	12.65	38.75	21.62	24.88	6.23	6.11
6/9/19	5.53	22.30	1.85	11.70	10.85	45.85	21.78	25.13	6.27	6.11
6/10/19	10.25	18.30	1.50	8.95	14.75	39.50	21.86	25.13	6.13	6.10

Table 11 (cont'd)

Date	NO3-N (mg/L)		NH3-N (mg/L)		TN (mg/L)		TP-P (mg/L)		pH	
	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest
6/11/19	8.50	19.65	1.95	11.45	14.65	42.63	23.58	26.22	6.21	6.16
6/12/19	6.83	20.20	1.75	10.70	10.90	47.45	24.82	25.83	6.24	6.12
6/13/19	5.68	17.55	1.75	11.55	10.70	45.63	23.97	28.14	6.28	6.27
6/14/19	8.02	21.15	2.25	11.30	13.45	42.00	22.60	27.77	7.13	6.94
6/15/19	11.80	22.45	1.75	11.70	16.25	47.20	27.54	27.44	6.14	6.04
6/16/19	12.30	22.30	2.00	9.80	16.50	40.43	25.47	27.31	5.95	5.89
6/17/19	4.71	12.90	2.00	8.30	9.38	39.18	24.13	26.09	6.05	6.09
6/18/19	2.89	16.45	2.05	11.30	6.70	37.03	24.67	27.87	6.17	6.09
6/19/19	1.93	12.95	2.05	12.95	5.79	38.88	24.51	27.17	6.24	6.12
6/20/19	3.56	17.52	1.40	14.25	10.12	47.30	24.49	27.56	6.33	6.09
6/21/19	8.36	19.45	1.85	15.00	14.35	47.58	24.95	27.61	6.17	6.04
6/22/19	13.72	19.68	2.15	9.95	18.85	41.55	25.06	27.38	5.94	5.91
6/23/19	7.66	18.23	1.65	7.50	19.20	36.15	23.64	26.74	5.94	5.94
6/24/19	2.52	16.67	2.05	12.15	7.50	43.90	22.55	25.22	6.13	6.12
6/25/19	2.06	12.38	1.90	9.85	8.93	41.70	24.96	28.03	6.19	6.04
6/26/19	0.64	9.60	1.95	9.35	6.06	32.90	25.24	28.00	6.30	6.12
6/27/19	0.65	11.97	2.25	12.35	4.20	32.68	26.25	29.25	6.22	6.11
6/28/19	3.33	12.56	2.20	10.25	8.71	43.55	25.83	28.42	6.15	6.11
6/29/19	0.62	9.00	2.60	10.85	5.90	34.98	23.72	29.71	6.00	5.95
6/30/19	0.66	4.51	2.65	9.00	6.66	42.08	25.58	26.73	6.10	6.02
7/1/19	2.10	5.07	3.55	7.70	12.05	48.18	23.90	28.7	6.09	6.15
7/2/19	0.68	6.08	2.55	10.15	4.67	26.80	26.2	29.38	6.20	6.10
7/3/19	0.69	8.39	3.00	9.95	3.98	26.30	27.51	28.72	6.22	6.08
7/4/19	0.60	9.18	2.75	6.90	5.24	23.80	23.04	26.38	6.11	5.99
7/5/19	0.69	11.60	3.30	10.15	5.70	34.20	23.10	26.37	6.16	6.05
7/6/19	1.99	10.60	3.20	6.80	7.06	23.90	25.16	27.12	6.12	6.00
7/7/19	0.73	7.02	2.55	6.70	5.38	21.80	25.80	26.87	6.12	6.02
7/8/19	0.62	5.41	3.00	10.55	6.89	28.80	24.46	29.6	6.11	6.07
7/9/19	0.78	7.14	2.70	11.65	7.83	30.90	28.4	30.64	6.21	6.21
7/10/19	0.70	6.42	2.75	9.35	8.79	28.35	25.03	29.8	6.14	6.09
7/11/19	0.70	10.30	2.60	10.55	5.09	24.90	27.9	29.58	6.11	6.08
7/12/19	0.61	9.62	3.30	9.24	2.26	29.10	27.80	30.59	6.02	6.08
7/13/19	2.14	8.33	2.75	9.65	6.70	25.90	27.61	28.03	5.96	5.96
7/14/19	1.53	6.27	2.50	9.70	6.41	25.70	24.95	28.16	5.99	5.99
7/15/19	0.67	6.61	2.65	7.75	5.51	25.50	26.35	29.01	6.01	6.14
7/16/19	0.67	8.93	2.80	11.90	6.15	31.40	28.99	31.05	6.10	6.04
7/17/19	0.74	7.03	3.40	12.10	6.53	30.30	30.27	31.52	6.12	6.08
7/18/19	0.58	10.30	3.10	11.70	4.37	27.40	29.21	32.27	6.07	5.98
7/19/19	0.66	9.15	3.25	11.90	4.49	28.00	29.50	32.01	6.16	6.02
7/20/19	0.76	8.94	2.80	9.65	5.16	18.77	24.98	29.66	6.00	5.99

Table 11 (cont'd)

Date	NO3-N (mg/L)		NH3-N (mg/L)		TN (mg/L)		TP-P (mg/L)		pH	
	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest
7/21/19	0.75	9.25	2.90	9.00	4.41	21.40	27.05	28.62	5.97	6.00
7/22/19	0.62	7.03	3.05	8.95	2.98	18.47	26.74	28.52	6.03	6.06
7/23/19	0.58	11.30	2.35	10.45	4.14	29.00	26.15	28.99	6.19	5.93
7/24/19	0.60	10.60	2.75	10.15	4.03	27.95	27.54	28.78	6.13	5.94
7/25/19	0.57	9.66	3.10	13.05	3.50	23.90	28.01	30.61	5.97	5.91
7/26/19	0.56	9.99	2.70	11.75	3.74	29.25	26.74	29.71	6.14	6.02
7/27/19	2.66	16.15	2.95	10.50	6.52	24.40	27.35	30.25	6.43	6.37
7/28/19	10.27	18.90	3.05	11.55	12.35	29.30	29.89	32.34	5.69	5.53
7/29/19	9.66	13.80	3.00	2.85	13.95	16.28	32.17	34.36	5.57	5.36
7/30/19	0.56	0.54	3.10	3.20	5.23	4.54	31.95	34.62	5.94	.
7/31/19	0.57	11.55	2.95	8.45	4.85	28.65	32.93	34.30	5.95	5.50
8/1/19	0.59	12.60	3.15	10.90	5.33	28.70	31.75	34.56	5.79	5.58
8/2/19	0.64	15.60	3.15	10.80	4.71	31.80	32.04	33.81	5.91	5.67
8/3/19	2.30	6.62	3.00	8.35	6.83	23.44	29.48	23.07	6.05	6.27
8/4/19	3.52	8.94	2.85	13.00	7.68	27.98	23.59	24.20	5.69	5.79
8/5/19	2.51	8.35	3.25	9.60	7.21	25.94	21.26	25.68	5.97	6.01
8/6/19	2.87	16.90	3.35	11.40	7.24	33.65	22.40	24.59	5.92	5.77
8/7/19	5.08	16.60	3.20	9.45	8.21	29.30	20.51	21.06	5.80	5.61
8/8/19	3.98	15.80	3.05	10.65	7.77	29.35	18.75	19.35	5.88	5.80
8/9/19	7.19	18.30	3.20	14.15	5.04	31.85	18.03	19.45	5.88	5.89
8/10/19	5.36	17.50	2.95	8.05	11.05	33.80	16.09	16.54	5.88	5.59
8/11/19	1.70	17.90	2.95	9.25	12.30	36.20	14.54	18.45	5.77	5.63
8/12/19	7.77	22.00	3.05	11.25	15.30	41.35	17.05	18.73	5.71	5.69
8/13/19	7.96	17.90	4.10	10.35	15.80	44.50	18.67	20.23	5.81	5.58
8/14/19	5.83	19.70	3.10	9.40	18.09	40.45	19.15	20.04	5.73	5.56

Table 12: Operational data for freshwater process.

Date	NO3-N (mg/L)		NH3-N (mg/L)		TN (mg/L)		TP-P (mg/L)		pH	
	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest
8/31/19	0.11	9.85	2.90	9.15	1.99	22.05	3.04	7.15	6.79	6.72
9/1/19	0.13	14.05	3.15	14.00	1.79	31.80	4.18	8.09	6.81	6.79
9/2/19	3.76	20.90	3.15	18.35	6.84	46.25	5.35	10.39	6.69	6.74
9/3/19	10.65	20.20	10.65	20.20	14.55	38.90	5.79	8.09	6.72	6.76
9/4/19	11.50	20.75	2.95	15.80	14.89	42.90	5.42	7.86	6.73	6.75
9/5/19	11.35	20.65	3.30	15.45	16.20	48.65	5.01	7.60	6.78	6.84
9/6/19	11.50	21.30	3.05	16.45	15.26	38.20	4.98	9.41	6.67	6.70
9/7/19	11.25	21.80	3.35	14.00	15.34	40.10	6.10	8.99	6.56	6.51
9/8/19	16.70	23.10	3.25	13.95	17.84	43.85	6.13	9.32	6.50	6.56
9/9/19	20.60	24.20	3.25	14.75	24.70	50.90	6.89	8.99	6.50	6.55
9/10/19	21.70	24.70	3.30	14.95	30.50	50.80	6.84	8.63	6.52	6.47
9/11/19	23.65	27.75	3.45	15.55	30.60	52.65	6.15	8.88	6.63	6.73
9/12/19	25.20	29.10	3.15	17.15	27.10	47.50	6.51	10.07	6.59	6.64
9/13/19	28.35	29.05	3.35	15.70	30.15	50.85	7.06	8.71	6.64	6.77
9/14/19	26.75	32.50	3.85	32.50	39.90	59.45	6.85	9.28	6.47	6.62
9/15/19	28.60	29.25	4.05	15.55	42.55	53.40	6.85	9.15	6.64	6.73
9/16/19	26.75	37.35	3.75	14.20	37.55	64.55	6.17	10.57	6.60	6.63
9/17/19	34.60	32.55	3.30	16.55	45.40	58.50	8.00	9.30	6.54	6.66
9/18/19	36.00	34.20	3.25	16.75	46.20	60.05	8.40	9.43	6.49	6.69
9/19/19	32.70	32.70	3.25	12.35	40.20	54.55	7.90	10.51	6.42	6.54
9/20/19	33.80	31.90	-0.50	12.50	38.80	56.20	8.09	9.48	6.54	6.61
9/21/19	29.40	19.47	-0.15	6.55	35.05	48.95	7.60	6.89	6.71	6.78
9/22/19	11.42	21.20	0.05	8.95	13.30	38.95	5.30	7.18	6.58	6.48
9/23/19	14.80	25.90	-0.05	12.45	20.45	48.70	5.60	9.63	6.58	6.59
9/24/19	17.51	23.70	-0.05	13.05	25.60	46.60	6.49	8.65	6.65	6.71
9/25/19	15.47	23.90	0.05	13.30	18.40	40.35	5.79	8.27	6.67	6.70
9/26/19	13.51	21.80	-0.25	12.15	16.34	41.05	5.60	8.11	6.65	6.72
9/27/19	11.97	22.40	0.10	15.05	14.27	42.05	5.30	8.42	6.53	6.54
9/28/19	14.03	23.70	0.55	10.50	16.49	41.65	5.30	8.45	6.59	6.66
9/29/19	10.17	19.99	0.20	11.85	12.72	36.90	5.35	7.98	6.69	6.76
9/30/19	9.51	31.00	0.20	27.15	13.08	67.50	5.17	10.74	6.74	7.12
10/2/19	9.65	20.30	0.50	12.90	12.85	36.50	4.80	7.51	6.80	6.82
10/3/19	8.44	20.50	0.30	14.85	11.10	38.55	4.86	8.57	6.52	6.57
10/4/19	11.65	21.75	0.25	13.55	14.93	40.90	5.40	8.39	6.64	6.67
10/5/19	11.25	20.35	0.85	12.70	13.54	39.35	3.92	8.70	6.56	6.56
10/6/19	9.46	20.20	0.70	11.30	11.89	36.40	4.99	7.99	6.63	6.55
10/7/19	10.50	15.45	0.70	8.65	13.62	32.25	5.42	10.82	6.59	6.44
10/8/19	10.45	20.55	0.85	13.85	12.55	37.20	9.93	13.35	6.64	6.72
10/9/19	9.32	19.90	1.40	14.30	11.73	36.15	8.12	10.73	6.64	6.69

