THESIS

EVALUATION OF WASTEWATER AS A NUTRIENT SOURCE FOR THE CULTIVATION OF THE MODEL CYANOBACTERIUM *SYNECHOCYSTIS* sp. PCC6803

Submitted by

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ABSTRACT

EVALUATION OF WASTEWATER AS A NUTRIENT SOURCE FOR THE CULTIVATION OF THE MODEL CYANOBACTERIUM *SYNECHOCYSTIS* sp. PCC6803

The rising demand for more sustainable and renewable energy sources has led to the development of using photosynthetic microalgae and cyanobacteria for biofuel feedstocks. Microalgae and cyanobacteria offer an attractive solution over the cellulosic and lignocellulosic feedstocks of first and second generation biofuels that compete for arable land, nutrients and water necessary for sufficient food crop production. It has been proposed for several decades that wastewater could be a sustainable and affordable source of water and nutrients. Phycoremediation of wastewater by microalgae as biofuel feedstocks could provide beneficial environmental health impacts by preventing eutrophication of fresh water supplies. Many eukaryotic microalgae have been grown in diluted and/or modified wastewaters. The growth of cyanobacteria on wastewaters has not been nearly as well characterized. Cyanobacteria grown on wastewaters could be an ideal feedstock for advanced biofuels, since cyanobacteria have a more extensively established molecular toolbox for genetic engineering.

The first aim of this thesis was to evaluate wastewater centrate as a growth medium for the cultivation of the cyanobacterium *Synechocystis* sp. strain PCC6803 (Chapter 1). Centrate was collected from the Drake Water Reclamation Facility (Fort Collins, CO) and filter sterilized to allow axenic culturing of PCC6803 under controlled laboratory conditions. PCC6803 was grown in up to 21% filtered centrate diluted in sterile water; while higher concentrations completely inhibited growth. Nitrogen drawdown from centrate by PCC6803 was then analyzed. Surprisingly, the drawdown of nitrogen from the centrate media correlated poorly with the

amount of cyanobacterial biomass. The cell densities of cultures grown in centrate were all significantly lower than that of PCC6803 grown in BG-11 indicating that diluted centrate does not provide adequate nutrients to support optimal growth. Abiotic precipitation of nitrogen was then determined to dominate the removal of nitrogen from the cultivation media. Furthermore, it suggested that centrate lacks a critical nutrient to support robust growth of PCC6803.

The second aim of this thesis was to augment the nutrient composition of wastewater in order to optimize PCC6803 growth and nutrient removal (Chapter 2). A series of bioassays were performed to elucidate the limiting macronutrient in centrate. Adding 304 μM Na_2SO_4 – equivalent to the concentration of SO_4^{2-} in BG-11 media – yielded final cell densities that were only 4% lower than those observed in cultures grown in the synthetic, standard media (BG-11). Exogenous supplementation of Na_2SO_4 also improved total dissolved nitrogen (TN) drawdown for centrate grown PCC6803 cultures. In Na_2SO_4 amended centrate, PCC6803 was able to grow to significantly higher cell densities, permitting the removal of 69% of the TN in diluted centrate. Transcript abundance of the sulfate transporters encoded by the *spbA-cysTWA* operon were found to be upregulated when grown in centrate, confirming that PCC6803 experienced S-limitation during growth on this media.

Hydrogen sulfide gas (H_2S) is an undesirable product of the biological nutrient removal process due to its pungent odor. Currently, H_2S produced at the DWRF is vented to biofilters consisting of wood chips and compost where sulfide oxidizing microbes convert sulfide into elemental sulfur. Therefore, endogenously sourced sulfur supplementation from H_2S into centrate could provide a viable sulfur source to support the cultivation of PCC6803. We have shown that sulfur supplementation improves the phycoremediation of nutrients in centrate. Cultivation of PCC6803 on centrate supplemented with endogenously sourced sulfur provides an industrially feasible method for combining wastewater treatment with advanced biofuel production.

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1. GENERAL INTRODUCTION

1.1 The need to reduce Greenhouse Gas Emissions

1.1.1 Anthropogenic effects on climate change

The year 2016 saw record high global surface greenhouse gas (GHG) concentrations; record high annual surface, sea and lower troposphere temperatures; as well as record high ice melt and glacier retreat [1]. Each year has set new record highs in many of these categories throughout the 21st Century, providing continued evidence for a drastically changing global climate [1]. It is important to note on the outset that slow cyclical changes of the global climate have been measured over long periods of time with glacial periods lasting around 100k years and the warmer interglacial periods lasting 10-12k years [2]. These glacial-interglacial cycles have historically been the natural result of changes in atmospheric conditions resulting in fluctuations in the amount of solar radiation absorbed by and emitted back from the Earth's surface [2, 3]. Climate change has significant effects on nearly every aspect of life on Earth. The Intergovernmental Panel on Climate Change (IPCC) summarized the scientific community's recent major findings of the influences attributed to climate change to include impacts on: terrestrial and marine ecosystems, natural disasters (drought, floods, wildfires, and extreme weather events), and the reciprocal effects on food production, human health, and economics [4]. An important question to ask is whether the recently observed evidence of pronounced climate change is following in the same historical pattern.

Increasing average annual temperatures across the globe are one of the major indicators of climate change. Climate models suggest that for the first 700 years of the 2nd millennium (1000 CE to 1700 CE) the temperature of the Northern Hemisphere steadily decreased a total of 0.3°C, maintained temperature from 1700 -1900, and then increased in temperature by nearly 1°C over

the next 93 years [3]. Interestingly, the 20th century saw a climate anomaly in which there was a rise in the rate of temperature increase from 0.07°C decade^{-1} to 0.22°C decade^{-1} after the 1960s [5, 6]. In congruence with these models, data collected from several satellites over the period of 1978-2016 demonstrate a high rate of temperature increase in the lower troposphere of 0.174°C decade^{-1} [7]. From these studies, we can recognize that there have been dramatic changes in the direction and rate of temperature change since the 18th century.

Human activity since the Industrial Revolution of the 18th century has been attributed to the increasing rates of global warming. A recent study by Paeth *et al.* (2017) used discriminant analysis coupled with Bayesian classification to assess the anthropogenic effects on climate change using 11 total indices of climate change. The authors found that anthropogenic influence is contributing to a significant rate of change in the increasing global temperatures since 1980 [8]. In fact, of all the climate science literature, 97% of the peer-reviewed studies that takes a stance on the cause of the shift in climate change support that human activities are directly impacting climate change [9, 10]. Benestad *et al* (2016) tried to replicate the experiments of 38 of the 3% of publications which present results that don't support anthropogenic global warming, but have been highly viewed by the public and have been cited for government policies. The authors found that all 38 of the chosen publications contained flaws in the analysis, statistics, physics, and/or provided misrepresentation of results from previous studies, thereby negating the original authors' findings [11]. These studies present just a few examples of the literature available supporting the stance that there is a strong anthropogenic effect on climate change.

Human activities contribute to the greenhouse gas emissions related to the observed climate change. The effect of greenhouse gasses on increasing the temperature of Earth's surface was first described in 1896 by Svante Arrhenius when he developed an equation that described the direct relationship between atmospheric CO_2 concentrations and surface temperatures [12]. In

the United States, carbon dioxide (CO_2), nitrous oxide (N_2O), and methane (CH_4) are the three primary greenhouse gasses emitted annually; with CO_2 emissions from the combustion of fossil fuels accounting for nearly 77% of all GHG emissions in 2015 [13]. Anthropogenic emissions of CH_4 and N_2O largely come from the industries of agriculture, fossil fuels, and waste treatment [14]. As noted in a 2014 IPCC climate change report, the total annual GHG emissions nearly doubled between 1970 and 2010 [4]. Additionally, the report showed that the average rate of GHG emissions increased by 60% after 2000 (compared to the annual GHG emissions rate determined from 1970 to 2000) [4]. Annual anthropogenic emissions of greenhouse gasses are on the rise, and are thus directly causing the global warming observed in the present climate change.

1.1.2 Demonstration of the need for a sustainable transport fuel

The combustion of fossil fuels is one of the largest contributors to GHG emissions [4, 13]. Combustible fossil fuels include coal, natural gas, and petroleum – with natural gas and petroleum both used for transportation. In the United States, over 75% of the CO_2 emitted in 2015 was from combustion of fossil fuels, with about half that being emitted from the transport sector [13]. Road transport has the greatest impact on global warming over other modes of transportation [15]. Based on findings by the International Energy Agency, the transport sector will remain dependent on fossil fuels as long as they remain cheaper and more energetically favorable to alternative fuel sources [16]. So, the emission of GHG from the combustion of fossil fuels for road based transportation is a significant driver in climate change.

Legislation and government policies have a significant impact on the production and use of biofuels. Per the final rule of the Renewable Fuel Standard (RFS2), 36 billion gallons of renewable fuels will be required to be blended into transport fuel by 2022 (75 FR 14670, EPA). As Hill and colleagues outline (Hill et al. 2016), this rule requires supplementing transport fuels with 15 billion gallons of conventional biofuels, 16 billion gallons of cellulosic biofuels, 4 billion

gallons of advanced biofuels, and 1 billion gallons of biomass-based diesel. These are predicted the EPA to reduce green-house gas emissions by 23, 73, 15, and 110 million metric tons, respectively, by 2022 [17]. This assessment provides support for the need to further revolutionize sustainability efforts by substantially reducing the carbon footprint of current state of the art biofuel production methods.

1.1.3 Renewable liquid transportation fuels

Biofuels for transportation are already in production. In regards to biomass based sources of renewable energy, liquid biofuels (as biomass inputs for fuel ethanol and biodiesel production) accounted for 22% of the renewable energy produced [18]. Liquid biofuels for use in the transport sector are produced from a variety of renewable feedstocks. Conventional biofuels, also termed 'first generation biofuels,' are those produced from agricultural food crops [19]. In the most simple of processes, sugars and starches from these sources are fermented to produce bioethanol by yeast enzymes [20]. Bioethanol can then be supplement into gasoline, or used as a complete replacement for gasoline [21, 22]. Additionally, transesterification of triglycerides from food crops can be catalytically or enzymatically driven to produce biodiesel (reviewed further below) [23]. It has, however, been demonstrated that the production of biofuels from cellulosic feedstocks compete with the food supply, both in regards to crop production and land/resource utilization[24].

Lignocellulosic biomass has also been considered for biofuel feedstocks to eradicate the problem of food competition. Lignocellulosic components of crops are not digestible by human enzymes [25, 26], and can therefore be reallocated towards energy production as second generation biofuels [27]. Conversion of lignocellulosic materials into liquid biofuel fractions requires energy intensive multi-step processing, including pretreatment [28-31] followed by thermochemical and/or enzymatic processes [32-36]. Methane (biogas) can also be produced

from anaerobic digestion of the organic wastes [37]. Thus far, the conversion processes are not efficient or sustainable enough to effectively produce large volumes of biofuels from lignocellulosic feedstocks [38].

The shortcomings of the first two generations of biofuels led to a set of criteria to be developed for what a cost-effective biofuel should have. In order to be considered effective feedstocks for biofuel production, organisms need to be able to grow quickly and produce large quantities of neutral lipids, oils, H_2, ethanol, or other biofuel feedstocks using nutrient and water sources that do not compete with agriculture. Additionally, organisms should be capable of growing on land/waters not allocated for agricultural use. In order to make biofuel as economical as possible, organisms that are able to produce additional value-added products such as nutraceuticals, biopolymers, proteins, etc., through metabolism of industrial and municipal wastes should also be considered [39, 40].

1.1.4 Biofuels from photosynthetic microbes

To accommodate the above defined criteria, mass cultivation of photoautotrophic microorganisms – eukaryotic microalgae and prokaryotic cyanobacteria (collectively called photosynthetic microbes, here) – have been investigated for the development of third generation biofuel feedstocks. Photosynthetic microbes can convert sunlight and atmospheric CO_2 into biomass with minimal nutrient requirements [41]. Photosynthetic microbes with high lipid accumulation capacities are sought after for the production of biodiesel, while others are being investigated for other biofuels [42] (reviewed further in 2.1) and value added commodities[43-46]. Microorganisms have the benefit of rapid growth and biomass accumulation, increased lipid production [47, 48], decreased nutrient requirements, and, in many cases, are easier to genetically transform than terrestrial plants [49]. Ultimately, biofuels produced from photosynthetic

microorganisms could be produced without affecting – or competing against – agricultural food production, and would have a reduced environmental impact [50].

1.2 Overview of biofuels produced by photosynthetic microbes

1.2.1 Fatty acid derived biofuels from biomass

Microalgae produce lipids for the formation of membranes and as energy stores. The biosynthesis pathways for fatty acids and other lipid production have been well characterized. Through consecutive condensation and elongation reactions, the two carbons of the acetate group of acetyl coenzyme A (acetyl-CoA) become elongated to C16 or C18 carbon chained fatty acids. These lipids are the feedstock for biodiesel production [39].

Storage lipids – such as the neutral lipid triacylglycerol (TAG) – are converted into fatty acid methyl esterases (FAMEs) and glycerol through transesterification using enzymatic or chemical catalysts for biodiesel production. Enzymatic catalysts rely on lipases to hydrolyze triglycerides to partial glycerides, which can then be converted to free fatty acids before forming methyl esters with the addition of methanol [51]. Chemical catalysts refer to either acid- or alkali-catalysts. Acid catalysts – sulfuric, phosphoric, hydrochloric, and organic sulfonic acids – have been observed to be slower in the catalysis of transesterification by alkali-catalysts [52]. Sodium and potassium hydroxides, carbonates, and many alkoxides are examples of alkalis used in transesterification. While alkali-catalyzed reactions can occur 4000 times faster than acid-catalyzed reactions, moisture can dramatically affect the quality and volume of final products due to saponification reactions in the presence of water [53]. Clearly, several factors of lipid transesterification to FAMEs can influence the rate and therefor cost of production.

Biosynthesis of storage lipids can be driven under stress conditions. Nitrogen starvation has been well studied and reviewed to directly affect lipid metabolism in favor of the accumulation of lipids [54-57]. Silica starvation has also been demonstrated to cause an increase

in lipid accumulation in diatoms [58]. High light intensity grown microalgae have been shown to shift formation of the polar membrane lipids to neutral storage lipids [59]. Temperature can affect the saturation and accumulation of lipids, but these changes vary greatly by species [39]. Therefore, altering the environment during the cultivation of photosynthetic microbes can affect the biosynthesis of neutral lipids for biofuel production.

1.2.2 Bioethanol production from microalgal feedstocks

Bioethanol production from microalgae can be accomplished by traditional saccharification/fermentation of carbohydrates, direct fermentation, or through genetic engineering (described in 2.1.3). Under optimal growth conditions (sufficient light and nutrients), microalgae can divide rapidly to generate large volumes of biomass containing large fractions of starch. Starch can be extracted by either mechanical or enzymatic methods which must then be hydrolyzed to monosaccharides in a process called saccharification. This process is driven either by acids and amylase enzymes. Monosaccharides can then be fermented into ethanol by yeast [60, 61]. Direct fermentation through the oxidation of stored starch by some microalgae can also occur during dark anaerobic conditions [62].

1.2.3 Genetically engineered microalgae and cyanobacteria

Fourth generation biofuels – those derived from genetically engineered photosynthetic microbes – are considered the most promising future of biofuel production [40]. Synthetic design of engineered strains aims to overcome the native regulatory pathways in order to amplify production of desired fuel substrates without negatively impacting growth rates. Engineered photosynthetic microbes are being designed to produce bioethanol (described above), biobutanol, enhanced fatty acid production, and isoprene production for use in transport fuels and other energy sources [63].

Bioethanol production from photosynthetic microbial cells *in situ* requires pyruvate decarboxylase and alcohol dehydrogenase to drive production of ethanol from pyruvate. Deng and Coleman (1998) demonstrated the first successful transformation of cyanobacteria to produce and excrete ethanol by cloning in pyruvate decarboxylase and alcohol dehydrogenase genes into the cyanobacterium *Synechococcus* sp. PCC7942 [64].

Metabolic pathways for butanol production – a higher chain alcohol that has been considered as a potential gasoline substitute – have been engineered via two different pathway manipulations in microalgae strains. A keto acid synthesis pathway has been transformed into *Synechococcus elongatus* sp. PCC7942, allowing the culture to produce isobutyraldehyde at a consistent rate of 2,500 $\mu g\ l^{-1}\ h^{-1}$over 9 days. Isobutyraldehyde was then converted into isobutanol – when alcohol dehydrogenase was also transformed in the mutant – at a reported rate of 450mg l^{-1} over 6 days [65]. *S. elongatus* has also been transformed to produce 1-butanol via a modified Co-A dependent 1-butanol production pathway. When photosystem II was inhibited in these mutants, *S. elongatus* was able to produce 1-butanol at a rate of 78.33 $\mu g\ l^{-1}\ h^{-1}$[66]. Clearly, genetic engineering of cyanobacteria can successfully produce biofuels of interest.

1.3 Commercialization of biofuels from photosynthetic microbes

1.3.1 Cost of production

The economic limitations of commercializing biofuel production using photosynthetic microbes have been well reviewed. Chisti (2012) estimated that production of photosynthetic microbe biomass (40% oil content/dry weight) will have to cost less than $0.25 per kg in order to compete with petroleum at a cost of $100/barrel [67]. These costs will have to be reduced further given the current cost of oil at ~$51.46/barrel (October 2017 average price, Nasdaq.com). A 2012 study investigating the actual cost of producing the green alga *Scenedesmus almeriensis* in a medium-scale plant in Spain calculated a cost of €69.3/kg dry biomass (equivalent to $89.07/kg

in 2012 U.S. dollars using the 2012 annual average exchange rate of €0.77803/$1.00). While *S. almeriensis* is cultivated for the high lutein and zeaxanthin (xanthophylls) content for nutraceuticals [68] and fishmeal [69], and not lipids or other biofuel feedstocks, the production method used relevant cultivation methods in tubular photobioreactors [70]. In order to produce photosynthetic microbes for biofuel at a cost equivalent to that of petroleum fuels every aspect of cultivation must be further developed and optimized including: strain and bioreactor design, harvesting of biomass, processing of biomass to convert lipids/desired substrates into final products, and extraction technologies of fuel isolates [70].

1.3.2 Improved processing methods

As algae have dramatically reduced cellulose content than terrestrial plants, conversion methods of lignocellulosic feedstocks would not be efficient processes for algal conversion to biofuels. Traditional lipid extraction technologies for biodiesel production (oil press, solvent extraction, supercritical fluid extraction, and ultrasound) are not currently cost effective, efficient, or sustainable enough to practically compete with petroleum based fuels [67, 71]. Hydrothermal liquefaction (HTL) however, could provide a cost effective conversion of algae – including the prominent composition of macromolecules such as proteins and polysaccharides – into energy dense bio-oil [72].

Investigators at the Pacific Northwest National Laboratory developed a Biomass Assessment Tool [73, 74] to compare the resource requirements for algal biofuel production between lipid extraction methods and hydrothermal liquefaction. The study was based on *Chlorella* sp. (a green alga) being used to meet the renewable diesel targets set by the Energy Independence and Security Act of 2007. The 2014 model estimates that processing by HTL would require 50% less land, 33% less freshwater and 85% less saline groundwater, and a 44% reduction in nitrogen (N) and phosphorus (P) demand relative to resources required for the same

biofuel production volume prepared by lipid extraction methods. When optimizing the combination of technologies, as well as nutrient reallocation from waste streams (municipal wastewaters and/or animal manure for N and P, and flue gasses for CO_2) to microalgal cultivation, the model predicts near complete satisfaction of the nutrient demands for both targeted biofuel production quotas [75, 76].

Improvements to HTL processes have been made since the 2014 analysis just described. A more efficient pretreatment process of microalgae for macromolecule extraction has been established by Hu et al. (2017) [76]. A low temperature NaOH/urea solvent was used to disrupt the cell walls of *Chlorella vulgaris* prior to HTL. The authors report that NaOH/urea pre-treatment yielded more bio-crude oil compared to traditional pre-treatment methods (although statistics for significance was not reported). Additionally, the bio-crude oil was shown to have better flow properties (lower viscosity) using the new pre-treatment method based on GC-MS analysis of major compounds and FT-IR analysis of functional groups [76].

1.3.3 Nutrient requirements and sources

The Redfield ratio of 106:16:1 describes the molar C:N:P elemental composition of phytoplankton in the deep ocean and demonstrates that nitrogen and phosphorus are rate limiting nutrients of algal growth[77]. As microalgae growth requires nutrients in addition to C, N, and P, mathematical models have been developed – founded on the Michaelis-Menton-Monod uptake, Droop's growth, and Liebig's law of the minimum functions – to predict growth rates based on available nutrient stoichiometry [78, 79]. In order to support maximal growth rates, nutrients must be supplied in excess concentrations to avoid growth limitation by nutrient deficiency [80, 81].

1.3.3.1 Carbon

Elemental carbon content of microalgae has been measured at ~50% biomass, primarily in the forms of carbohydrates and lipids [82]. Carbon may be supplied as inorganic carbon as CO_2 or HCO_3^- for photoautotrophic growth [83], or as organic carbon (primarily glucose) for species capable of performing heterotrophic or mixotrophic growth [84, 85]. CO_2 supplied from the atmosphere through aeration is energy intensive and thus costly[83]; so is purchasing pure CO_2 at industrial scales, which accounts for ~50% of the total production cost [67]. As coal burning power plants have been identified as a major emitter of CO_2, the capture and recycling of inorganic carbon from the flue gasses for microalgal cultivation has been explored.

Chen et al. (2012) developed a novel photosynthetic bioreactor (PBR) system, supplied with flue gas from a power plant as the carbon source, to grow the cyanobacterium *Spirulina platensis*. The ~ 30,000 L system (with a footprint of only 100 m^3) had a demonstrated net CO_2 fixation rate of 74kg CO_2 per year after total energy consumption of the PBR was subtracted. When scaled up, this reactor system was projected to fix CO_2 at a rate of 74 tons per hectare per year. In addition to performing carbon fixation of flue gasses from a coal power plant, the authors demonstrated the simultaneous production of a nutraceutical by *Spirulina. Spirulina platensis* produce polysaccharides whose bioactivity was elucidated in this study to positively influence the mammalian immuno-inflammatory signal transduction pathway by stimulating the release of the cytokine TNF-α [86].

More recently, in a response to a review published by Cheah *et al*. (2015) [87], Kim and Lee (2016) grew the green alga *Nannochloris* sp. LB1999 using trona mineral ($Na3(CO3)(HCO3)\cdot 2H2O$) as a cheap buffer alternative to counter the reported inhibitory acidifying effects of SO_2 and nitrogen oxides (NO_x). Flue gas composition was simulated by adjusting the concentrations of CO_2, SO_2, NO, and HCl. The use of trona relieved any toxic

effects of the non-carbon gasses as demonstrated by similar growth rates of *Nannochloris* sp. in all flue gas combinations tested [88]. These two studies demonstrate the opportunity to sequester CO_2 emitted from prominent GHG emitters, such as coal power plants.