Table 12 (cont'd)

Date	NO3-N (mg/L)		NH3-N (mg/L)		TN (mg/L)		TP-P (mg/L)		pH	
	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest	pre-harvest	post-harvest
10/10/19	5.94	18.60	1.60	16.00	8.63	36.20	5.12	8.89	6.68	6.62
10/11/19	10.45	20.40	1.35	14.85	13.80	42.50	5.85	8.10	6.67	6.74
10/12/19	7.58	19.05	1.65	12.35	9.98	34.40	5.29	7.51	6.80	6.87
10/13/19	8.36	21.05	1.80	15.55	11.43	39.45	5.06	8.88	6.74	6.70
10/14/19	21.20	31.85	8.30	30.00	31.20	63.70	5.95	14.76	6.63	6.75
10/18/19	0.32	13.95	0.05	10.25	2.88	34.20	3.20	7.03	6.50	6.45
10/20/19	0.65	14.45	0.15	7.40	3.26	36.00	2.48	6.67	6.75	6.67
10/21/19	2.48	9.38	0.85	6.40	5.51	24.80	4.29	6.74	6.72	6.69
10/22/19	6.71	19.25	-0.05	13.70	8.62	33.05	5.34	8.39	6.70	6.74
10/23/19	10.50	21.30	0.10	13.15	12.35	41.15	5.38	8.26	6.64	6.70
10/24/19	13.35	21.80	0.30	13.70	14.65	43.80	5.55	8.71	6.63	6.70
10/25/19	15.15	23.00	0.30	13.50	17.50	46.95	5.74	8.39	6.67	6.76
10/26/19	15.40	25.15	0.25	11.45	13.68	43.80	5.40	8.19	6.67	6.74
10/27/19	18.55	25.90	0.25	11.95	13.25	43.20	5.51	8.26	6.71	6.76
10/28/19	20.55	22.55	0.15	12.60	14.07	45.00	5.97	6.66	6.79	6.69
10/29/19	21.50	26.25	0.60	13.35	19.15	50.10	6.25	7.98	6.58	6.71
10/30/19	20.30	25.70	0.45	12.20	22.99	43.95	5.76	7.70	6.66	6.72
10/31/19	20.10	27.85	0.25	15.15	22.40	47.20	5.59	9.29	6.41	6.46
11/1/19	24.75	26.15	0.45	13.25	28.50	50.65	6.10	8.83	6.63	7.06
11/2/19	23.60	26.40	1.55	13.60	26.60	47.10	5.96	9.77	6.60	6.67
11/3/19	18.95	24.60	1.35	15.00	22.10	45.10	6.41	9.53	6.67	6.70
11/4/19	22.25	30.15	1.35	12.50	27.45	44.70	6.75	10.25	6.61	6.82
11/5/19	26.50	27.55	1.30	13.80	33.90	49.65	6.69	8.48	6.53	6.60
11/6/19	24.00	27.05	1.60	14.10	31.20	42.45	6.20	8.71	6.67	6.86
11/7/19	25.55	28.20	1.50	14.75	31.85	46.70	7.60	8.83	6.57	6.69
11/8/19	26.20	32.65	1.45	14.70	29.95	46.45	6.23	8.99	6.51	6.59
11/9/19	27.40	27.70	1.95	12.10	29.20	40.10	6.25	8.08	6.51	6.65
11/10/19	23.15	26.65	1.90	14.15	25.25	44.35	5.97	9.25	6.59	6.70
11/11/19	24.65	30.35	1.90	11.15	26.55	41.95	7.06	9.76	6.59	6.72
11/12/19	27.55	30.10	1.85	15.00	31.15	48.25	7.31	9.01	6.57	6.69
11/13/19	26.70	28.75	1.90	13.35	30.00	45.35	6.48	8.53	6.72	6.79
11/14/19	25.10	29.15	2.00	14.55	28.70	47.30	6.62	9.07	6.71	6.82
11/15/19	24.25	27.00	2.15	14.55	33.95	49.10	6.23	9.07	6.76	6.87

Table 13: Illumina OTU classification key. *****

Key	Domain	Phylum	Class	Order	Family	Genus	Species
F1	Bacteria	Bacteria_[ ]	Bacteria_[ ]	Bacteria_[ ]	Bacteria_[ ]	Bacteria_[ ]	Bacteria_[ ]
F2	Bacteria	Actinobacteria	Actinobacteria_[ ]	Actinobacteria_[ ]	Actinobacteria_[ ]	Actinobacteria_[ ]	Actinobacteria_[ ]
F3	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetales_[ ]	Actinomycetales_[ ]	Actinomycetales_[ ]
F4	Bacteria	Bacteroidetes	Bacteroidetes_[ ]	Bacteroidetes_[ ]	Bacteroidetes_[ ]	Bacteroidetes_[ ]	Bacteroidetes_[ ]
F5	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cytophagales_[ ]	Cytophagales_[ ]	Cytophagales_[ ]
F6	Bacteria	Bacteroidetes	Cytophagia	Cytophagales	Cyclobacteriaceae	Cyclobacteriaceae_[ ]	Cyclobacteriaceae_[ ]
F7	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriales_[ ]	Flavobacteriales_[ ]	Flavobacteriales_[ ]
F8	Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Flavobacteriaceae	Flavobacteriaceae_[ ]	Flavobacteriaceae_[ ]
F9	Bacteria	Bacteroidetes	Sphingobacteriia	Sphingobacteriales	Sphingobacteriaceae	Sphingobacteriaceae_[ ]	Sphingobacteriaceae_[ ]
F10	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirae]_[ ]	[Saprospirae]_[ ]	[Saprospirae]_[ ]	[Saprospirae]_[ ]
F11	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	[Saprospirales]_[ ]	[Saprospirales]_[ ]	[Saprospirales]_[ ]
F12	Bacteria	Bacteroidetes	[Saprospirae]	[Saprospirales]	Chitinophagaceae	Chitinophagaceae_[ ]	Chitinophagaceae_[ ]
F13	Bacteria	Cyanobacteria	Cyanobacteria_[ ]	Cyanobacteria_[ ]	Cyanobacteria_[ ]	Cyanobacteria_[ ]	Cyanobacteria_[ ]
F14	Bacteria	Cyanobacteria	Chloroplast	Chlorophyta	Chlorophyta_[ ]	Chlorophyta_[ ]	Chlorophyta_[ ]
F15	Bacteria	Firmicutes	Firmicutes_[ ]	Firmicutes_[ ]	Firmicutes_[ ]	Firmicutes_[ ]	Firmicutes_[ ]
F16	Bacteria	Firmicutes	Bacilli	Bacilli_[ ]	Bacilli_[ ]	Bacilli_[ ]	Bacilli_[ ]
F17	Bacteria	Firmicutes	Bacilli	Bacillales	Bacillales_[ ]	Bacillales_[ ]	Bacillales_[ ]
F18	Bacteria	Firmicutes	Clostridia	Clostridiales	Ruminococcaceae	Bacteroides	cellulosolvens
F19	Bacteria	Planctomycetes	Planctomycetia	Gemmatales	Isosphaeraceae	Isosphaeraceae_[ ]	Isosphaeraceae_[ ]
F20	Bacteria	Proteobacteria	Proteobacteria_[ ]	Proteobacteria_[ ]	Proteobacteria_[ ]	Proteobacteria_[ ]	Proteobacteria_[ ]
F21	Bacteria	Proteobacteria	Alphaproteobacteria	Alphaproteobacteria_[ ]	Alphaproteobacteria_[ ]	Alphaproteobacteria_[ ]	Alphaproteobacteria_[ ]
F22	Bacteria	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	Brevundimonas	Brevundimonas_[ ]
F23	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Rhizobiales_[ ]	Rhizobiales_[ ]	Rhizobiales_[ ]
F24	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Bradyrhizobiaceae	Bradyrhizobiaceae_[ ]	Bradyrhizobiaceae_[ ]
F25	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylobacteriaceae	Methylobacteriaceae_[ ]	Methylobacteriaceae_[ ]
F26	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	Phyllobacteriaceae_[ ]	Phyllobacteriaceae_[ ]
F27	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacterales_[ ]	Rhodobacterales_[ ]	Rhodobacterales_[ ]
F28	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodobacterales	Rhodobacteraceae	Rhodobacteraceae_[ ]	Rhodobacteraceae_[ ]
F29	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillales_[ ]	Rhodospirillales_[ ]	Rhodospirillales_[ ]
F30	Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Rhodospirillaceae	Rhodospirillaceae_[ ]	Rhodospirillaceae_[ ]
F31	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadales_[ ]	Sphingomonadales_[ ]	Sphingomonadales_[ ]
F32	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Erythrobacteraceae	Erythrobacteraceae_[ ]	Erythrobacteraceae_[ ]
F33	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonadaceae_[ ]	Sphingomonadaceae_[ ]
F34	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	Novosphingobium_[ ]
F35	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Novosphingobium	nitrogenifigens
F36	Bacteria	Proteobacteria	Alphaproteobacteria	Sphingomonadales	Sphingomonadaceae	Sphingomonas	Sphingomonas_[ ]
F37	Bacteria	Proteobacteria	Betaproteobacteria	Betaproteobacteria_[ ]	Betaproteobacteria_[ ]	Betaproteobacteria_[ ]	Betaproteobacteria_[ ]
F38	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiales_[ ]	Burkholderiales_[ ]	Burkholderiales_[ ]
F39	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae	Comamonadaceae_[ ]	Comamonadaceae_[ ]
F40	Bacteria	Proteobacteria	Gammaproteobacteria	Gammaproteobacteria_[ ]	Gammaproteobacteria_[ ]	Gammaproteobacteria_[ ]	Gammaproteobacteria_[ ]
F41	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Xanthomonadaceae_[ ]	Xanthomonadaceae_[ ]
F42	Bacteria	Verrucomicrobia	Verrucomicrobia_[ ]	Verrucomicrobia_[ ]	Verrucomicrobia_[ ]	Verrucomicrobia_[ ]	Verrucomicrobia_[ ]
F43	Bacteria	Verrucomicrobia	Verrucomicrobiae	Verrucomicrobiales	Verrucomicrobiaceae	Verrucomicrobiaceae_[ ]	Verrucomicrobiaceae_[ ]

*: _ [ ] means the OTU is unclassified.