1.3.3.2 Nitrogen and phosphorus

Nitrogen and phosphorus are growth limiting nutrients accounting for 1-14% and 0.5-3.3% biomass content of microalgae, respectively [89]. Which of the nutrients, nitrogen or phosphorus, is completely limiting has been thoroughly debated; however it was concluded by Guildford and Hecky (2000) that, regardless of marine or freshwater ecosystem, either nitrogen or phosphorus can be limiting depending on the total nitrogen(TN):total phosphorus(TP) ratio of the environment [90], further supporting similar observations made earlier by Smith in 1982 [91].

Nitrogen is essential for the production of nucleic acids, amino acids, and pigments in microalgae. As reviewed by Flores and Herrero (2005), many cyanobacteria use ATP-binding-cassette-type uptake permease transporters for nitrate, nitrite, urea, glutamine, and arginine uptake into the cell; while ammonia permeases can directly transport ammonia. Once in the cell, nitrate is reduced to nitrite, followed by further reduction of nitrite into ammonia. Ammonia is then condensed with glutamate to form glutamine, the nitrogen storage compound [92].

Phosphorus is an essential nutrient required for nucleic acid, membrane phospholipid, and ATP synthesis. Sources of phosphorus for microalgae – all produced from phosphate-rock – include potassium-, sodium-, and ammonium-phosphates. Microalgae typically take up phosphorous as orthophosphate, requiring phosphatase enzymes [83]. Chisti estimates that for roughly 100 tons of algal biomass production 1 ton of phosphorous would be required for growth, and to account for phosphate interactions with metal ions [93].

The sustainability of traditional nitrogen and phosphorus sources for algal cultivation are both relatively poor. Immediate concerns are directed toward global food production – requiring

nitrogen and phosphorus fertilizers – which will predictably have a demand greater than the supply[94]. Nitrogen fertilizers – namely ammonium/ammonia – for microalgae production are largely produced using the Haber-Bosch process. This process produces ammonia using natural gas as a hydrogen source for the synthesis reaction with an amine solution. In doing so, Daghash (2012) has estimated the generation of 785-999 kg CO_2 per ton of ammonia produced – a considerable contribution of greenhouse gas emissions [95]. Phosphorus-rock is a non-renewable mined mineral, the known global reserve for which is expected to become depleted by the end of this century. Apart from geopolitical and food security concerns, environmental sustainability is at risk due to carbon emissions from processing and transportation of phosphate, the toxic production of radioactive phosphogypsum, as well as the toxic release of heavy metals [94]. To reduce the need for nitrogen and phosphorus fertilizers, wastewater has been proposed as a viable source of nutrients for algal growth [67, 75, 83, 93, 94].

1.4 Wastewater treatment and biological nutrient removal

1.4.1 Influence of nitrogen and phosphorus on aquatic systems

Point and nonpoint sources of nitrogen and phosphorus can greatly influence both freshwater and marine ecosystems. Some point sources of nutrients include: wastewater effluent, waste disposal site runoff, animal feedlot runoff, mining and oil field runoff, and overflow from storm and sanitary sewers; while nonpoint sources include agricultural runoff (from irrigation and pastures/range), atmospheric deposition, and anthropogenic alterations of land [96]. Nitrogen and phosphorus are growth limiting nutrients for autotrophs and the eutrophication – nutrient enrichment of water supplies – stimulates rapid development of algal blooms [97].

Algal blooms in both freshwater and marine ecosystems are detrimental to aquatic life, can be harmful to humans, and cause an economic burden [98]. Nitrogen-ammonium can cause acidification of water systems through biological nitrogen uptake by phytoplankton when

sufficient phosphate is present or through nitrification when ammonium is present in high concentrations[99]. Eutrophic conditions promote algal blooms which have been shown to directly cause hypoxic (and anoxic) waters, leading to physiological disturbances and death of aquatic life [96-98, 100, 101]. Algal blooms comprised of certain dinoflagellates and cyanobacteria are capable of producing toxins such as neuro- and hepatotoxins [44, 96, 98, 102, 103], affecting both fish and human health. In U.S. freshwater systems alone, it is conservatively estimated that eutrophication causes $2.2 billion in damages annually [104, 105].

1.4.2 The Clean Water Act and Colorado Regulation 85

Federal regulation of wastewater discharge is documented under the Federal Water Pollution Control Act (first passed in 1948 with amendments in 1972 and 1977) – renamed as the Clean Water Act – and aims to improve the quality of the U.S.'s water supplies. Under the Clean Water Act, wastewater discharge into water streams became illegal without a permit granted under the National Pollutant Discharge Elimination System. The program provides the EPA with the authority and framework to regulate wastewater treatment, requiring strict standards for nutrient discharge. Thus, the overall goal of wastewater treatment plants is to remove pollutants and contaminants from wastewaters in order to preserve the integrity of domestic fresh water supplies for safe use [106].

The U.S. EPA, under the Clean Water Act, requires state and local governments to design, finance, operate, maintain, and regulate wastewater treatment facilities. Colorado municipal wastewater treatment plants obtain discharge permits according to Colorado Regulation 85 (CR85). Permits limit the annual median of discharged effluent to nutrient concentrations of 7.0 mg L^{-1} Total Inorganic Nitrogen (TIN = [nitrate-N] + [nitrite-N] + [ammonia-N]) and 0.7 mg L^{-1} Total Phosphorus for wastewater discharge permits obtained after May 31, 2012; while discharge facilities with permits obtained prior to that date have more

relaxed limitations set to 15.0 mg L^{-1} Total Inorganic Nitrogen and 1.0 mg L^{-1} Total Phosphorus [107]. Meeting nutrient discharge limits poses challenges for wastewater treatment facilities, requiring advanced bioremediation processes and technology.

1.4.3 Biological nutrient removal processes for N and P removal

1.4.3.1 Current state-of-the-art biological nutrient removal technology

Current state-of-the-art technology for wastewater treatment aims to remove nutrients such as nitrogen and phosphorus prior to discharge in order to preserve freshwater systems. Wastewater treatment facilities incorporate biological nutrient removal (BNR) systems to reduce the nutrient load of the effluent by means of microbial metabolism. Several types of BNR systems exist, but they all function to reduce the total nitrogen and total phosphorus in accordance with EPA standards. The productivity of BNR systems rely on a series/cycle of aerobic and anaerobic digestion, nitrification, and denitrification by different microbial communities under controlled environments [106, 108].

Anaerobic and aerobic digestion cycles allow phosphorus accumulating organisms (PAOs) to remove phosphorus from the influent. PAOs convert volatile fatty acids (organic carbon source) to polyhydroxyalkanoates (PHAs), a stored energy source during anaerobic conditions. Under aerobic conditions, PAOs use the energy stored in PHAs via oxidation to take up phosphorus and phosphate from the wastewater. Some varieties of PAOs are denitrifying and use nitrate to remove phosphorus under anoxic conditions [109].

Removing nitrogen from wastewater influent is a multi-step process. Initially, organic nitrogen is converted into ammonia-nitrogen by non-PAO heterotrophs under aerobic conditions. Then, ammonia-nitrogen is removed though nitrification. Nitrification oxidizes ammonia to nitrate and nitrite using nitrifying autotrophic bacteria. Reactors for nitrification must be controlled at the level of temperature, solids retention time, dissolved oxygen, alkalinity, and pH

for optimal growth of the ammonia oxidizing bacteria. Following nitrification, denitrification must take place to reduce nitrate to nitrogen gas. Heterotrophic bacteria perform this reduction under anoxic conditions when rapidly biodegradable organic matter is available [110].

1.4.3.2 Drake Water Reclamation Facility employs an A^2O BNR system

The Drake Water Reclamation Facility (DWRF) located in Fort Collins, CO uses an Anaerobic – Anoxic – Oxic (A^2O) BNR system for the removal of nitrogen and phosphorus [111]. As Figure 1.1 below depicts, municipal wastewaters enter the treatment plant as the primary influent. Following removal of large obstructive materials – such as trash, personal hygiene products, rocks, and debris– through large bar screens (not depicted), liquid influent travels to a primary settling tank (also not shown for simplicity) to allow organic solids to settle. The settled solids are then transferred to anaerobic digestion tanks for phosphorus removal by PAO. Within the anaerobic digestion tanks the solids from primary settling is mixed with centrate (supernatant following centrifugation of the digested sludge from anaerobic digestions) and return activated sludge (settled solids from the downstream secondary clarifiers). The mixed liquors (wastewater and suspended activated sludge) from the anaerobic digestion tanks are pumped to an anoxic zone for denitrification (microbial oxidation of ammonia into nitrate). Following denitrification, the suspension is moved to an oxic (aerobic) zone for nitrification (microbial oxidation of nitrate to nitrogen gas). The mixed liquor is recycled through the anoxic and anaerobic zones for optimum nitrogen removal. Effluent then travels to a secondary clarifier to separate water and sludge. Water is disinfected (formerly through chlorination, now through ultra-violet irradiation) and then discharged into the Fossil Ridge Ditch for irrigation. Meanwhile, the sludge is pumped back to the anaerobic digestion tanks. Anaerobically digested sludge is removed from the system through a dewatering process whereby digested sludge is centrifuged to separate the liquid fraction (centrate) from the solids. The solids are shipped to the Meadow

Springs Ranch to be used as top soil, while centrate is held in a holding tank and slowly released back to the head of the plant [111].

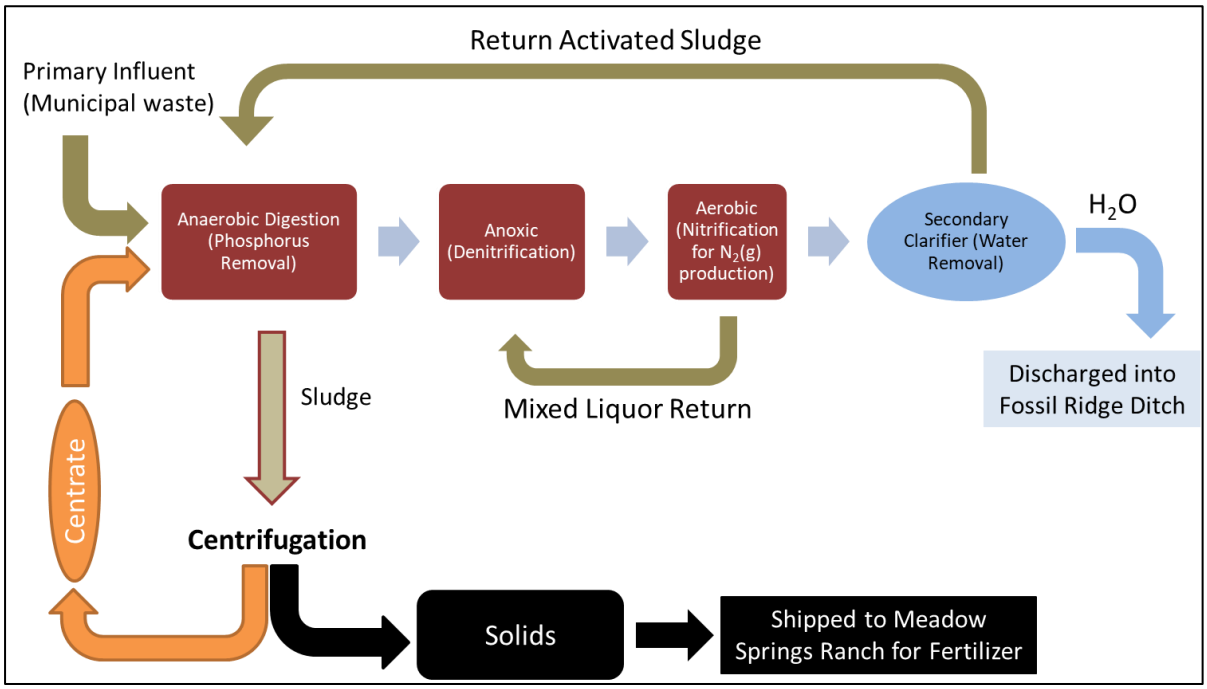

Figure 1.1: Flow chart of the A^2O BNR process used by the Drake Water Reclamation Facility

1.4.4 Costs and challenges of wastewater treatment

1.4.4.1 Upgrade/maintenance costs

Fitting wastewater treatment facilities with the current state of the art biological nutrient removal systems is an expensive endeavor. According to the U.S. EPA Office of Water, upgrading existing wastewater treatment facilities is a cheaper alternative to building new plants using the best available technologies. Estimates (in 2006 Dollars) predict that for new construction of a facility capable of treating 25,000 gallons per day (gpd) would cost $19.34/gpd with operating and maintenance (O&M) costs of $2.10/gpd; whereas to upgrade an existing facility would cost only $3.72/gpd and $0.67/gpd for O&M. The associated costs per gallon for wastewater treatment – for both new construction and upgrades - could be further decreased by

increasing the volume of wastewater treated. Increasing the volume four times to 100,000 gpd would reduce new construction costs to $8.50/gpd and $0.94/gpd for O&M, while the cost to upgrade would drop to $1.47/gpd with an O&M cost of $0.25/gpd [109].