Table 14: Illumina sequencing abundance under recirculation conditions. *

Key	Recirculation Sample Date														
	5/7	5/14	5/21	5/28	6/4	6/11	6/18	6/25	7/2	7/9	7/16	7/23	7/30	8/6	8/13
F1	144	122	167	115	102	31	30	56	71	77	68	73	33	57	75
F2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F3	0	0	0	0	0	0	2	2	2	0	1	3	2	6	3
F4	2	2	40	79	21	26	15	8	50	89	41	57	59	131	143
F5	14	24	51	111	112	28	40	42	48	60	67	78	54	73	227
F6	0	0	0	0	0	0	0	0	0	0	0	0	0	2	0
F7	3	0	0	0	0	0	0	0	0	0	0	0	4	0	2
F8	0	0	6	0	0	0	0	0	0	0	0	0	3	0	0
F9	0	0	4	9	5	2	4	6	24	13	17	25	8	9	29
F10	0	0	0	0	1	0	0	0	0	0	0	2	0	0	0
F11	2	0	31	41	1	0	0	0	0	2	0	0	0	3	6
F12	38	59	260	458	403	172	221	442	620	1159	527	560	459	528	415
F13	10422	9876	20939	15833	16157	16671	15235	11089	12175	10960	12215	13740	16278	14152	12606
F14	3	6	4	11	10	4	2	8	4	5	2	6	4	6	0
F15	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F16	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
F17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F18	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F19	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F20	1228	2017	2897	1900	1797	615	676	1260	1327	1312	931	1030	862	2388	832
F21	58	43	68	81	60	42	69	164	82	119	108	124	135	67	74
F22	0	2	10	9	9	7	4	10	12	11	8	9	34	31	27
F23	373	463	823	629	450	210	157	219	160	159	174	201	159	127	99
F24	110	37	60	74	49	33	124	211	192	77	43	46	29	26	66
F25	0	0	0	0	0	0	0	0	4	0	0	0	0	0	5
F26	12	11	7	4	2	6	12	35	17	13	11	15	28	8	11
F27	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F28	0	0	0	0	0	0	0	0	2	0	0	0	0	0	0
F29	0	2	5	6	13	4	6	4	3	4	7	3	74	17	7
F30	7	17	11	9	8	7	2	8	12	44	23	57	43	37	36
F31	0	2	3	2	0	0	0	0	0	0	0	0	0	0	2
F32	0	0	0	0	0	0	4	0	0	0	0	0	2	8	15
F33	0	0	7	16	7	5	4	10	10	4	15	8	0	0	7
F34	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F35	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F36	0	0	0	0	0	0	0	0	0	3	2	0	3	0	9
F37	13	78	135	430	207	93	58	84	67	181	188	141	107	86	132
F38	1	25	37	49	19	21	9	10	28	22	14	14	39	21	34
F39	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
F40	0	0	4	0	4	1	5	4	2	5	0	6	0	9	14
F41	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
F42	0	0	0	0	0	2	0	0	7	3	0	0	0	0	0
F43	0	0	0	0	2	0	2	0	0	0	3	4	0	0	0

*: See Table 13 for OTU identification key.

Table 15: Illumina sequencing abundance under freshwater conditions. *

Key	Freshwater Sample Date								
	9/3	9/10	9/17	9/24	10/8	10/22	10/29	11/5	11/12
F1	94	136	145	150	301	982	892	434	194
F2	0	0	0	0	0	0	2	0	0
F3	7	4	5	5	5	0	1	2	2
F4	18	58	40	33	117	98	152	147	71
F5	122	145	69	68	51	124	72	86	56
F6	0	0	0	0	0	0	0	0	0
F7	0	0	0	0	0	0	0	0	0
F8	19	14	21	7	10	8	23	31	4
F9	9	14	21	17	24	22	15	9	0
F10	0	0	0	9	6	24	29	57	20
F11	4	27	17	26	25	0	64	118	46
F12	499	604	534	580	134	372	342	216	113
F13	15480	14262	11458	13744	14727	10098	15774	17288	6313
F14	0	0	0	0	0	0	0	0	0
F15	0	0	0	2	0	0	0	0	0
F16	0	0	0	0	0	0	0	0	0
F17	0	0	0	2	3	0	0	0	0
F18	3	2	0	0	3	0	4	0	0
F19	2	0	0	3	0	0	0	0	0
F20	226	141	557	244	68	47	66	137	164
F21	99	171	120	150	89	76	80	64	48
F22	55	111	85	21	5	4	8	38	23
F23	243	280	371	336	228	164	271	210	306
F24	18	26	14	15	39	11	14	11	5
F25	0	0	0	0	0	0	0	0	0
F26	36	26	18	16	33	25	24	9	17
F27	0	3	2	0	0	1	0	3	0
F28	0	0	0	0	0	0	0	0	0
F29	48	29	60	118	14	14	15	22	0
F30	75	92	102	117	31	25	42	18	13
F31	1	5	13	19	53	42	78	62	69
F32	20	6	5	12	2	0	1	0	0
F33	42	68	31	20	47	25	27	42	22
F34	0	0	2	0	0	0	0	0	0
F35	3	4	0	0	0	0	0	0	0
F36	13	6	6	2	0	0	5	2	0
F37	1350	576	239	113	54	51	31	27	0
F38	86	166	627	168	199	473	557	423	307
F39	12	0	0	0	0	0	0	0	0
F40	3	2	0	9	2	4	14	7	0
F41	25	26	27	28	30	32	33	34	35
F42	0	21	3	1	0	3	0	0	0
F43	0	23	24	18	2	20	28	29	8

*: See Table 13 for OTU identification key.

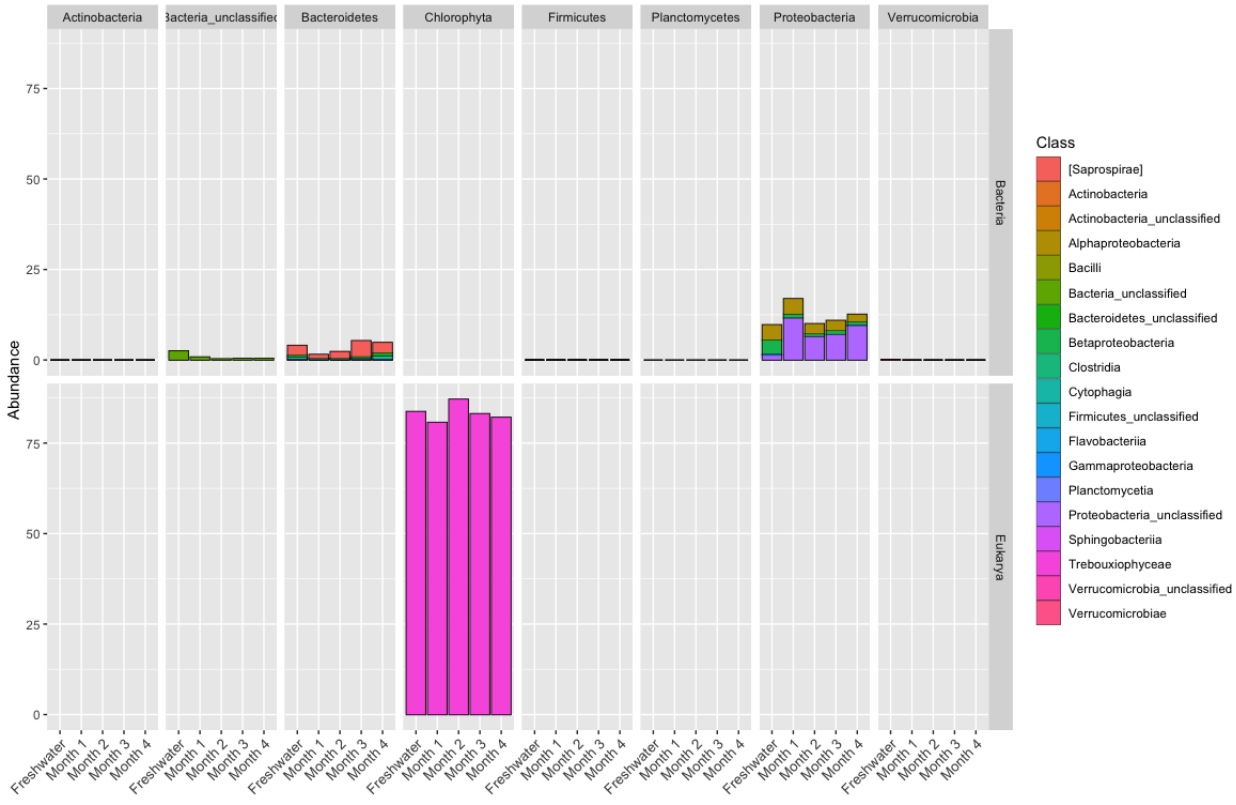

Figure 8: Overall microbial community for freshwater and recirculation samples.

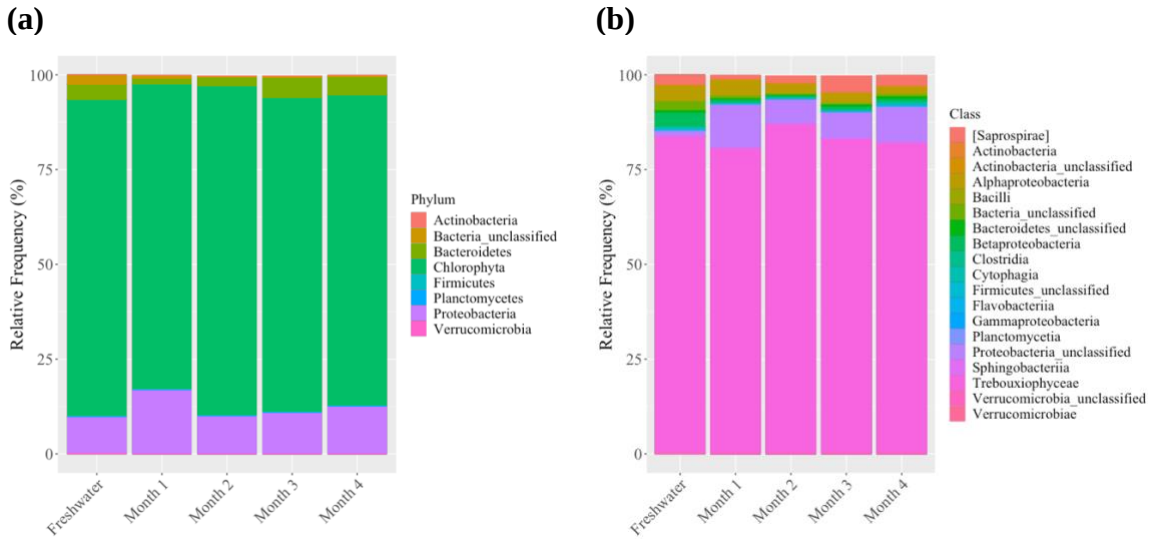

Figure 9: Microbial community abundance **a.)** at phylum level. **b.)** at class level.

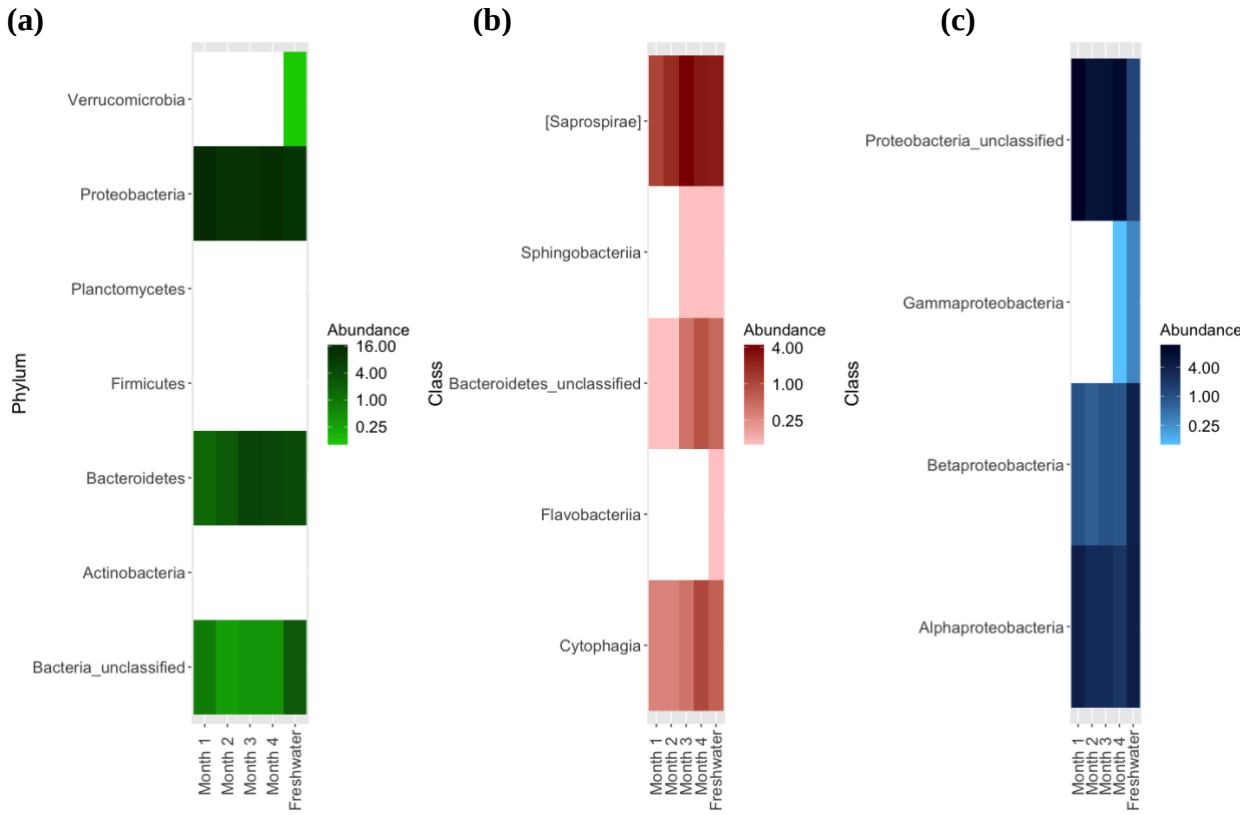

Figure 10: Heatmaps of microbial community abundance **a.)** at bacterial phylum **b.)** within Bacteroidetes phylum and **c.)** within Proteobacteria phylum.

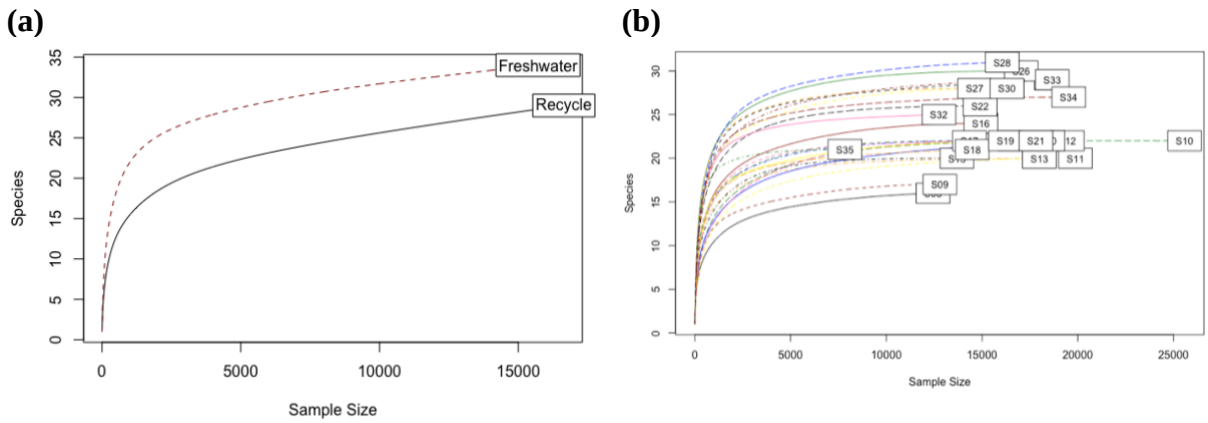

Figure 11: Rarefaction curves **a.)** Recirculation and freshwater conditions and **b.)** Individual recirculation (S8-22) and freshwater (S23-35) samples.

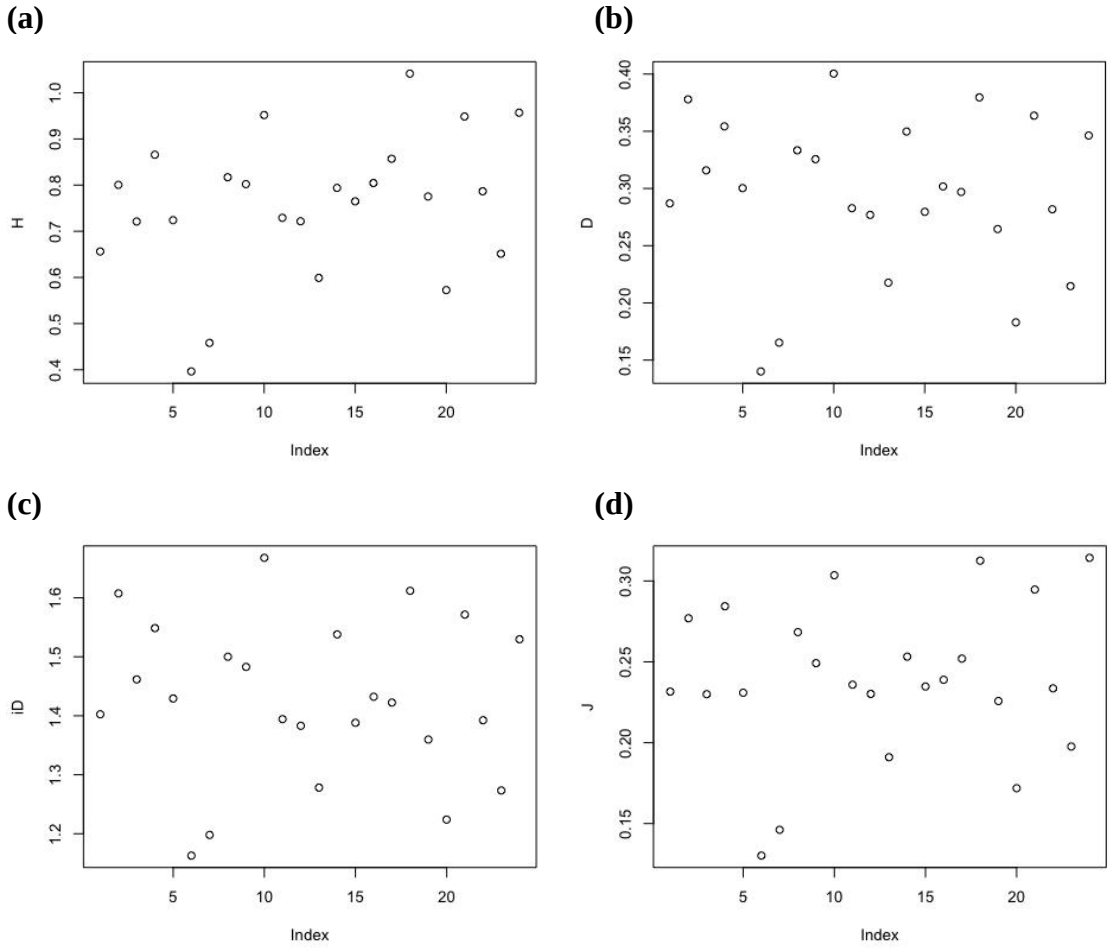

Figure 12: Scatter plots for community indices **a.)** Shannon's Index **b.)** Simpson's Index **c.)** Inverse Simpson's Index **d.)** Pielou's Index

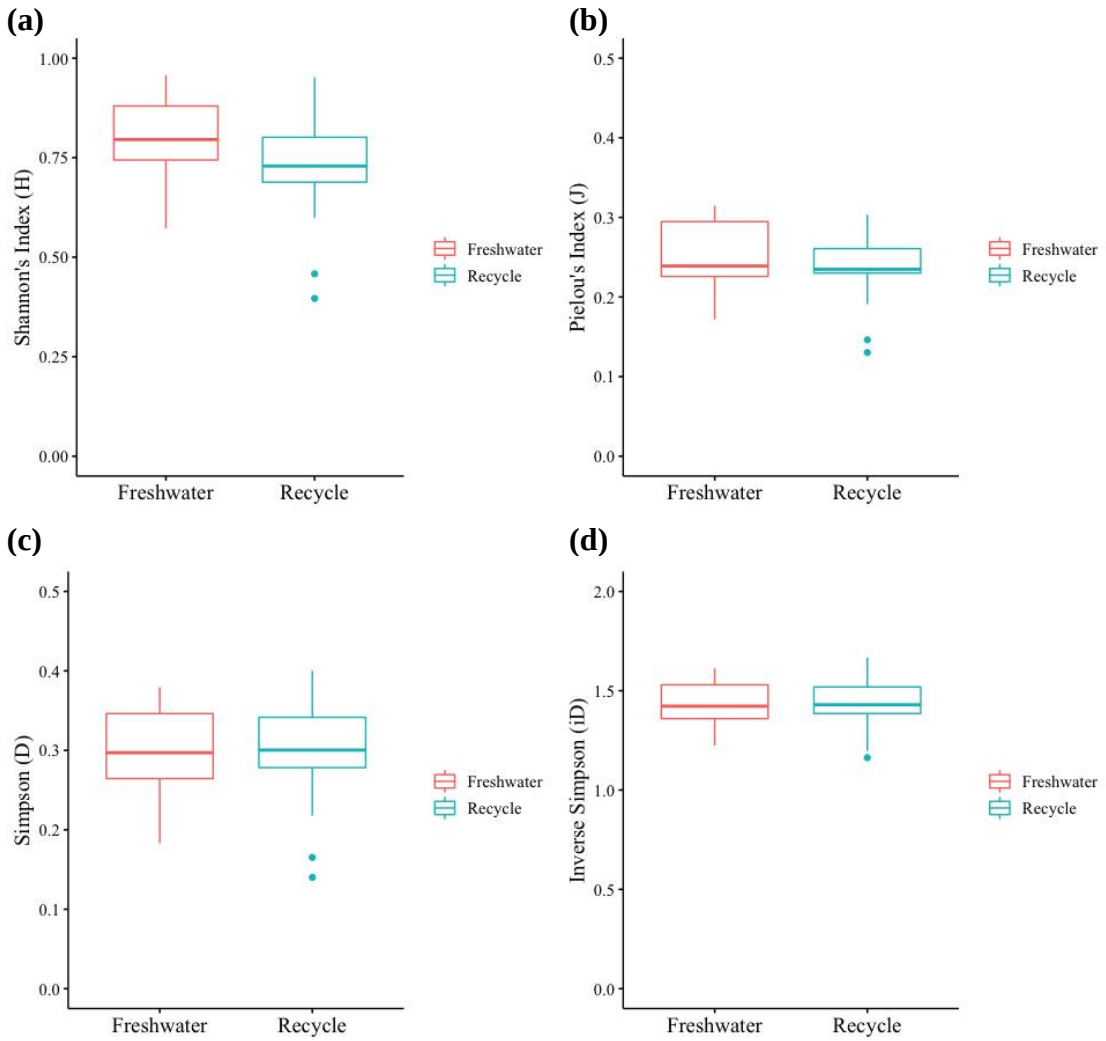

Figure 13: Boxplots for microbial community indices comparing recirculation and freshwater conditions **a.)** Shannon's Index **b.)** Pielou's Index **c.)** Simpson's Index **d.)** Inverse Simpson's Index

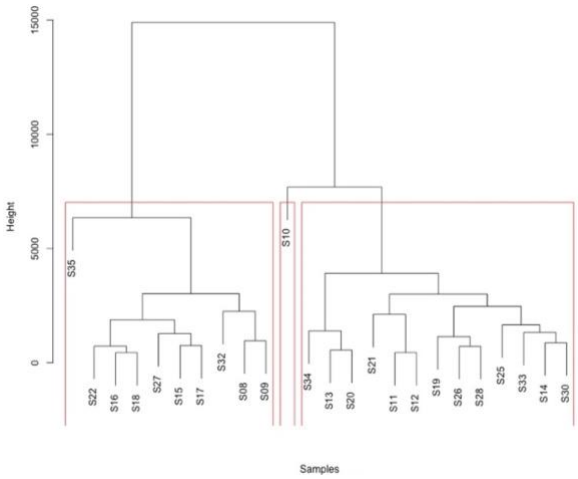

Figure 14: Dendrogram of individual recirculation (S8-22) and freshwater (S23-35) samples.