1.4.4.2 Environmental Footprint: GHG and toxic products

Greenhouse gas emissions of methane (CH_4) and nitrous oxide (N_2O) produced during anaerobic digestion and anoxic denitrification, respectively, greatly contributes to the environmental footprint of wastewater treatment facilities. Between domestic/municipal and industrial wastewater treatment facilities, CH_4 emissions totaled 148 MMT of CO_2 Eq in the U.S. during 2015. N_2O emissions during the same year totaled $5.0x10^6$ metric tons of CO_2e from just domestic wastewater treatment [13]. The total environmental footprint of wastewater treatment facilities can be reduced by capturing the biomethane gas produced as a fuel source [112].

Managing the generation and discharge of toxic substances from the biological nutrient removal system is another challenge for wastewater treatment plants. Wastewater centrate, the liquid fraction resulting from the dewatering process of the anaerobic digestion effluent, is a concentrated source of nutrients that cannot be discharged into receiving streams per EPA regulations. Centrate composition varies by geographical location, season, treatment facility, and the nutrient removal processes used [113-117]. Struvite crystal (chemical formula: $MgNH_4PO_4{\bullet}6H_2O$) formation is a common problem that challenges many wastewater plants. Crystals form spontaneously under alkaline conditions, blocking transfer pipes and equipment following anaerobic digestion, as well as forming large crystal deposits in centrate storage tanks [118, 119].

1.4.5 Bioremediation of wastewater using photosynthetic microbes

Wastewater could be a source of nutrients and water to support microalgal growth. Microalgal species would further reduce the nutrient load on wastewater treatment facilities, and

thus would serve as an additional treatment step to allow additional water discharge. Several studies have already been performed demonstrating the bioremediation potential of eukaryotic microalgae growing on wastewater.

Wang *et al.* (2010) evaluated a wild-type *Chlorella* sp. grown on filtered wastewaters taken from different points of a wastewater treatment process flow in St. Paul, Minnesota. Wastewater nutrient characteristics were analyzed, with centrate from sludge centrifugation containing the highest concentrations of ammonia-nitrogen, total phosphorus, total nitrogen, and chemical oxygen demand. Growth curves of *Chlorella* in different wastewaters showed the greatest growth rate and cell density in, as well as the greatest nutrient evolution, when grown in centrate [120].

These results were consistent with studies performed later by Cho et al. (2013) who also measured FAME production following cultivation in diluted wastewaters. Unsurprisingly, the wastewater fraction with the lowest nitrogen availability yielded a significantly higher FAME content over cultures grown in higher nitrogen concentrations [113].

Microalgal species have been compared for their productivity on centrate by Morales-Amaral *et al.* (2015) who compared the ability of the freshwater green algae *Muriellopsis* sp. and *Pseudokirchneriella subcapitata* to grow in centrate without nutrient supplementation. *Muriellopsis* was concluded to be the more ideal strain between the two given a higher centrate tolerance, greater nitrogen removal rates, and more biomass accumulation over *P. subcapitata* [117]. The same group compared two types of open cultivation reactors – thin layer and raceway – for scaled-up production of *Scenedesmus* sp. using centrate as the nutrient source. In this work, the thin layer reactor produced the greater biomass productivity with higher photosynthetic efficiency, using wastewater as the nutrient source for a final minimal production cost estimated at 0.9€ per kg of biomass (approaching the cost recommended previously by Chisti) [121].

Other waste sources for large scale cultivation of photosynthetic microbes have been evaluated by Ji *et al*. (2015). Industrial wastewaters generated from the food sector were used to grow the green alga *Scenedesmus obliquus*. The productivity on minimal growth media supplemented with food wastewater provided higher biomass accumulation, lipid productivity, and nutrient removal efficiency than cultivation in normal growth media [122]. In another study by the same group, municipal wastewater was combined with food wastewater and power plant flue gasses to grow *S. obliquus* mixotrophically [85]. The addition of flue gas containing CO_2 further enhanced the growth and productivity of *S. obliquus* compared to the first study [85, 122]. In conclusion, several nutrients can be sourced from various waste sectors.

1.5 Summary

In order to reduce greenhouse gas emissions, renewable fuel sources need to be pursued. These fuel sources have the potential to come from microalgae given their areal productivity, simple growth requirements, and ability to grow in conditions that would not be productive for terrestrial plants. Additionally, biofuels from microalgae would not compete against resources necessary for food crop production. In order to make algal biofuels more economically viable, cultivation and harvesting processes, conversion processes, nutrient sources, and strain design need to be further optimized. Wastewaters have been shown as a sustainable source of nutrients to support microalgal growth for biofuel production. Growing microalgae on wastewaters would improve the nutrient removal of wastewaters, which is highly regulated by the EPA. Several studies have begun to evaluate eukaryotic microalgal species growth on various wastewaters, but very few have been performed using cyanobacteria. Cyanobacteria would have the additional benefit of being more amenable to genetic modifications; which has been shown to make valuable biofuels such as butanol. Therefore, a comprehensive study evaluating cyanobacterial growth on wastewater should be performed.

2. CHAPTER 2: EVALUATION OF USING WASTEWATER CENTRATE FOR THE CULTIVATION OF *SYNECHOCYSTIS* SP. PCC6803

2.1 Introduction

Wastewater is currently being investigated as a nutrient source for biofuel feedstocks of microalgae and cyanobacteria. In order to produce commercially relevant volumes of biofuel for the transport sector: water, carbon, nitrogen, and phosphorus must be supplied exogenously. Municipal wastewater provides a water source that does not directly compete with freshwater supplies for humans. Additionally, wastewater cannot be used for direct agricultural fertilizer so its use in biofuel production will not compete with established industries. In 2002, the National Research Council estimated that 5.6 million dry tons of sewage sludge – rich in nitrogen, phosphorus, and carbohydrates [123] – was produced from wastewater treatment in the United States [124]. Several studies have indicated that following anaerobic digestion, the liquid removed from sludge (centrate) has the highest concentration of nitrogen and phosphorus within the wastewater treatment process [125-128]. These plant/algal nutrients are concentrated to the extent that the U.S. EPA Clean Water Act prohibits the discharge of centrate into receiving water bodies to reduce the risk of eutrophication [129]. Therefore, centrate provides a source of water, nitrogen, phosphorus, and potentially other nutrients for microalgal growth.

Several studies have been performed evaluating the productivity of eukaryotic microalgae grown on wastewaters. *Chlorella* sp., *Scenedesmus* sp., *Nannochloropsis* sp., and *Chlamydamonas* sp., are among the species most actively used, as they have fairly minimal nutrient requirements and provide high lipid yields [113, 117, 121, 125, 127, 130-136]. In addition to productivity measurements, investigators also evaluate the ability for eukaryotic algae to remove dissolved nutrients from the wastewater [120, 137]. Furthermore, pilot-scale

experiments of eukaryotic microalgae grown in raceway ponds using wastewater as the water and nutrient source have shown that wastewater based systems can be scaled up to industrially relevant conditions [116, 121].

However, there is a large gap in the literature describing the use of prokaryotic cyanobacteria in wastewater systems for potential biofuel production. Uma *et al.* (2002) showed that *Oscillatoria willei* BDU130511 reduced the bacterial and coliform populations in raw sewage, and could therefore have a role in wastewater disinfection [138]. El-Sheekh *et al.* (2014) went one step further by demonstrating that *Nostoc muscorum* and *Anabaena subcylindrica* could be used to treat municipal and industrial wastewaters, as they were able to greatly reduce the nutrient load (specifically phosphorus, nitrate, and ammonia) of the wastewater effluent [139]. Lynch *et al.* (2015) evaluated seven different native cyanobacterial strains (*Synechococcus* sp. *Snowella litoralis, Microcystis* sp., *Scenedesmus* sp., two *Synechococcus* sp., and *Microcystis* sp.) and *Synechocystis* sp. PCC6803 for their ability to accumulate biomass, remediate nutrients, and produce neutral lipids on synthetic wastewater [140]. Yamamoto *et al.* (2014) grew *Aphanothece clathrata* and *Microcystis wesenbergii* on activated sludge filtrate and measured chemical oxygen demand (COD) production rate as an estimator for biomethane production [141].

Few studies have been performed using the model cyanobacterium *Synechocystis* sp. PCC6803 grown on wastewaters. Prior to the work described by Lynch *et al.* (2015) above, Cai *et al.* (2013) compared the lipid production of *Synechocystis* sp. PCC6803 to the eukaryote *Nannochloropsis salina* grown in artificial sea water amended with anaerobic digestion effluent; finding that *Synechocystis* sp. PCC6803 grew faster than *N. salina*, but had lower lipid productivities [142]. Collectively, these experiments demonstrate the feasibility of using

cyanobacteria – specifically *Synechocystis* sp. PCC6803 – for wastewater treatment and potentially for biofuel production.

There is no common consensus on methods and practices for using wastewater as a nutrient source for microalgae production. Investigators commonly neglect to describe comprehensive evaluations of the nutrient content and chemical composition of the wastewaters being used [143]. This is a relevant problem for the reproducibility of results as wastewaters can vary in chemical composition due to factors such as the type of wastewater treatment process, the local and geographical location of the facility, and even seasonal fluctuations in temperature [115, 121]. Additionally, several researchers have used a synthetic or artificial wastewater for their studies using the measured concentrations of only a few nutrients from the wastewater, and supplementing in key micronutrients and adjusting the pH for their organism of interest [144-147]. This approach minimizes the impact of a variety of uncharacterized dissolved organic molecules, and dissolved metals which vary within wastewater. Synthetic media systems might be too benign for relevant physiological measurements [143]. Accurate characterization and reporting of the wastewaters used in the published studies is crucial to further advancing the field.

The focus of Chapter 1 of this thesis is to develop a method to adequately characterize the growth of the model cyanobacterium, *Synechocystis* sp. PCC6803, on wastewater centrate. Initial efforts focused on developing a reliable and repeatable technique for creating centrate based culture media for PCC6803. Next, a method to consistently evaluate and characterize the growth of PCC6803 on centrate was developed. These techniques were then applied to the examination of the centrate remediation capacity (nutrient removal) of PCC6803.