Table 16: Process compounds, mass flow rate, temperature, chemical exergy rate, physical exergy rate, and total exergy rate of individual compounds for microalgae cultivation under recirculation conditions. *

Process Step	Compound	Mass flow rate (kg/d)	Temp. (K)	Specific chemical exergy (kJ/kg)	Chemical exergy rate (W)	Specific enthalpy (kJ/kg)	Reference specific enthalpy (kJ/kg)	Reference state Temp. (K)	Specific Entropy (kJ/kg·K)	Reference specific Entropy (kJ/kg·K)	Physical exergy rate (W)	TOTAL EXERGY RATE (W)
Inputs	Water	4.20	298.15	50	2.43	-	-	-	-	-	-	**2.43**
	NH4NO3	0.08	298.15	3685	3.29	-	-	-	-	-	-	**3.29**
	KH2PO4	0.010	298.15	368	0.04	-	-	-	-	-	-	**0.04**
	K2HPO4	0.021	298.15	453	0.11	-	-	-	-	-	-	**0.11**
	MgSO4*7H20	0.013	298.15	353	0.05	-	-	-	-	-	-	**0.05**
	CaCl2	0.005	298.15	946	0.05	-	-	-	-	-	-	**0.05**
	FeCl3	0.015	298.15	1406	0.24	-	-	-	-	-	-	**0.24**
	DOW (antifoaming)	0.060	298.15	2.353	0.00	-	-	-	-	-	-	**0.00**
	CO2	15.8	298.15	452	82.6	-	-	-	-	-	-	**82.6**
	Solar (irradiation/electricity)	-	-	-	-	-	-	-	-	-	8958	**8958**
	Electricity for flue gas pump	-	-	-	-	-	-	-	-	-	146	**146**
	Electricity for water refill pump	-	-	-	-	-	-	-	-	-	2.8	**2.8**
	Electricity for harvesting pump	-	-	-	-	-	-	-	-	-	2.8	**2.8**
	Electricity for centrifuge	-	-	-	-	-	-	-	-	-	10.4	**10.4**
	Electricity for storage tank pump	-	-	-	-	-	-	-	-	-	1.0	**1.0**
	Electricity for centrifuge pump	-	-	-	-	-	-	-	-	-	1.0	**1.0**
Outputs	Water	3.00	298.15	50	1.74	-	-	-	-	-	-	**1.74**
	NH4NO3	0.0005	298.15	3685	0.02	-	-	-	-	-	-	**0.02**
	KH2PO4	0.0001	298.15	368	0.00	-	-	-	-	-	-	**0.00**
	K2HPO4	0.0001	298.15	453	0.00	-	-	-	-	-	-	**0.00**
	MgSO4*7H20	0.0015	298.15	353	0.01	-	-	-	-	-	-	**0.01**
	CaCl2	0.0009	298.15	946	0.01	-	-	-	-	-	-	**0.01**
	FeCl3	0.0004	298.15	1406	0.01	-	-	-	-	-	-	**0.01**
	DOW (antifoaming)	0.060	298.15	2.353	0.00	-	-	-	-	-	-	**0.00**
	CO2	15.4	298.15	452	80.5	-	-	-	-	-	-	**80.5**
	Biomass	0.23	298.15	21732	57.9	-	-	-	-	-	-	**57.9**
	Water in Biomass	1.2	298.15	50	0.69	-	-	-	-	-	-	**0.69**

*: "-" means that the value was not considered in the exergy efficiency calculation.

Table 17: Process compounds, mass flow rate, temperature, chemical exergy rate, physical exergy rate, and total exergy rate of individual compounds for microalgae cultivation under freshwater conditions. *

Process Step	Compound	Mass flow rate (kg/d)	Temp. (K)	Specific chemical exergy (kJ/kg)	Chemical exergy rate (W)	Specific enthalpy (kJ/kg)	Reference specific enthalpy (kJ/kg)	Reference state Temp. (K)	Specific Entropy (kJ/kg·K)	Reference specific Entropy (kJ/kg·K)	Physical exergy rate (W)	TOTAL EXERGY RATE (W)
Inputs	Water	500.00	298.15	50	289.4	-	-	-	-	-	-	**289.4**
	NH4NO3	0.10	298.15	3685	4.39	-	-	-	-	-	-	**4.39**
	KH2PO4	0.014	298.15	368	0.06	-	-	-	-	-	-	**0.06**
	K2HPO4	0.027	298.15	453	0.14	-	-	-	-	-	-	**0.14**
	MgSO4*7H20	0.014	298.15	353	0.06	-	-	-	-	-	-	**0.06**
	CaCl2	0.005	298.15	946	0.06	-	-	-	-	-	-	**0.06**
	FeCl3	0.024	298.15	1406	0.40	-	-	-	-	-	-	**0.40**
	DOW (antifoaming)	0.060	298.15	2.353	0.00	-	-	-	-	-	-	**0.00**
	CO2	15.8	298.15	452	82.6	-	-	-	-	-	-	**82.6**
	Solar (irradiation/electricity)	-	-	-	-	-	-	-	-	-	8958	**8958**
	Electricity for flue gas pump	-	-	-	-	-	-	-	-	-	146	**146**
	Electricity for water refill pump	-	-	-	-	-	-	-	-	-	2.8	**2.8**
	Electricity for harvesting pump	-	-	-	-	-	-	-	-	-	2.8	**2.8**
	Electricity for centrifuge	-	-	-	-	-	-	-	-	-	10.4	**10.4**
	Electricity for storage tank pump	-	-	-	-	-	-	-	-	-	1.0	**1.0**
	Electricity for centrifuge pump	-	-	-	-	-	-	-	-	-	1.0	**1.0**
Outputs	Water	498.9	298.15	50	288.7	-	-	-	-	-	-	**288.7**
	NH4NO3	0.03	298.15	3685	1.34	-	-	-	-	-	-	**1.34**
	KH2PO4	0.01	298.15	368	0.02	-	-	-	-	-	-	**0.02**
	K2HPO4	0.01	298.15	453	0.05	-	-	-	-	-	-	**0.05**
	MgSO4*7H20	0.0176	298.15	353	0.07	-	-	-	-	-	-	**0.07**
	CaCl2	0.00	298.15	946	0.00	-	-	-	-	-	-	**0.00**
	FeCl3	0.0050	298.15	1406	0.08	-	-	-	-	-	-	**0.08**
	DOW (antifoaming)	0.0600	298.15	2.353	0.00	-	-	-	-	-	-	**0.00**
	CO2	15.4	298.15	452	80.5	-	-	-	-	-	-	**80.5**
	Biomass	0.22	298.15	21922	55.8	-	-	-	-	-	-	**55.8**
	Water in Biomass	0.88	298.15	50	0.51	-	-	-	-	-	-	**0.51**

*: "-" means that the value was not considered in the exergy efficiency calculation.

APPENDIX B: R CODE FOR PLOTTING AND ANALYSIS

Non-metric multidimensional scaling (condition)

```
## NMDS analysis for Microalgae Cultivation (Lumped)
## Wei Liao, March 10, 2020
## Carly Daiek, March 20, 2020 update

# Loading Library and Tables ----------------
# Load "vegan" and "MASS" libraries in R

library(vegan)
library(MASS)

# Load data files, make sure the data files are saved as macintosh .csv and follow the sample format

species <- read.csv(file.choose(), head = TRUE, row.names = 1)
env <- read.csv(file.choose(), head = TRUE, row.names = 1)
performance <- read.csv(file.choose(), head= TRUE, row.names = 1)

#Statistical analysis ----------------------
species.mds <- metaMDS(species, trace=FALSE)
ef.sp <- envfit(species.mds, env, permu=999)
perf.sp <- envfit(species.mds, performance, permu=999)
species.mds
ef.sp
perf.sp

# Plotting NMDS chart -------------------
plot(species.mds, display="sites", type="points")
with(env, ordiellipse(species.mds, Recycle, kind= "se", draw="polygon", col="green", alpha=50,
label=TRUE,border=NA, conf=0.95))

# With significant performance data
ef.perf <- envfit(species.mds, performance[,c(2,5)], permu=999)
plot(ef.perf, col="red", cex=0.8)

# With significant microbial community data
ef.perf <- envfit(species.mds, performance[, c(9,16,17,20)], permu=999)
plot(ef.perf, col="blue", cex=0.8)
```

Non-metric multidimensional scaling (recirculation dynamics)

```
## NMDS analysis for Microalgae Cultivation (Dynamic)
## Wei Liao, March 10, 2020
## Carly Daiek, March 20, 2020 update

# Loading Library and Tables ----------------
# Load "vegan" and "MASS" libraries in R

library(vegan)
library(MASS)
```

```
# Load data files, make sure the data files are saved as macintosh .csv and follow the sample format

species <- read.csv(file.choose(), head = TRUE, row.names = 1)
env <- read.csv(file.choose(), head = TRUE, row.names = 1)
performance <- read.csv(file.choose(), head= TRUE, row.names = 1)

# Statistical analysis ----------
species.mds <- metaMDS(species, trace=FALSE)
ef.sp <- envfit(species.mds, env, permu=999)
perf.sp <- envfit(species.mds, performance, permu=999)
species.mds
ef.sp
perf.sp

# Plotting NMDS chart ----------
plot(species.mds, display="sites", type="points", xlim=c(-1,1))
with(env, ordiellipse(species.mds, Month, kind= "se", draw="polygon", col="darkred", alpha=50,
label=TRUE,border=NA, conf=0.95))

# With significant performance data
ef.perf <- envfit(species.mds, performance[, c(1)], permu=999)
plot(ef.perf, col="red", cex=0.8)

# With significant microbial community data
ef.perf <- envfit(species.mds, performance[, c(9,15,20)], permu=999)
plot(ef.perf, col="blue", cex=0.8)
```

Biomass statistics and plotting

```
## Algal Cultivation: Dynamic analysis of Recirculation vs. Freshwater
## BIOMASS
## Wei Liao
## Carly Daiek, February 2020 update

# Loading Library and Tables ----------
library (MASS)
library(ggplot2)
library(grid)
library(gridExtra)
library(ggpubr)
library(plyr)
library(RVAideMemoire)
library(DescTools)
library(PMCMRplus)
library(inferr)

# Installing the font package ----------
library(extrafont)
font_import() #It may take a few minutes to import.
loadfonts(device="win")

# PROGRAM TO PLOT BAR CHART WITH STANDARD DEVIATION ----------
#++++++++++++++++++++++++
# Function to calculate the mean and the standard deviation
```

```
# for each group
#+++++++++++++++++++++++++
# data : a data frame
# varname : the name of a column containing the variable
#to be summarized
# groupnames : vector of column names to be used as
# grouping variables
data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      sd = sd(x[[col]], na.rm=TRUE))
  }
  data_sum<-ddply(data, groupnames, .fun=summary_func,
                  varname)
  data_sum <- rename(data_sum, c("mean" = varname))
  return(data_sum)
}

# ANALYSIS----------------------------------------------------------------

## the .txt file needs to be saved as the type of "Tab delimited".
##choose the metadata_biomass_dynamic, should be .txt
con <-file.choose(new = FALSE)
mastermetadata <- read.table(con, header = T, row.names = 1,na.strings=c("","NA"," ","  "))
metadata <- mastermetadata
metadata$month <- factor(metadata$month)

# STEP 1 Assumption 1: normality of each group
#Biomass Productivity
byf.hist(biomass_productivity~month, density=TRUE, sep=FALSE, data = metadata)
byf.shapiro(biomass_productivity~month, data = metadata) #Shapiro-Wilk Test
#All months are normal

# STEP 2 Checking assumption 2: variance
#Biomass Productivity
infer_levene_test(data=metadata, biomass_productivity, group_var = "month") #Levene F test
#All months have equal variance

## STEP 3a Run a regular ANOVA if BOTH assumptions met
ANOVA_BiomassProductivty <- aov(biomass_productivity~month, data=metadata)
summary (ANOVA_BiomassProductivty) #Some or all means signficantly different if P < 0.05
# residual plots to spot unequal variance, lack of normality of residuals, & outliers
with (metadata, par (mfrow=c(2,2)))
plot (aov(biomass_productivity~month, data=metadata))
#At least one month is significantly different

## Step 3b IFF your ANOVA was significant, run a post hoc
## Tukey's HSD for ALL pairwise
TukeyHSD(ANOVA_BiomassProductivty)

# PLOTTING ----------------------------------------------------------------
## ORGANIZE DATA FOR PLOTTING

# Biomass productivity
BiomassProductivity <- data_summary(metadata, varname="biomass_productivity",
```

```
                        groupnames=c("month"))
BiomassProductivity$month=as.factor(BiomassProductivity$month)

## PLOTTING
#Grouped bar plot (Nutrient reduction by Month)
values <- BiomassProductivity[,2]
sd <- BiomassProductivity[,3]
condition <- c("Freshwater", "1", "2", "3", "4")
df <- data.frame(values, condition)

box_1 <- ggplot(df, aes(x=factor(condition), y=values)) +
  geom_bar(stat="identity", position=position_dodge(), colour="black")+
  geom_errorbar(aes(ymin=values-sd, ymax=values+sd), width=0.2, position=position_dodge(0.9))+
  ylab("Biomass Productivity (g/L/day)") + ylim(0, 0.4)+ labs(title = "", subtitle=NULL) +
  xlab ("Month") +
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=16, family="Times New Roman"),
        axis.text.y=element_text(size=16, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.title=element_blank(),
        legend.text = element_text(size = 14, family="Times New Roman"),
        legend.position = "top")
box_1
```