2.2 Materials and Methods

2.2.1 Culture conditions, growth media, and growth measurements

The cyanobacterium *Synechocystis* sp. PCC6803 (PCC6803, herein), generously gifted by Dr. Jianping Yu of the National Renewable Energy Laboratory (NREL), was maintained axenically in BG-11 pH8 growth media modified from [148] as follows: 17.6mM $NaNO_3$, 304µM $MgSO_4$, 245µM $CaCl_2$, 31µM Citric Acid, 22.9µM $C_2H_8O_7 \bullet xFe_3^+ \bullet yNH_3$, 3.4µM EDTA, 1.05mM K_2HPO_4, 189µM Na_2CO_3, 46.3µM H_3BO_4, 14.4µM $MnCl_2$, 0.7µM $ZnSO_4 \bullet 7H_2O$, 1.9µM Na_2MoO_4, 0.3µM $CuSO_4 \bullet 5H_2O$, 0.2µM $Co(NO_3)_2 \bullet 6H_2O$; buffered with 10mM Tes-NaOH buffer (pH 8). All chemicals used for the BG-11media were laboratory grade and purchased from either Thermo FisherScientific® or Millipore Sigma®. PCC6803 was also maintained on 1.5% agar BG-11 plates containing the molar concentrations of the components listed above with the addition of 1mM $Na_2S_2O_3 \bullet 5H_2O$. Unless otherwise noted, PCC6803 cultures were incubated in a Percival Model E30B (Perry, IA) Incubator under 165 µmol photons m^{-2} s^{-1} of continuous photosynthetically active radiation at 30°C. Liquid cultures were grown in 125ml Erlenmeyer flasks on an orbital shaker (VWR, Model 3500).

To monitor growth of cultures, *in-vivo* chlorophyll fluorometry (IVF) [149] was used as a proxy for biomass accumulation. A Turner Designs Trilogy® Fluorometer fitted with the blue light module (460nm LED, 441-482nm excitation, 660-710nm emission) was used to measure *in vivo* chlorophyll *a* fluorescence (1ml of culture in a 10x10mm 4ml optical acrylic cuvette (Sarstedt Ag & Co; Nümbrecht, Germany) or in a 2ml Eppendorf tube with the Turner 12mm round vial adapter. Density of the cultures (cells ml^{-1}) was also monitored using a Reichart Bright-Line Hemacytometer with Neubauer ruling (Hausser Scientific; Horsham, PA) under 400x magnification with a bright field microscope.

The relative exponential growth rates were calculated for PCC6803 growing in different media by calculating the slopes from the natural log transformation of exponentially growing culture densities.

2.2.2 Evaluation of PCC6803 tolerance to wastewater centrate

The supernatant from the wastewater dewatering process of anaerobically digested sludge (centrate) was collected directly from a decanter centrifuge (Alfa Laval/Sharples® Model DS 706) at the City of Fort Collins Drake Water Reclamation Facility (Fort Collins, CO) in 250ml plastic bottles.

2.2.2.1 Biotic factors influencing wastewater centrate toxicity

To test if microorganisms from the biological nutrient removal process remained in centrate and could survive on the nutrients provided by BG-11 media, 200µl of raw (non-filtered, non-sterilized) centrate was pipetted alongside 200µl PCC6803 onto 1.5% agar BG-11 medium. Additionally, 200µl of each PCC6803 and raw centrate were mixed on a 1.5% agar BG-11 plate to test if native microorganisms directly inhibited the growth of PCC6803. For liquid cultures, 25mls of BG-11 was supplemented with 25mls raw centrate, and inoculated with 1ml of PCC6803 in late exponential phase. Plates and liquid cultures were incubated as described above for five days before being photographed (Canon PowerShot SX260 HS). Positive growth of contaminants was scored as colonies or lawns that did not appear in the PCC6803 alone treatment.

2.2.2.2 Sterilization of centrate by pH and temperature treatment

To remove native microorganisms from centrate, pH and temperature treatment of centrate was conducted using a modified method from [150, 151]**.** Centrate (pH ~7.5-8, measured with ColorpHast® pH-indicator strips) was vacuum filtered through a 0.2µm filter (Thermo Scientific™ Nalgene™ Rapid-Flow™ 75mm Bottle Top Filter-500ml) to remove any remaining

solids and large particles. In a separate set of experiments, the pH of centrate was reduced to pH 2 using 3ml 12M HCl and then kept in a 40°C water bath (Fischer Scientific Model 2329) for 24 hrs. The pH was titrated back to pH ~7.5-8 with 2.5ml 5M NaOH and then kept at 4°C until experimental use.

2.2.2.3 PCC6803 tolerance to centrate in BG-11 media

To determine the maximum concentration of centrate PCC6803 could tolerate under optimal conditions, centrate was diluted with BG-11 media at 11 concentrations between 10% and 100% centrate. PCC6803 was also grown on diluted centrate + BG-11 + 1.5% agar plates (Centrate plates henceforth). Centrate was filtered as described above, diluted to the desired concentrations with 18.2 MΩ H_2O, and added directly to melted BG-11 + agar before plates were poured.

2.2.2.4 Abiotic factors influencing wastewater centrate toxicity

Flocculating polymers are added during the centrifugation process of dewatering to promote the flocculation of small particles to form larger solids. HydroFloc 1687 cationic flocculant polymer (proprietary blend, AQUA BEN CORP) in solution (~0.2% w/v in water) was generously provided by the Drake Water Reclamation Facility (Fort Collins, CO). The flocculant was diluted to 5% and 10% (v/v) in BG-11 media. This is the typical concentration range used by DWRF during dewatering. The flocculant supplemented growth media was inoculated with 500µl of PCC6803 culture in mid-exponential growth. Growth was monitored by measuring and recording chlorophyll fluorescence twice daily. Photographic images were taken after 10 days of incubation.

2.2.2.5 Chemical changes of centrate

The pollution control lab at the DWRF conducts weekly analysis of the ammonia (NH_3) concentration in centrate from both the north and south centrifuges using EPA Method 350.1

"Determination of Ammonia Nitrogen by Semi-automated Colorimetry". Weekly analysis results for ammonia were generously provided by Link Mueller of the DWRF, and are presented as a function of time through the entire duration of this project.

A compound microscope was used to observe a sample of 0.2μm filtered centrate following 24 hrs of incubation at 4°C. Images were taken of precipitates observed at 600x magnification.

2.2.2.6 Maximum concentration of centrate supporting growth

Eight hours post centrate collection, centrate was diluted in sterile water to final concentrations of 3, 7, 9, 15, 17, 19, 21, 23, 25% v/v. PCC6803 in mid exponential growth phase (OD_{750} 0.4) was diluted to $2.5x10^6$ cells/ml. Cells were cultured in 15ml of media in 50ml flasks. A positive control of PCC6803 in BG-11 was also included using the same starting cell density. Cultures were incubated under 165 μmol photons m^{-2} s^{-1} of continuous light at 30°C on an orbital shaker (VWR, Model 3500). The relative chlorophyll fluorescence was measured daily until stationary phase was reached. All treatments were repeated in triplicate.

2.2.3 Nitrogen removal capacity of PCC6803 grown on just centrate

2.2.3.1 Culture conditions

Wastewater centrate was collected and treated as previously described. 8 hours post collection, centrate was diluted in sterile water to final concentrations of 9,19, and 25% v/v. PCC6803 WT in early exponential (OD_{750} 0.15) was diluted to $2.5x10^6$ cells ml^{-1}. Aliquots of culture were spun down at 3220g for 15 min, and the supernatant removed. Pellets were re-suspended in 5ml of the centrate concentrations described above and then distributed into 100ml bottles containing 95mls of the respective centrate media. Cell counts were performed again, indicating an average cell density for each culture to be $2.13x10^6$ cells*ml^{-1}. 25ml of each inoculated centrate dilution were distributed to each of 4x 125ml flasks. A positive control of

PCC6803 in BG-11 (n=4) was also included using the same starting cell density. Cultures were incubated under 165 µmol photons m^{-2} s^{-1} of continuous light at 30°C on an orbital shaker. Aliquots of each culture were pipetted into a 2ml Eppendorf tube with 1% Lugols iodine solution, and the cell densities were calculated using cell counts with a haemocytometer daily.

2.2.3.2 Centrate media collection and sample dilutions

Pre-treatment (Day_0) samples of the centrate media were collected prior to inoculation with PCC6803 as follows. 10ml of each centrate dilution prior to inoculation with PCC6803 was spun down in an acid washed (10% HCl) 15ml conical tube with 1% Tween by volume at 3220x g, 4°C for 10 minutes as a standardized collection method. In order to keep within the autoanalyzer's linear detection range (0.5mg ml^{-1} to 5 mg ml^{-1}) for nitrogen, and assuming an initial ammonia-nitrogen concentration of 850 mg ml^{-1} (average ammonia-nitrogen concentration of DWRF centrate measured November 2016 through February 2017), 9% centrate samples were diluted 1:16, 19% centrate samples were diluted 1:40, and 25% centrate samples were diluted 1:80 in DI water to a final volume of 40mls.

The centrate media was harvested following the incubation period as follows. 15mls of each culture were spun down in an acid washed (10% HCl) 15ml conical tube with 1% Tween by volume at 3220g, 4°C for 20 minutes to promote complete pelleting of the cells. 10ml of the 9% and 19% centrate cultures supernatant, and 500µl of the 25% centrate cultures were transferred into acid washed 40ml glass vials, and brought to a final volume of 40mls with DI water for final dilutions of 1:4 and 1:80, respectively. The remaining cell pellets were re-suspended in a 3.5% w/v NaCl solution, pelleted again by centrifugation, and the pellet re-suspended in 1.1ml fresh NaCl solution. A 100µl sample was pulled for cell counts with 1% Lugol's iodine solution added. Cells were flash frozen at -80°C and preserved for future analysis.

An additional experiment was performed to quantify the amount of nitrogen that naturally precipitates out of centrate without the addition of PCC6803. The experimental design and centrate concentrations followed that described in 1.3.1 above without the addition of PCC6803 culture.

2.2.3.3 Total Nitrogen and Total Organic Carbon analysis

To determine the amount of nitrogen removed from the centrate media by PCC6803, total nitrogen analysis was performed on pre- and post-treatment samples using a Shimadzu TOC-L Autoanalyzer with the Total Nitrogen Module (TNM-L). The method described in Caballero *et al.*., 2016 was used with the changes in media collection described above.

2.2.4 Statistics

Statistical analysis was performed using Sigma Plot (v 1.3, Systat Software Inc.) on independent experiment replicates. A one-way ANOVA was used to compare relative exponential growth rates of PCC6803 in different centrate concentrations with Tukey's post-hoc test. An unpaired t-test was used to compare the average TN values of Day_0 and Day_{14} centrate media samples (without PCC6803). For both statistical tests, samples with $p<0.05$ were considered statistically different and data are presented as the means ±1 standard deviation (SD) throughout the chapter unless otherwise noted.

2.3 Results

2.3.1 Evaluation of PCC6803 tolerance to Centrate

2.3.1.1 Biotic Factors Influencing Wastewater Centrate Toxicity

Raw centrate contained native microorganisms capable of growing in BG-11 medias. I compared a lawn of PCC6803 growing on BG-11 + agar medium (Figure 2.1A) to raw centrate plated onto BG-11 + agar medium (Figure 2.1B). The raw centrate contained numerous species of microorganisms capable of growing on BG-11 media. This was based off of the visual

observation of the various size, color, and textures of the microbial colonies observed (Figure 2.1B). When raw centrate was mixed with PCC6803 culture, clear plaques formed within the lawn of our cyanobacteria indicating the presence of an inhibiting species or toxin (Figure 2.1C). Native microorganisms were also able to grow in BG-11 liquid media, as I observed the growth of brown and white flocculants that did not resemble PCC6803 (Red box in Figure 2.1D). Isolated native bacterial colonies that were re-plated on BG-11 agar medium and incubated in complete darkness (wrapped in foil) were capable of growing on BG-11 + agar without an organic carbon source (Figures 2.1E-G).

2.3.1.2 Sterilization of centrate to remove native microorganisms

In an attempt to remove the native microorganisms (described above) from the centrate prior to use as a media supplement, centrate pre-treatment methods described in [152] were replicated. While no contamination was observed in cultures growing in the treated centrate, the treatment allowed PCC6803 to grow in 100% treated centrate (Figure 2.2**),** suggesting some chemical modification generated by this treatment. I therefore only filter sterilized centrate in the subsequent experiments.

2.3.1.3 PCC6803 tolerance to centrate in BG-11 media

The growth of PCC6803 in filtered centrate diluted with BG-11 media was monitored by *in vivo* chlorophyll fluorescence. The maximum centrate concentration tolerated by PCC6803 was 18% centrate in BG-11 media (pink line in Figure 2.3A). Growth was completely inhibited from 20% through 100% centrate; however in other replicate experiments PCC6803 tolerated up to 20% centrate (Data not shown).

Tolerance to centrate concentration was also tested on BG-11 agar plates. PCC6803 was able to grow on 15% centrate plates, but was inhibited on 25% centrate plates (Figure 2.3B).

While the 15% centrate plates showed growth as lawns/smears, independent colonies were not observed.

2.3.1.4 Abiotic factors influencing centrate toxicity

Flocculant polymers are added by the DWRF to increase the solid yields during dewatering of digested sludge. We tested if HydroFloc flocculating polymer in solution added directly into BG-11 media would influence centrate toxicity to PCC6803. As Figure 2.4A shows, both 5% and 10% flocculant in BG-11 yielded longer lag phases, but both cultures were able to recover and reach nearly the same relative chlorophyll fluorescence yield as the control culture (Figure 2.4B).

2.3.1.5 Chemical changes of centrate

The DWRF takes weekly measurements of the total ammonia nitrogen in centrate that reveal changes in centrate nutrient composition. All of the weekly sampling points over the course of the project are presented in Figure 2.5, where the blue dotted line indicates the mean ammonia concentration of 887.62mg $NH_3\,L^{-1}$ ± 74.54 mg $NH_3\,L^{-1}$. Nearly 74% of the sample measurements fell in the range of 800-900 mg $NH_3\,L^{-1}$. Two data points reached NH_3 concentrations above 1100 mg $NH_3\,L^{-1}$. In addition to weekly fluctuations, large seasonal oscillations in ammonia concentration were detected.

2.3.1.6 Maximum concentration of centrate supporting growth

To test centrate as the sole nutrient source for PCC6803 growth, 0.2μm filtered centrate was diluted in sterile water to final concentrations of 3% up to 25% centrate. After 3 days of incubation, cultures grown in low centrate concentrations (3,7, & 9%) had reached stationary phase (Figure 2.6A), but had 8.9, 4.7, and 3.8 times lower relative fluorescence (RFU) values, respectively, than BG-11 grown cultures at the same time point (Figure 2.6C). The 3, 7, and 9% centrate cultures had a one day lag phase; whereas 15, 17, and 19% centrate cultures had a lag

phase of 2-2.5 days, and 21% centrate cultures had nearly a 5-day lag phase. The 15, 17, and 19% centrate grown cultures reached RFU values that were 2.0, 1.6, and 1.8 times lower RFUs than BG-11, respectively. The 21% centrate cultures had the longest lag phase before entering into exponential growth and had 2.7 times lower chlorophyll fluorescence than BG-11 grown cultures. No growth was observed in the 23 and 25% centrate cultures.

Additionally, BG-11 grown cultures had a significantly higher relative exponential growth rate compared to all centrate grown cultures (Figure 2.6B). The relative exponential growth rate of BG-11 cultures was 1.93 ± 0.18 day^{-1} which was significantly higher than all centrate grown cultures ($p<0.001$, one-way ANOVA). 3% centrate cultures had an average relative exponential growth rate of 0.864 day^{-1} (± 0.03 day^{-1}) and was not statistically different from the relative growth rates of 7-21% centrate cultures ($p>0.05$, one-way ANOVA). 7, 9, and 15% centrate grown cultures had significantly higher relative growth rates (1.07 ± 0.03 day^{-1}, 1.09 ± 0.01 day^{-1}, 1.08 ± 0.07 day^{-1}, respectively) compared to 17, 19, and 21% centrate grown cultures (0.65 ± 0.25 day^{-1}, 0.57 ± 0.27 day^{-1}, 0.57 ± 0.05 day^{-1}), respectively) as determined by a one-way ANOVA ($p<0.05$).

2.3.2 Nitrogen removal from centrate

2.3.2.1 Bioremediation of Nitrogen from centrate by PCC6803

PCC6803 was grown in diluted centrate to estimate the amount of nitrogen the cells are able to remove from centrate. Total dissolved nitrogen (TN) analysis was performed on the media only.

BG-11 grown cultures grew faster and to higher final culture densities compared to centrate grown cultures, as observed above (Figure 2.7A & B). Cultures grown in 9% centrate once again had a shorter lag phase relative to the higher centrate concentrations, although in this experiment the difference in lag phase was 3 days compared to the 1 day observed previously.

Despite the longer lag phase, 19% centrate cultures reached a significantly higher final culture density than 9% cultures. Once again, cultures did not survive 25% centrate. The final cell densities of the cultures in 9, 19, and 25% centrate at the time of harvest were $1.56x10^7$ $\pm 3.86x10^6$ cells ml^{-1}, $2.25x10^7$ $\pm 3.51x10^6$ cells ml^{-1}, and $9.13x10^5$ $\pm 3.86x10^4$ cells ml^{-1}, respectively. PCC6803 grown in BG-11 grew to $5.57x10^8$ $\pm 3.7x10^7$ cells ml^{-1}. The final culture density of BG-11 grown cultures was over a full order of magnitude greater than centrate grown cultures; and there were 31.4 and 23.1 times more cells in BG-11 grown cultures than in 9 and 19% centrate grown cultures, respectively. Additionally, the number of cells in 25% centrate was reduced to half of that in the inoculum by the end of the incubation period.

The total dissolved nitrogen (TN) in the media was reduced for all three centrate dilutions (Figure 2.7C). For cultures grown in 9, 19, and 25% centrate, the total dissolved nitrogen (TN) of centrate was reduced by 8.95 ±1.87 mg N L^{-1}, 42.24 ±2.58 mg N L^{-1}, and 49.78 ±17.17 mg N L^{-1}, respectively. These TN values describe a decrease of 14.4%, 31.3%, and 29.4% TN in the 9%, 19%, and 25% centrate cultures, respectively. The reduced TN did not correlate with the change in cell densities of the cultures in 9, 19, and 25% centrate, which were $1.35x10^7$, $2.03x10^7$, and $-1.2x10^6$ cells ml^{-1}, respectively. The cell densities of cultures grown in centrate were all significantly lower than that of PCC6803 grown in BG-11 – which grew to $5.57x10^8$ $\pm 3.7x10^7$ cells ml^{-1} – indicating that diluted centrate does not provide adequate nutrients to support optimal growth. Conversely to the TN reduction, total organic carbon (TOC) increased in all three centrate dilutions (Figure 2.7D). TOC increased by 2.05 ±0.03 times, 1.56 ±0.29 times, and 1.65 ±0.36 times following incubation for 9%, 19%, and 25% centrate culture media, respectively.

2.3.2.2 Nitrogen precipitation out of centrate without PCC6803 inoculum

Nitrogen precipitates out of centrate – along with equimolar concentrations of phosphate and magnesium – as struvite crystals [118]. Struvite can be found in both centrate, as well as in

the solid fractions following sludge centrifugation (Figure 2.8B). We hypothesized that the unexpected reduction in nitrogen in all centrate dilutions – particularly 25% centrate in which the culture did not survive – is due to the precipitation of struvite.

We repeated the experiment described in 3.2.1 above, without the addition of PCC6803 inoculum. For each of the three centrate dilutions tested (9%, 19%, and 25% centrate), the TN was significantly reduced by 51.5% ± 8.1%, 56.2% ±4.7%, and 43.3% ± 3.6%, respectively after 14 days (Figure 2.8A).

Struvite crystals have frequently been observed in filtered centrate stored at 4°C after 24 hours (Figure 2.8C), as well as at the bottom of flasks following incubation (images not shown). Crystals were viewed under 600x magnification and images captured (Figure 2.8D) for reference. PCC6803 was grown in diluted centrate to estimate the amount of nitrogen the cells are able to remove from centrate. Total dissolved nitrogen (TN) analysis was performed on the media only.

BG-11 grown cultures grew faster and to higher final culture densities compared to centrate grown cultures, as observed above (Figure 2.7A & B). Cultures grown in 9% centrate once again had a shorter lag phase relative to the higher centrate concentrations, although in this experiment the difference in lag phase was 3 days compared to the 1 day observed previously. Despite the longer lag phase, 19% centrate cultures reached a significantly higher final culture density than 9% cultures. Once again, cultures did not survive 25% centrate. The final cell densities of the cultures in 9, 19, and 25% centrate at the time of harvest were 1.56×10^7 $\pm 3.86 \times 10^6$ cells ml^{-1}, 2.25×10^7 $\pm 3.51 \times 10^6$ cells ml^{-1}, and 9.13×10^5 $\pm 3.86 \times 10^4$ cells ml^{-1}, respectively. PCC6803 grown in BG-11 grew to 5.57×10^8 $\pm 3.7 \times 10^7$ cells ml^{-1}. The final culture density of BG-11 grown cultures was over a full order of magnitude greater than centrate grown cultures; and there were 31.4 and 23.1 times more cells in BG-11 grown cultures than in 9 and

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Struvite crystals have frequently been observed in filtered centrate stored at 4°C after 24 hours (Figure 2.8C), as well as at the bottom of flasks following incubation (images not shown). Crystals were viewed under 600x magnification and images captured (Figure 2.8D) for reference.

2.4 Discussion

2.4.1 Evaluation of centrate as a media source

The first objective of this study was to gain a better understanding of centrate as a potential nutrient source for culturing *Synechocystis* sp. PCC6803. The literature describes the chemical and biological composition of centrate being dependent on numerous factors. Factors include: the source of the wastewater; the physical, chemical, and biological nutrient processes by which the influent is treated; as well as the geographical region in which the wastewater is being treated [115, 116, 153-156]. Given the tremendous variability in the reported centrate compositions – as well as the tendency for researches to produce unrealistic artificial wastewater centrate for consistent laboratory experiments – I sought to perform an array of studies to produce a standard method for using centrate sourced from A^2O wastewater treatment plants.

2.4.1.1 Competition by native microorganisms

Early observations of centrate grown cultures becoming quickly contaminated led to the hypothesis that native flora had survived the dewatering process and could tolerate centrate. Standard BG-11 agar plates were used to assess the biological properties of centrate. Even on the minimal BG-11 media, where the only organic carbon is agar, several clearly distinct types of bacteria were detected. Liu *et al.* (2017) also recognized diverse microbial communities present in centrate, leading the group to isolate and identify a native *Acinetobacter* sp. within their centrate sample [157]. Rather than inhibiting growth, however, *Acinetobacter* sp. developed a synergistic cooperation with a lab strain of *Chlorella* sp. Yamamoto *et al.* (2016), however, showed that anti-microbial treatment of wastewater was necessary to prevent the inhibitory

effects of native microorganisms on the cyanobacteria *Aphanothece clathrata* and *Microcystis wesenbergii*. Opposing results from my experiments and throughout the literature might be indicative of various microbial communities amongst the wastewaters tested.