Operational statistics and plotting

```
## Algal Cultivation: Dynamic analysis of Recirculation vs. Freshwater
## Operational
## Wei Liao
## Carly Daiek, February 2020 update

# Loading Library and Tables ----------------------------------------------
library(MASS)
library(ggplot2)
library(grid)
library(gridExtra)
library(ggpubr)
library(plyr)
library(RVAideMemoire)
library(DescTools)
library(PMCMRplus)
library(inferr)

# Installing the font package ---------------------------------------------
library(extrafont)
font_import() #It may take a few minutes to import.
loadfonts(device="win")

# ANALYSIS----------------------------------------------------------------
## the .txt file needs to be saved as the type of "Tab delimited".
##choose the metadata_operational_dynamic, should be .txt
con <-file.choose(new = FALSE)
mastermetadata <- read.table(con, header = T, row.names = 1,na.strings=c("","NA"," ","  "))
metadata <- mastermetadata
```

```
metadata$month <- factor(metadata$month)

## STEP 1 Assumption 1: normality
#TN CONSUMED: all months normal
byf.hist(TN_reduction~month, density=TRUE, sep=FALSE, data = metadata)
byf.shapiro(TN_reduction~month, data = metadata) #Shapiro-Wilk Test
#TP CONSUMED: 1st month non-normal, all other months normal
byf.hist(TP_reduction~month, density=TRUE, sep=FALSE, data = metadata)
byf.shapiro(TP_reduction~month, data = metadata)
#NH3 CONSUMED: Freshwater non-normal, all other months normal
byf.hist(NH3_N_reduction~month, density=TRUE, sep=FALSE, data = metadata)
byf.shapiro(NH3_N_reduction~month, data = metadata)
#NO3 CONSUMED: Freshwater non-normal, all other months normal
byf.hist(NO3_N_reduction~month, density=TRUE, sep=FALSE, data = metadata)
byf.shapiro(NO3_N_reduction~month, data = metadata)

## STEP 2 Assumption 2:Variance
#TN CONSUMED: unequal variance
infer_levene_test(data=metadata, TN_reduction, group_var = "month") #Levene F test
#TP CONSUMED: unequal variance
infer_levene_test(data=metadata, TP_reduction, group_var = "month")
#NH3 CONSUMED: equal variance
infer_levene_test(data=metadata, NH3_N_reduction, group_var = "month")
#NO3 CONSUMED: unequal variance
infer_levene_test(data=metadata, NO3_N_reduction, group_var = "month")

## STEP 3 Non-Parametric Alternative to ANOVA
## KRUSKAL-WALLIS TEST if normality tests fail AND transforms cannot fix
kruskal.test(TN_reduction~month, data=metadata) #KW test--> shows signficant difference
kruskal.test(TP_reduction~month, data=metadata) #KW test --> shows signficant difference
kruskal.test(NH3_N_reduction~month, data=metadata) #KW test --> shows significant difference
kruskal.test(NO3_N_reduction~month, data=metadata) #KW test --> shows significant difference

#####GRAPH BOX PLOT (USE W/ KW TEST)
# KW indirectly compares medians, use box plots to visualize
ggplot(metadata, aes(x = month, y = TN_reduction, fill = month)) + geom_boxplot()
ggplot(metadata, aes(x = month, y = TP_reduction, fill = month)) + geom_boxplot()
ggplot(metadata, aes(x = month, y = NH3_N_reduction, fill = month)) + geom_boxplot()
ggplot(metadata, aes(x = month, y = NO3_N_reduction, fill = month)) + geom_boxplot()

## Post Hoc Tests for KW
## Conover Test
kwAllPairsConoverTest(TN_reduction~month, p.adjust="bonf", data=metadata) #all pairwise
kwAllPairsConoverTest(TP_reduction~month, p.adjust="bonf", data=metadata) #all pairwise
kwAllPairsConoverTest(NH3_N_reduction~month, p.adjust="bonf", data=metadata) #all pairwise
kwAllPairsConoverTest(NO3_N_reduction~month, p.adjust="bonf", data=metadata) #all pairwise

# PROGRAM TO PLOT BAR CHART WITH STANDARD DEVIATION ----------------------
#+++++++++++++++++++++++++
# Function to calculate the mean and the standard deviation
# for each group
#+++++++++++++++++++++++++
# data : a data frame
# varname : the name of a column containing the variable
#to be summarized
# groupnames : vector of column names to be used as
```

```
# grouping variables
data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      sd = sd(x[[col]], na.rm=TRUE))
  }
  data_sum<-ddply(data, groupnames, .fun=summary_func,
                  varname)
  data_sum <- rename(data_sum, c("mean" = varname))
  return(data_sum)
}

# PLOTTING----------------------------------------------------------------
# Data sorting
data1 <- metadata

#ORGANIZE DATA FOR PLOTTING
# CONSUMPTION DATA
# TN reduction
TNreduction <- data_summary(metadata, varname="TN_reduction",
                        groupnames=c("month"))
TNreduction$month=as.factor(TNreduction$month)
# TP reduction
TPreduction <- data_summary(metadata, varname="TP_reduction",
                        groupnames=c("month"))
TPreduction$month=as.factor(TPreduction$month)
# NH3 reduction
NH3reduction <- data_summary(metadata, varname="NH3_N_reduction",
                         groupnames=c("month"))
NH3reduction$month=as.factor(NH3reduction$month)
# NO3 reduction
NO3reduction <- data_summary(metadata, varname="NO3_N_reduction",
                         groupnames=c("month"))
NO3reduction$month=as.factor(NO3reduction$month)

## PLOTTING
#Grouped bar plot (Nutrient reduction by Month)
values1 <- c(TNreduction[,2],TPreduction[,2], NH3reduction[,2],NO3reduction[,2])
sd1 <- c(TNreduction[,3],TPreduction[,3], NH3reduction[,3],NO3reduction[,3])
condition1 <- rep(c("Freshwater", "1", "2", "3", "4") , 4)
nutrient1 <- c(rep("TN",5), rep("TP",5), rep("NH3-N",5), rep("NO3-N",5))
df1 <- data.frame(values1, condition1, nutrient1)

box_1 <- ggplot(df1, aes(x=factor(condition1), y=values1, fill=nutrient1)) +
  geom_bar(stat="identity", position=position_dodge(), colour="black")+
  geom_errorbar(aes(ymin=values1-sd1, ymax=values1+sd1), width=0.2, position=position_dodge(0.9))+
  ylab("Nutrient Consumed (mg/L/day)") + ylim(0, 40)  + labs(title = "", subtitle=NULL) +
  xlab("Month")+
  theme(title=element_text(size=20, family="Times New Roman"),
        axis.text.x = element_text(size=16, family="Times New Roman"),
        axis.text.y=element_text(size=16, family="Times New Roman"),
        axis.title.y = element_text(size = 20, family="Times New Roman"),
        axis.title.x=element_text(size=20, family="Times New Roman"),
        legend.title=element_blank(),
        legend.text = element_text(size = 16, family="Times New Roman"))+
```

```
  scale_fill_manual(values=c("#999999", "#E69F00", "#56B4E9", "111111"))
box_1
```

Part A (condition)

```
## Metagenomic analysis
## Algal Cultivation: Lumped analysis of Recirculation vs. Freshwater
## Part A
## Wei Liao
## Carly Daiek, February 2020

# Loading Library and Tables ----------------------------------------------
library(vegan)
library(phyloseq)
library(MASS)
library(ggplot2)
library(grid)
library(gridExtra)
library(ggpubr)
## the .txt file needs to be saved as the type of "Tab delimited".
## Gene frequency data from QIIME2

##Choose the Frequency_Table_average should be a .txt
con <- file.choose(new = FALSE)
##Now choose the Frequency_Table_Taxanomy should be .txt
con1 <-file.choose(new = FALSE)

## Now we create the data.frame used for Frequency Table.
Frequency_Table <- read.table(con, header = T, row.names = 1)
Frequency_Table_taxonomy <- read.delim(con1, header = T, row.names = 1)

## Alpha Diversity ---------------------------------------------------------
t.Frequency.table <- t(Frequency_Table)  # Transpose the data
class(t.Frequency.table) # Check the class of the table

#Rarefaction -------------------------------------------------
col <- c("black", "darkred", "forestgreen", "orange", "blue", "yellow", "hotpink")
lty <- c("solid", "dashed", "longdash", "dotdash")
pars <- expand.grid(col = col, lty = lty, stringsAsFactors = FALSE)
ra <- rarecurve(t.Frequency.table, step = 20, col =col,lty = lty, cex = 0.6) # Rarefaction Curve
```