I observed plaque formation in a lawn of PCC6803 (Figure 2.1B) which suggests that viruses or allelopathic chemicals are present in centrate that inhibit PCC6803 growth. Plaque formation could be indicative of the presence of viruses that attack prokaryotic species (phages). Jamal *et al.* (2017) isolated a bacteriophage (of the *Siphoviridae* family) from municipal wastewater that significantly inhibited the growth of *Pseudomonas aeruginosa*-2995 [158]. Viruses in wastewater that specifically infect cyanobacteria (cyanophages) have been described by Cannon and Stanley (1977). The authors identified an LPP-cyanophage that was present in wastewater year round, and attacked the filamentous cyanobacterial generas: *Lyngbya, Phormidium, and Plectonem* [159]. Additionally, plaque formation could be caused by toxic metabolites produced from native microorganisms. Shunmugam *et al.* (2013) discovered that *Nostoc* XPORK14A produces a non-protein metabolite called M22 that directly inhibits the growth of PCC6803 [160]. Further exploration of the plaques formed in lawns of PCC6803 spiked with raw centrate could provide valuable insight into factors contributing to centrate toxicity. Minimizing centrate toxicity will be important for mass cultivation of PCC6803 in order to maximize the efficacy of using cyanobacteria to remediate nutrients and as a biofuel feedstock.

I used chemical and physical treatments to sterilize centrate for use as a growth media. The pH and temperature treatment of centrate allowed PCC6803 to grow in undiluted centrate. This was dramatically different from our preliminary growth measurements of PCC6803 in filtered or untreated centrate, where concentrations above 20% lead to growth inhibition (Figure 2.2). The pH treatment of centrate could be affecting the chemical structures of potentially toxic substances; considering that acids can degrade organic compounds by catalyzing hydrolysis

reactions [161]. As shown by Zhao *et al.* (2013), buffer solutions containing different concentrations of HCl prepared at different pH's chemically degraded an organic pharmaceutical drug used for cancer treatment by hydrolysis reactions at 37°C. The pH of the solution dictated the location of the hydrolysis reaction on the parent compound, resulting in three potential degradant products [162]. This experiment provides evidence that HCl treatment of pH 1.0 at 37°C chemically altered the organic compound studied. Elemental analysis and TN analysis, could provide evidence to elucidate broad chemical composition changes, while reverse-phase high-performance liquid chromatography (RP-HPLC) and liquid chromatography-mass spectrometry (LC-MS)[162] could be used to identify putative toxin degradation during the extreme pH shifts. Regardless, I opted to pursue filtration as a means to sterilize centrate for the remainder of the experiments to lessen any chemical modifications associated with heat/chemical treatment.

2.4.1.2 Growth inhibition by flocculating polymers

I tested the addition of a synthetic flocculant polymer – used by DWRF during dewatering – in normal BG-11 media on the growth of PCC6803. The two concentrations of flocculant both resulted in an increased lag phase over the BG-11 control culture. While AQUABEN HydroFloc 1687 is proprietary, it is assumed to be anionic in nature to remove metals during dewatering steps. Cationic polymers have been shown to be toxic to many aquatic species, whereas anionic polymers are typically not toxic – except to algae which require relatively high concentrations of cationic metals as nutrients[163]. Roselet *et al.* (2017) showed that the use of a synthetic polyacrylamide-based flocculant generated dose-dependent interference with the photosynthetic capacity of *Chlorella vulgaris* [164]. Whether the prolonged lag phase observed with PCC6803 grown with flocculant is due to metal limitations in the media (due to the anionic polymer) or directly by unknown components of the polymer is an open question at this time.

2.4.1.3 Centrate composition is subject to seasonal changes

Centrate from the Drake Water Reclamation Facility undergoes seasonal fluctuations in chemical composition. Weekly ammonia-nitrogen analysis performed by the DWRF Pollution Control Lab shows lower ammonia-nitrogen concentrations during the colder months, while the summer months had a greater centrate ammonia-nitrogen content (Figure 2.4). Important to note is that the DWRF was undergoing several facility upgrades between 2015 and 2017 that might have affected annual average ammonia concentrations. Recalling that the goal of wastewater treatment is to remove nutrients from the discharged water, higher nitrogen ammonia-nitrogen content in the centrate would indicate greater and more successful nitrate reduction from the influent. The seasonal – and presumably temperature – dependent fluxes in centrate composition were also identified by Herrera (2009) who performed monthly chemical analysis on centrate produced in Reno, NV [115]. Year-round use of centrate for biomass production could be complicated by climates with extreme changes of the seasons and weather. Chapanova *et al.* (2007) adjusted the temperatures of an aerobic reactor used in wastewater treatment and evaluated the resulting changes in nutrient remediation [165]. The authors showed that a shift in temperature from 25-30°C down to 5°C reduced the ammonium removal rate by a factor of 5. Colorado can see average monthly temperature fluctuations from -8°C up to 32°C (usclimatedata.com) Therefore, in climates with large seasonal shifts in temperature, the nutrient composition of wastewater should be expected to fluctuate greatly and would in turn affect annual productivity of both bioremediation and biofuel feedstock production.

The average ammonia concentration in undiluted centrate is likely toxic to PCC6803. Ammonia concentrations of 850 mg NH_3 L^{-1} have been shown to inhibit PCC6803 growth by destroying the manganese cluster in the PSII oxygen-evolving complex [166]. In non-diluted centrate, the average ammonia concentration between Fall of 2015 through Summer of 2017 was

almost 900 mg $NH_3\,L^{-1}$ (Figure 2.4). In dilutions of centrate permitting the growth of PCC6803 (<20% centrate, see figure 2.2), I would expect an average ammonia concentration of <180 mg $NH_3\,L^{-1}$, which is well below the toxic ammonia concentration. Since PCC6803 cannot survive in undiluted centrate, centrate will most certainly need to be diluted for full scale production of PCC6803 biomass on centrate.

2.4.2 Characterization of PCC6803 growth on centrate

The second major objective of this work was to illustrate how PCC6803 grew on centrate as the sole nutrient source. Since wastewater is being explored as a cheap and sustainable source of nutrients for microalgal growth, it is important to demonstrate that the organism of interest can indeed grow on wastewater without requiring excess nutrient supplementation to achieve high biomass [167]. The mutualistic benefit of bioremediation of nutrients from wastewater by microalgae and cyanobacteria for the production of biomass for biofuel can be elucidated by measuring the change in nutrient composition of centrate after use as a growth media.

2.4.2.1 Dynamic between nutrient availability and centrate toxicity

Insight into the balance between nutrient limitation and centrate toxicity was provided by growing PCC6803 in a dilution series of centrate. I measured relative chlorophyll fluorescence (RFU) as a proxy for biomass accumulation. In centrate dilutions below 10%, I observed a reduced lag phase compared to higher centrate concentrations, and a step-like decrease in the maximum chlorophyll fluorescence at stationary phase as the concentration of centrate in water decreased below 10%. This interaction may be explained by a study performed by John Caperon (1968), who demonstrated that under various nitrate concentrations – where nitrate was the limiting nutrient – the growth rate of *Isochrysis galbana* was determined by the nitrate uptake rate; while the final cell density was dependent on the initial concentration of nitrate [168].

Caperon is describing Liebig's Law of the minimum, which could also be describing the interactions of PCC6803 grown on different concentrations of centrate are used.

I observed the duration of the lag phase of PCC6803 increased as centrate concentration increased until PCC6803 growth was completely inhibited. The observation of a lag phase is in contrast to what was observed by Cai *et al.* (2013) who grew *Synechocystis* sp. PCC6803 on artificial wastewater and did not observe a lag phase, rather a consistent increase in the biomass accumulation from the initial inoculation [142]. I would expect though, that artificial wastewater would not contain uncharacterized toxic substances; which might explain the lack of a lag phase in Cai *et al.*'s observations. Wang *et al.* (2010) evaluated the biomass accumulation of a wild-type *Chlorella* sp. on wastewater from different points of wastewater treatment, but growth curves of culture grown on filtered centrate did not show a lag phase [120]. The authors report that the absence of a lag phase is most probably due to the adaptive abilities of the wild-type *Chlorella* sp. used in their experiments. Each of these experiments have their limitations though for large scale bioremediation of wastewater and advanced biofuel production; as artificial wastewater was used in the first experiment and a wild-type eukaryotic microalgae were used in the second.

PCC6803 cultures did not survive in centrate dilutions above 21%. This finding is similar to that observed for *Nannochloropsis gaditana* by Ledda *et al.* (2015) who showed optimal biomass accumulation in and optimal nitrogen removal from 20% centrate [116]. However, *Scenedesmus* sp., *Muriellopsis* sp., *Pseudokirchneriella subcapita* have all been shown to tolerate centrate concentrations up to 40 and 50% centrate, but above these concentrations, ammonia toxicity was suggested to inhibit growth [117, 121]. As mentioned above, the ammonia concentration in diluted centrate was not anticipated to contribute to toxicity for PCC6803, given

the previously reported ammonia tolerance of 850mg N L^{-1} [166]. Clearly, centrate composition can become toxic within a narrow range of centrate concentrations.

2.4.2.2 TN analysis of centrate media shows complex dynamic of nutrient precipitation and nutrient utilization

I evaluated the ability of PCC6803 to remove nitrogen from centrate media. PCC6803 did not survive in 25% centrate, but total dissolved nitrogen analysis of the centrate media suggested the greatest amount of nitrogen reduction compared to the centrate prior to being inoculated. I performed rough calculations to determine the expected nitrogen removal (mg N L^{-1}) from the system by multiplying the final cell density of the culture (cells L^{-1}) by the nitrogen content per cell ($1.1x10^{-10}$ mg N $cell^{-1}$ from TOC/TN analysis of *Synechocystis,* Bjoern Andersson: manuscript in preparation). Using these calculations, I estimated that 9% centrate grown cultures should remove 1.47 mg N L^{-1}, 19% centrate grown cultures should remove 2.18 mg N L^{-1}, and 25% centrate cultures should add back 0.127 mg N L^{-1}due to cell death. In contrast to these predicted values, 9 mg N L^{-1}, 42 mg N L^{-1}, and 49 mg N L^{-1} was removed from the media, respectively. These calculations revealed that nitrogen was removed in excess of what cells should have been able to use in all centrate dilutions based on cell densities and nitrogen content per cell.

I tested if the presence of PCC6803 significantly influences the removal of nitrogen by incubating dilutions of centrate without inoculum and then measured total dissolved nitrogen. The TN was reduced by ~50% for all dilutions in the absence of culture, making it clear that the excess nitrogen removed from the media in the previous experiment was not due to the uptake of NH_3 by PCC6803. This data suggests that PCC6803 prevents nitrogen precipitation. Nitrogen precipitates out of centrate as magnesium ammonium in alkaline conditions, which is a common characteristic of centrate [169]. PCC6803 could be influencing the centrate by utilizing any

bioavailable phosphate or magnesium which would in turn affect the availability of nutrients in relation to the 1:1:1 molar ratio of magnesium : nitrogen: phosphorus to produce struvite [170]. Another consideration is that pH of the media affects the solubility of struvite ions in solution, such that struvite is soluble in acidic conditions [171]. Oxygenation of centrate by PCC6803 during oxygenic photosynthesis could prevent the removal of dissolved CO_2 [172], which would thereby reduce the pH, and thus increase the solubility of struvite in solution. A pH test at the end of the exponential growth phase would have been useful for elucidating the mechanism by which PCC6803 prevents struvite precipitation.