Part A (recirculation dynamics)

```
## Metagenomic analysis
## Algal Cultivation: Dynamic analysis of Recirculation vs. Freshwater
## Part A
## Wei Liao
## Carly Daiek, February 2020

# Loading Library and Tables ----------------------------------------------
library(vegan)
library(phyloseq)
library (MASS)
```

```
library(ggplot2)
library(grid)
library(gridExtra)
library(ggpubr)
## Gene frequency data from QIIME2

# Installing the font package ------------------------------------------------
library(extrafont)
font_import() #It may take a few minutes to import.
loadfonts(device="win")

#IMPORT DATA
## the .txt file needs to be saved as the type of "Tab delimited".
##Choose the Frequency_Table should be a .txt
con <- file.choose(new = FALSE)
##Now choose the Frequency_Table_Taxonomy should be .txt
con1 <-file.choose(new = FALSE)

Frequency_Table <- read.table(con, header = T, row.names = 1)
Frequency_Table_taxonomy <- read.delim(con1, header = T, row.names = 1)

# Alpha Diversity ------------------------------------------------------------

## Now we create the data.frame used for Frequency Table.
## Now we create a matrix object with the data frame

t.Frequency.table <- t(Frequency_Table)  # Transpose the data
class(t.Frequency.table) # Check the class of the table

# Alpha diversity analysis indexes
#First Shannon
H <- diversity(t.Frequency.table, index = "shannon", MARGIN = 1, base = exp(1))
#Then Simpson
D <- diversity(t.Frequency.table, "simpson", MARGIN = 1, base = exp(1))
#Third inverse Simpson
iD <- diversity(t.Frequency.table, "inv")
# The last is Pielou's evenness
J<-H/log(specnumber(t.Frequency.table))
##List all indexes
IN <- cbind(H,D,iD,J)
write.csv(IN, "diversity.csv")

##Let's plot H, D, iD, and J
par(mfrow=c(2,2))
plot(H)
plot(D)
plot(iD)
plot(J)

## Estimate Chao1 and ACE
estimateR(t.Frequency.table)

# ANOVA for Alpha Diversity ---------------------------------------------

# using the H, D, iD, and J data to generate "alphadiversity.txt" to run one way ANOVA
# choose the alpha diversity data should be .txt (tab delimited)
```

```
con3 <-file.choose(new = FALSE)
alphadiversity <- read.table(con3, header = T, row.names = 1)
alphadiversity$recycle <- factor(alphadiversity$recycle) ##Factor Statement

#ANOVA of H index
fit1 <- aov(H~recycle, data = alphadiversity)
summary(fit1) #Provide P-value
Tukey1 <- TukeyHSD(fit1, conf.level=0.95) #Tukey multiple comparison
Tukey1 #Plot Tukey results

#ANOVA of D index
fit2 <- aov(D~recycle, data = alphadiversity)
summary(fit2) #Provide P-value
Tukey2 <- TukeyHSD(fit2, conf.level=0.95) #Tukey multiple comparison
Tukey2 #Plot Tukey results

#ANOVA of iD index
fit3 <- aov(iD~recycle, data = alphadiversity)
summary(fit3) #Provide P-value
Tukey3 <- TukeyHSD(fit3, conf.level=0.95) #Tukey multiple comparison
Tukey3 #Plot Tukey results

#ANOVA of J index
fit4 <- aov(J~recycle, data = alphadiversity)
summary(fit4) #Provide P-value
Tukey4 <- TukeyHSD(fit4, conf.level=0.95) #Tukey multiple comparison
Tukey4 #Plot Tukey results

##boxplot of H and J and D and iD

box_1 <- ggboxplot(alphadiversity, x = "recycle", y = "H", color="recycle")+ ylab("Shannon's Index (H)") + ylim(0,
1)+
      theme(legend.position="right", axis.title.x = element_blank(),
          axis.text.y = element_text(size = 11, family="Times New Roman"),
          axis.text.x = element_text(size = 14, family="Times New Roman", angle = 0, hjust = 0.5),
          axis.title.y = element_text(size = 14, family="Times New Roman"),
          legend.text = element_text(size = 11, family="Times New Roman"),
          legend.title= element_blank(),
          legend.direction="vertical")

box_2 <- ggboxplot(alphadiversity, x = "recycle", y = "J", color="recycle")+ ylab("Pielou's Index (J)") + ylim(0,
0.5) +
  theme(legend.position="right", axis.title.x = element_blank(),
      axis.text.y = element_text(size = 11, family="Times New Roman"),
      axis.text.x = element_text(size = 14, family="Times New Roman", angle = 0, hjust = 0.5),
      axis.title.y = element_text(size = 14, family="Times New Roman"),
      legend.text = element_text(size = 11, family="Times New Roman"),
      legend.title= element_blank(),
      legend.direction="vertical")

box_3 <- ggboxplot(alphadiversity, x = "recycle", y = "D", color="recycle")+ ylab("Simpson (D)") + ylim(0, 0.5) +
  theme(legend.position="right", axis.title.x = element_blank(),
      axis.text.y = element_text(size = 11, family="Times New Roman"),
      axis.text.x = element_text(size = 14, family="Times New Roman", angle = 0, hjust = 0.5),
      axis.title.y = element_text(size = 14, family="Times New Roman"),
```

```
      legend.text = element_text(size = 11, family="Times New Roman"),
      legend.title= element_blank(),
      legend.direction="vertical")

box_4 <- ggboxplot(alphadiversity, x = "recycle", y = "iD", color="recycle")+ ylab("Inverse Simpson (iD)") +
ylim(0, 2) +
  theme(legend.position="right", axis.title.x = element_blank(),
      axis.text.y = element_text(size = 11, family="Times New Roman"),
      axis.text.x = element_text(size = 14, family="Times New Roman", angle = 0, hjust = 0.5),
      axis.title.y = element_text(size = 14, family="Times New Roman"),
      legend.text = element_text(size = 11, family="Times New Roman"),
      legend.title= element_blank(),
      legend.direction="vertical")
grid.arrange(box_1, box_2, box_3, box_4, nrow=2)

#Rarefaction ------------------------------------------------
col <- c("black", "darkred", "forestgreen", "orange", "blue", "yellow", "hotpink")
lty <- c("solid", "dashed", "longdash", "dotdash")
pars <- expand.grid(col = col, lty = lty, stringsAsFactors = FALSE)
ra <- rarecurve(t.Frequency.table, step = 20, col =col,lty = lty, cex = 0.6) # Rarefaction Curve
rad <- rad.lognormal(t.Frequency.table) # Rank of Abundance
rad1 <- plot(rad, xlab = "Rank", ylab = "Abundance") # Plotting the rank

# Beta diversity ----------------------------------------------------------
# Dendrogram --------------------------------------------------------------
par(mfrow=c(1,1))
distance <-vegdist(t.Frequency.table, method="euclidean") ## Generate distance matrix
cluster <- hclust(distance, method="complete", members = NULL) ## Production of Hierarchical Cluster Production
tree_m <- plot(cluster, xlab = "Samples", sub = NULL, main ="Dendrogram")
range(distance)
rect.hclust(cluster, k = 3, border = "red")
grp <- cutree(cluster, k = 3)
```

Part B (recirculation dynamics), statistics

```
## Metagenomic analysis
## Algal Cultivation: Dynamic analysis of Recirculation vs. Freshwater
## Part B
## Wei Liao, January, 2020 update
## Carly Daiek, February 2020 update

# Install "phyloseq" package
# source ('http://bioconductor.org/biocLite.R')
# biocLite('phyloseq')

# Loading Library and Tables ----------------------------------------------

library(vegan)
library (MASS)
library(ggplot2)
library(grid)
library(gridExtra)
library(ggpubr)
library(plyr)
library(RVAideMemoire)
```

```
library(DescTools)
library(PMCMRplus)
library(tadaatoolbox)
library(inferr)

#+++++++++++++++++++++++++
# Function to calculate the mean and the standard deviation
# for each group
#+++++++++++++++++++++++++
# data : a data frame
# varname : the name of a column containing the variable
#to be summarized
# groupnames : vector of column names to be used as
# grouping variables
data_summary <- function(data, varname, groupnames){
  require(plyr)
  summary_func <- function(x, col){
    c(mean = mean(x[[col]], na.rm=TRUE),
      sd = sd(x[[col]], na.rm=TRUE))
  }
  data_sum<-ddply(data, groupnames, .fun=summary_func,
                  varname)
  data_sum <- rename(data_sum, c("mean" = varname))
  return(data_sum)
}

## the .txt file needs to be saved as the type of "Tab delimited".
  ##Choose the Relative Frequency Table should be a .txt
con <- file.choose(new = FALSE)
##Now choose the Frequency Table Taxanomy should be .txt
con1 <-file.choose(new = FALSE)
##Now choose the Meta data table should be .txt
con2 <-file.choose(new = FALSE)

Frequency_Table <- read.table(con, header = T, row.names = 1)
Frequency_Table_taxonomy <- read.delim(con1, header = T, row.names = 1)

metadata <- read.table(con2, header = T, row.names = 1) #this table includes key OTU from the .csv files generated
in the following analysis
metadata$month <- factor(metadata$month)
metadata$recycle <- factor(metadata$recycle)

## Abundances ----------------------------------------------------

#Phyloseq
Full_Frequency <- cbind.data.frame(Frequency_Table, Frequency_Table_taxonomy)
Frequency <- otu_table(Frequency_Table,taxa_are_rows = TRUE) # Frequency Table production for phyloseq
TAX <- tax_table(as.matrix(Frequency_Table_taxonomy)) ## Taxanomy production for phyloseq
physeq <- phyloseq(Frequency, TAX) ##physeq document production
physeq0 <- tax_glom(physeq, taxrank=rank_names(physeq)[3], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
tax_table(physeq0)

## Overall abundances for Domain, Phylum, Class, Order, and Family ---------

# Abundance Plotbar Domain
physeqa <-tax_glom(physeq, taxrank=rank_names(physeq)[1], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
```

```
tablea <- otu_table(physeqa)
write.csv(tablea, "domain.csv")

#Abundance Plotbar Phylum
physeqa1 <-tax_glom(physeq, taxrank=rank_names(physeq)[2], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
tablea1 <- otu_table(physeqa1)
write.csv(tablea1, "Phylum.csv")

#Abundance Plotbar Class
physeqa2 <-tax_glom(physeq, taxrank=rank_names(physeq)[3], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
tablea2 <- otu_table(physeqa2)
write.csv(tablea2, "Class.csv")

## Abundance Plotbar Bacteria----------
#Abundance Plotbar Bacteria (Phylum)

physeq2 <-subset_taxa(physeq, Domain== "Bacteria")
physeq2_1 <-tax_glom(physeq2, taxrank=rank_names(physeq2)[2], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
table2_1 <- otu_table(physeq2_1)
write.csv(table2_1, "bacterialPhylum.csv")

##Abundance Plotbar Bacteroidetes (Class)
physeq3 <-subset_taxa(physeq, Phylum == "Bacteroidetes")
physeq3_1 <-tax_glom(physeq3, taxrank=rank_names(physeq3)[3], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
table3_1 <- otu_table(physeq3_1)
write.csv(table3_1, "BacteroidetesFamily.csv")

#Abundance Plotbar Proteobacteria (Class)
physeq5 <-subset_taxa(physeq, Phylum == "Proteobacteria")
physeq5_1 <-tax_glom(physeq5, taxrank=rank_names(physeq5)[3], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
table5_1 <- otu_table(physeq5_1)
write.csv(table5_1, "ProteobacteriaFamily.csv")

# ANOVA Eukarya (Domain)
Eukarya <- aov(Domain.Eukarya~month, data = metadata)
summary(Eukarya)

data_summary(metadata, varname="Domain.Eukarya",
                groupnames=c("month"))

# ANOVA Eukarya (Domain)
Bacteria <- aov(Domain.Bacteria~month, data = metadata)
summary(Bacteria)

data_summary(metadata, varname="Domain.Bacteria",
        groupnames=c("month"))

# ANOVA Proteobacteria (Phylum)
Proteobacteria <- aov(Phylum.Proteobacteria~month, data = metadata)
summary(Proteobacteria)

data_summary(metadata, varname="Phylum.Proteobacteria",
        groupnames=c("month"))

TukeyHSD(Proteobacteria) #If ANOVA reports P < 0.05, use TukeyHSD to detect differences between monthly
comparisons
```

```
#ANOVA Bacteroidetes (Phylum)
Bacteroidetes <- aov(Phylum.Bacteroidetes~month, data = metadata)
summary(Bacteroidetes)

data_summary(metadata, varname="Phylum.Bacteroidetes",
        groupnames=c("month"))

TukeyHSD(Bacteroidetes)

#ANOVA BacteriaUnclassified (Phylum)
BacteriaUnclassified <- aov(Phylum.BacteriaUnclassified~month, data = metadata)
summary(BacteriaUnclassified)

data_summary(metadata, varname="Phylum.BacteriaUnclassified",
        groupnames=c("month"))

TukeyHSD(BacteriaUnclassified)

# ANOVA Alphaproteobacteria (Class)
Alphaproteobacteria <- aov(Class.Alphaproteobacteria~month, data = metadata)
summary(Alphaproteobacteria)

data_summary(metadata, varname="Class.Alphaproteobacteria",
        groupnames=c("month"))

# ANOVA Betaproteobacteria (Class)
Betaproteobacteria <- aov(Class.Betaproteobacteria~month, data = metadata)
summary(Betaproteobacteria)

data_summary(metadata, varname="Class.Betaproteobacteria",
        groupnames=c("month"))

data_summary(metadata, varname="Class.Betaproteobacteria",
        groupnames=c("recycle"))

TukeyHSD(Betaproteobacteria)

# ANOVA Gammaproteobacteria (Class)
Gammaproteobacteria <- aov(Class.Gammaproteobacteria~month, data = metadata)
summary(Gammaproteobacteria)

data_summary(metadata, varname="Class.Gammaproteobacteria",
        groupnames=c("month"))

# ANOVA Proteobacteria_unclassified (Class)
ProteobacteriaUnclassified <- aov(Class.ProteobacteriaUnclassified~month, data = metadata)
summary(ProteobacteriaUnclassified)

data_summary(metadata, varname="Class.ProteobacteriaUnclassified",
        groupnames=c("month"))

TukeyHSD(ProteobacteriaUnclassified)

# ANOVA Bacteroidetes_unclassified (Class)
BacteroidetesUnclassified <- aov(Class.BacteroidetesUnclassified~month, data = metadata)
```

```
summary(BacteroidetesUnclassified)

data_summary(metadata, varname="Class.BacteroidetesUnclassified",
        groupnames=c("month"))

TukeyHSD(BacteroidetesUnclassified)

# ANOVA [Saprospirae] (Class)
Saprospirae <- aov(Class.Saprospirae~month, data = metadata)
summary(Saprospirae)

data_summary(metadata, varname="Class.Saprospirae",
        groupnames=c("month"))

TukeyHSD(Saprospirae)

# ANOVA Cytophagia (Class)
Cytophagia <- aov(Class.Cytophagia~month, data = metadata)
summary(Cytophagia)

data_summary(metadata, varname="Class.Cytophagia",
        groupnames=c("month"))

TukeyHSD(Cytophagia)

# ANOVA Flavobacteria (Class)
Flavobacteria <- aov(Class.Flavobacteria~month, data = metadata)
summary(Flavobacteria)

data_summary(metadata, varname="Class.Flavobacteria",
        groupnames=c("month"))

TukeyHSD(Flavobacteria)

# ANOVA Sphingobacteria (Class)
Sphingobacteria <- aov(Class.Sphingobacteria~month, data = metadata)
summary(Sphingobacteria)

data_summary(metadata, varname="Class.Sphingobacteria",
        groupnames=c("month"))

TukeyHSD(Sphingobacteria)
```

Part B (recirculation dynamics), plotting

```
## Metagenomic analysis
## Algal Cultivation: Dynamic analysis of Recirculation vs. Freshwater
## Part B - PLOTTING
## Wei Liao, Janurary, 2020 update
## Carly Daiek, February 2020 update

# Install "phyloseq" package
# source ('http://bioconductor.org/biocLite.R')
# biocLite('phyloseq')
```

```
# Loading Library and Tables ----------------------------------------------

library(vegan)
library(phyloseq)
library (MASS)
library(ggplot2)
library(grid)
library(gridExtra)
library(ggpubr)

# Installing the font package ---------------------------------------------
library(extrafont)
font_import() #It may take a few minutes to import.
loadfonts(device="win")

## the .txt file needs to be saved as the type of "Tab delimited".

##Choose the Relative Frequency Table should be a .txt
con <- file.choose(new = FALSE)
##Now choose the Frequency Table Taxanomy should be .txt
con1 <-file.choose(new = FALSE)

##Now choose the Meta data table should be .txt
##con2 <-file.choose(new = FALSE)
metadata <- read.table(con, header = T, row.names = 1)
Frequency_Table <- metadata
order <- c("Month 1", "Month 2", "Month 3", "Month 4", "Freshwater")
order <- factor(order,levels = c("Month 1", "Month 2", "Month 3", "Month 4", "Freshwater"))
names(Frequency_Table) <- order
Frequency_Table_taxonomy <- read.delim(con1, header = T, row.names = 1)

## Abundances ---------------------------------------------------

#Phyloseq
Full_Frequency <- cbind.data.frame(Frequency_Table, Frequency_Table_taxonomy)
Frequency <- otu_table(Frequency_Table,taxa_are_rows = TRUE) # Frequency Table production for phyloseq
TAX <- tax_table(as.matrix(Frequency_Table_taxonomy)) ## Taxanomy production for phyloseq
#SAM <- sample_data(metadata)
physeq <- phyloseq(Frequency, TAX) ##physeq document production
physeq0 <- tax_glom(physeq, taxrank=rank_names(physeq)[3], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
tax_table(physeq0)

p = plot_bar(physeq0, fill = "Class", facet_grid=Domain~Phylum, ) +
  theme(axis.title.x = element_blank(), axis.text.x = element_text(size = 5, angle = 45, hjust = 1)) +
  geom_bar(color = "black", size = .1, stat = "identity", position = "stack")
p


## Overall abundances for Domain, Phylum, Class, Order, and Family ---------

# Abundance Plotbar Domain
physeqa <-tax_glom(physeq, taxrank=rank_names(physeq)[1], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
tablea <- otu_table(physeqa)
tablea
write.csv(tablea, "domain.csv")
```

```
a = plot_bar(physeqa, fill = "Domain") +
  geom_bar(aes(color=Domain, fill=Domain), stat = "identity", position = "stack") +
  ylab("Relative Frequency (%)") + labs(title= "") +
  theme(legend.position="right", axis.title.x = element_blank(),
        axis.text.y = element_text(size = 12, family="Times New Roman"),
        axis.text.x = element_text(size = 10, family="Times New Roman", angle = 45, hjust = 1),
        axis.title.y = element_text(size = 14, family="Times New Roman"),
        legend.text = element_text(size = 11, family="Times New Roman"),
        legend.title= element_text(size = 12, family="Times New Roman"),
        legend.direction="vertical")
a

#Abundance Plotbar Phylum
physeqa1 <-tax_glom(physeq, taxrank=rank_names(physeq)[2], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
tablea1 <- otu_table(physeqa1)
#tablea1
write.csv(tablea1, "Phylum.csv")

a1 = plot_bar(physeqa1, fill = "Phylum") +
  geom_bar(aes(color=Phylum, fill=Phylum), stat = "identity", position = "stack") +
  ylab("Relative Frequency (%)") + labs(title= "") +
  theme(legend.position="right", axis.title.x = element_blank(),
        axis.text.y = element_text(size = 12, family="Times New Roman"),
        axis.text.x = element_text(size = 10, family="Times New Roman", angle = 45, hjust = 1),
        axis.title.y = element_text(size = 14, family="Times New Roman"),
        legend.text = element_text(size = 11, family="Times New Roman"),
        legend.title= element_text(size = 12, family="Times New Roman"),
        legend.direction="vertical")
a1

#Abundance Plotbar Class
physeqa2 <-tax_glom(physeq, taxrank=rank_names(physeq)[3], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
tablea2 <- otu_table(physeqa2)
tablea2
write.csv(tablea2, "Class.csv")

a2 = plot_bar(physeqa2, fill = "Class") +
  geom_bar(aes(color=Class, fill=Class), stat = "identity", position = "stack") +
  ylab("Relative Frequency (%)") + labs(title= "") +
  theme(legend.position="right", axis.title.x = element_blank(),
        axis.text.y = element_text(size = 12, family="Times New Roman"),
        axis.text.x = element_text(size = 10, family="Times New Roman", angle = 45, hjust = 1),
        axis.title.y = element_text(size = 14, family="Times New Roman"),
        legend.text = element_text(size = 11, family="Times New Roman"),
        legend.title= element_text(size = 12, family="Times New Roman"),
        legend.direction="vertical")
a2

grid.arrange(a,a1,a2,nrow=1)

## Abundance Plotbar Bacteria----------
#Abundance Plotbar Bacteria (Phylum)

physeq2 <-subset_taxa(physeq, Domain== "Bacteria")
physeq2_1 <-tax_glom(physeq2, taxrank=rank_names(physeq2)[2], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
```

```
table2_1 <- otu_table(physeq2_1)
table2_1
write.csv(table2_1, "bacterialPhylum.csv")

c = plot_bar(physeq2_1, fill = "Phylum") + geom_bar(aes(color=Phylum, fill=Phylum), stat = "identity",position =
"stack") +
  ylab("Relative Frequency (%)") + labs(title = "") +
  theme(legend.position="right", axis.title.x = element_blank(),
        axis.text.y = element_text(size = 12, family="Times New Roman"),
        axis.text.x = element_text(size = 10, family="Times New Roman", angle = 45, hjust = 1),
        axis.title.y = element_text(size = 14, family="Times New Roman"),
        legend.text = element_text(size = 11, family="Times New Roman"),
        legend.title= element_text(size = 12, family="Times New Roman"),
        legend.direction="vertical")
c

##Abundance Plotbar Bacteroidetes (Class)
physeq3 <-subset_taxa(physeq, Phylum == "Bacteroidetes")
physeq3_1 <-tax_glom(physeq3, taxrank=rank_names(physeq3)[3], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
table3_1 <- otu_table(physeq3_1)
table3_1
write.csv(table3_1, "BacteroidetesFamily.csv")

d = plot_bar(physeq3_1, fill = "Class")+ geom_bar(aes(color=Class, fill=Class), stat = "identity",position = "stack")
+
  ylab("Bacteroidetes Abundance (%)") + xlab("Samples") + labs(title = "") +
  theme(legend.position="right", axis.title.x = element_blank(),
        axis.text.y = element_text(size = 12, family="Times New Roman"),
        axis.text.x = element_text(size = 10, family="Times New Roman", angle = 45, hjust = 1),
        axis.title.y = element_text(size = 14, family="Times New Roman"),
        legend.text = element_text(size = 11, family="Times New Roman"),
        legend.title= element_text(size = 12, family="Times New Roman"),
        legend.direction="vertical")
d

#Abundance Plotbar Proteobacteria (Class)
physeq5 <-subset_taxa(physeq, Phylum == "Proteobacteria")
physeq5_1 <-tax_glom(physeq5, taxrank=rank_names(physeq5)[3], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
table5_1 <- otu_table(physeq5_1)
table5_1

write.csv(table5_1, "ProteobacteriaFamily.csv")

f = plot_bar(physeq5_1, fill = "Class")+ geom_bar(aes(color=Class, fill=Class), stat = "identity",position = "stack")
+
  ylab("Proteobacteria Abundance (%)") + xlab("Samples") + labs(title = "") +
  theme(legend.position="right", axis.title.x = element_blank(),
        axis.text.y = element_text(size = 12, family="Times New Roman"),
        axis.text.x = element_text(size = 10, family="Times New Roman", angle = 45, hjust = 1),
        axis.title.y = element_text(size = 14, family="Times New Roman"),
        legend.text = element_text(size = 11, family="Times New Roman"),
        legend.title= element_text(size = 12, family="Times New Roman"),
        legend.direction="vertical")
f

grid.arrange(d,f,nrow=1)
```

```
## Heatmap ----------------------
heatorder <- order

#Heatmap Phylum in bacteria
physeq9 <- subset_taxa(physeq, Domain== "Bacteria")
physeq9_1 <- tax_glom(physeq9, taxrank=rank_names(physeq9)[2], NArm=TRUE, bad_empty=c(NA, "", " ", "\t"))
i = plot_heatmap(physeq9_1, method = "NMDS", distance = "bray",
              sample.label = NULL, taxa.label = "Phylum", low = "#00cd00",
              high = "#003400", na.value = "white",
              max.label = 250, title = NULL, sample.order = heatorder, taxa.order = NULL,
              first.sample = NULL, first.taxa = NULL)+
       theme(legend.position="right",
       axis.title.x = element_blank(),
       axis.text.x = element_text(size = 10, angle = 45, hjust = 0.70),
       axis.title.y = element_text(size = 12),
       axis.text.y = element_text(size = 10),
       legend.text = element_text(size = 10),
       legend.title= element_text(size = 12),
       plot.title= element_text(size = 15))
i

#Heatmap family in bacteroidetes
physeq10 <- subset_taxa(physeq, Phylum == "Bacteroidetes")
physeq10_1 <- tax_glom(physeq10, taxrank=rank_names(physeq10)[3], NArm=TRUE, bad_empty=c(NA, "", " ",
"\t"))
j = plot_heatmap(physeq10_1, method = "NMDS", distance = "bray",
              sample.label = NULL, taxa.label = "Class", low = "#FFCCCB",
              high = "#8B0000", na.value = "white",
              max.label = 250, title = NULL, sample.order = heatorder, taxa.order = NULL,
              first.sample = NULL, first.taxa = NULL) +
           theme(legend.position="right",
            axis.title.x = element_blank(),
            axis.text.x = element_text(size = 10, angle = 45, hjust = 0.70),
            axis.title.y = element_text(size = 12),
            axis.text.y = element_text(size = 10),
            legend.text = element_text(size = 10),
            legend.title= element_text(size = 12),
            plot.title= element_text(size = 15))
j

#Heatmap Proteobacteria
physeq11 <- subset_taxa(physeq, Phylum== "Proteobacteria")
physeq11_1 <- tax_glom(physeq11, taxrank=rank_names(physeq11)[3], NArm=TRUE, bad_empty=c(NA, "", " ",
"\t"))
k = plot_heatmap(physeq11_1, method = "NMDS", distance = "bray",
              sample.label = NULL, taxa.label = "Class", low = "#66CCFF",
              high = "#000033", na.value = "white",
              max.label = 250, title = NULL, sample.order = heatorder, taxa.order = NULL,
              first.sample = NULL, first.taxa = NULL)+
           theme(legend.position="right",
            axis.title.x = element_blank(),
            axis.text.x = element_text(size = 10, angle = 45, hjust = 0.70),
            axis.title.y = element_text(size = 12),
            axis.text.y = element_text(size = 10),
            legend.text = element_text(size = 10),
            legend.title= element_text(size = 12),
```

```
        plot.title= element_text(size = 15))
k
grid.arrange(i,j,k,nrow=1)
```

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