2.5 Conclusions

The series of experiments presented in this work provided novel insight into using centrate as a media source for PCC6803. I first showed that centrate is host to many native microorganisms some of which may inhibit PCC6803. Centrate composition is variable throughout the year due to the effect of changing temperatures, which could make year-round cultivation on centrate a major challenge due to fluctuations in nutrient availability and the negative influence of low temperatures on cyanobacteria [173]. The final culture densities (based on *in vivo* fluorescence measurements) increased in increasing concentrations of centrate up to 17% centrate; suggesting that the nutrient concentration of centrate limits final biomass accumulation up to this level. Centrate becomes toxic at concentrations above 21%. Evaluating centrate for the presence of bacteriophages or toxic natural products would be very useful for future work. Elemental analysis of the centrate would provide insight into nutrients limiting biomass accumulation. Identifying limiting nutrients in centrate could improve the biomass accumulation of PCC6803 to industrially relevant concentrations; thereby making PCC6803 (or other photoautotrophic microbes) a more economically viable candidate for simultaneous wastewater remediation and biofuel production from centrate.

2.6 Figures

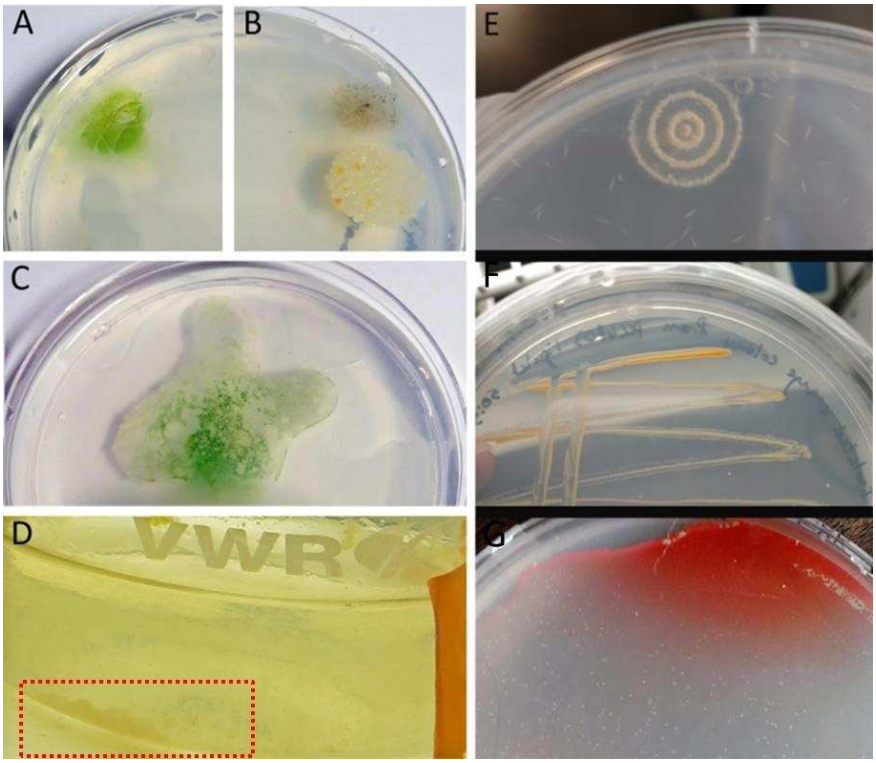

Figure 2.1: Native microorganisms in centrate capable of growing on BG-11 agar media. A) BG-11 agar plate with PCC6803. B) Native microorganisms in wastewater centrate. C) Plaque formation in PCC6803 mixed directly with centrate. D) Typical contamination observed in raw centrate diluted in BG-11 liquid media. The panels of E-G shows an array of the isolated contaminates from the raw centrate growing on BG-11 agar.

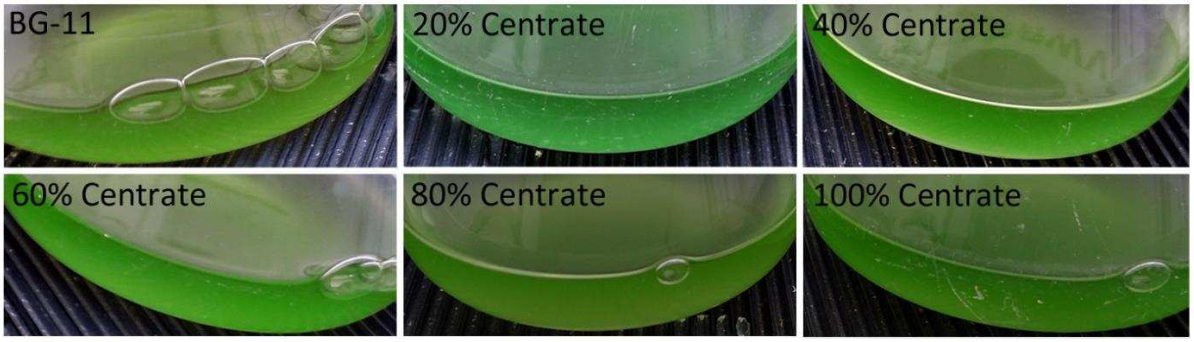

Figure 2.2 PCC6803 in pH and Temperature treated centrate. Images collected of PCC6803 in increasing concentrations of centrate diluted in BG-11 (n=1).

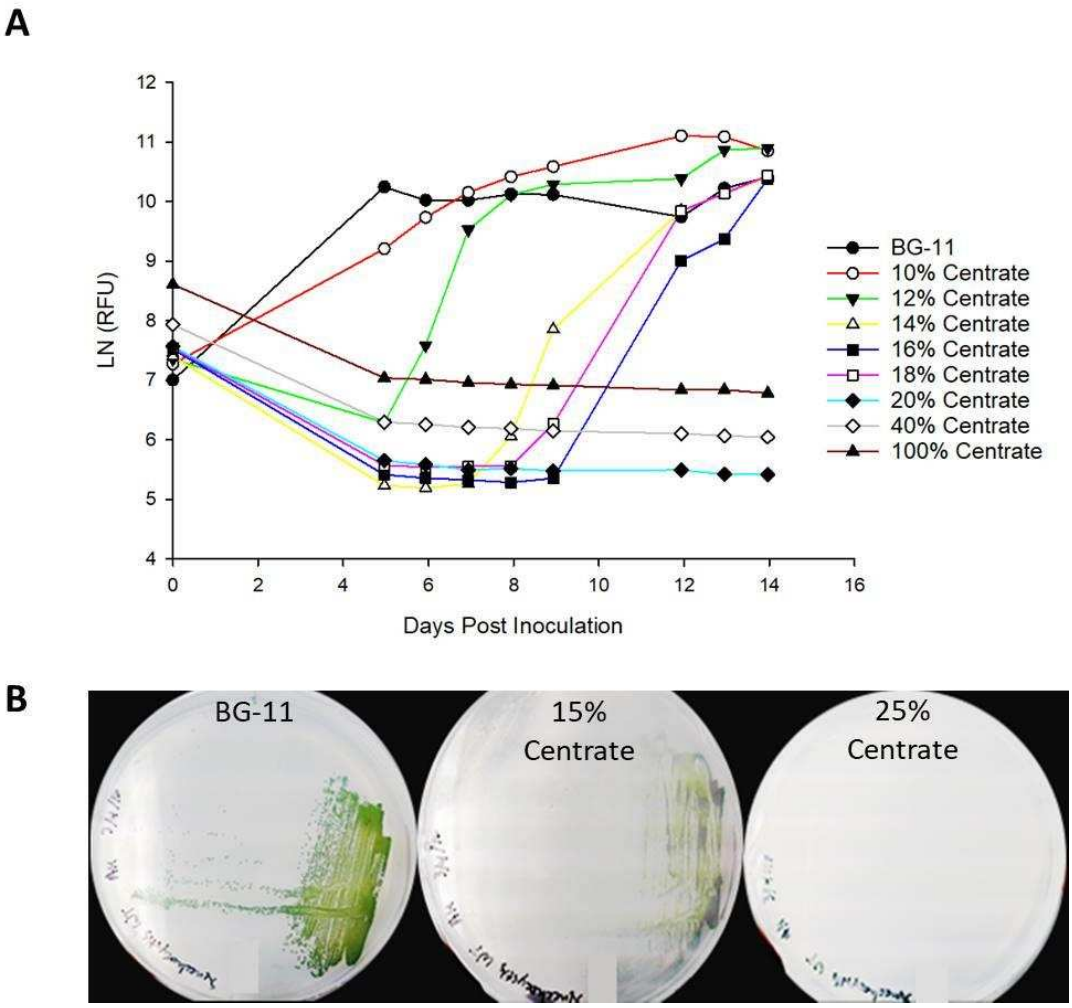

Figure 2.3: PCC6803 in centrate diluted with BG-11 media. A) graph of the natural log transformed growth curves of PCC6803 in centrate diluted with BG-11 media (n=1). B) Representative photographs collected of PCC6803 on centrate +BG-11 agar plates (n=3).

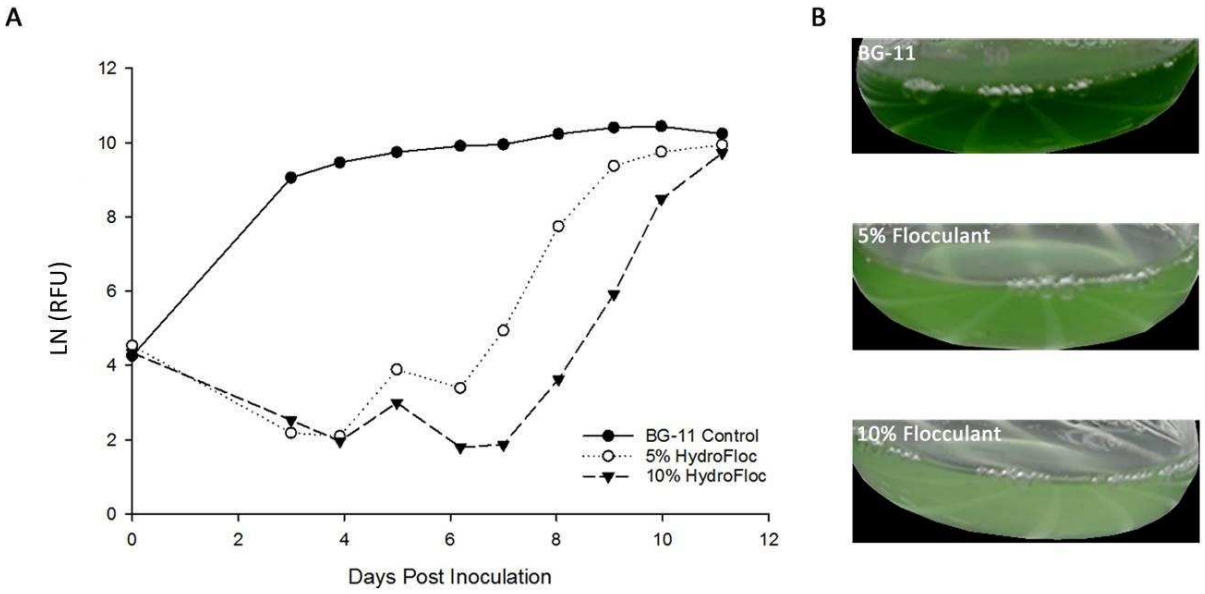

Figure 2.4: PCC6803 in BG-11 media + AQUABEN HydroFloc 1687. A) Growth curves for PCC6803 in BG-11 media supplemented with two dilutions of AQUABEN HydroFloc 1687 cationic flocculant polymer (n=1). B) Photographs of cultures after 10 days of incubation.

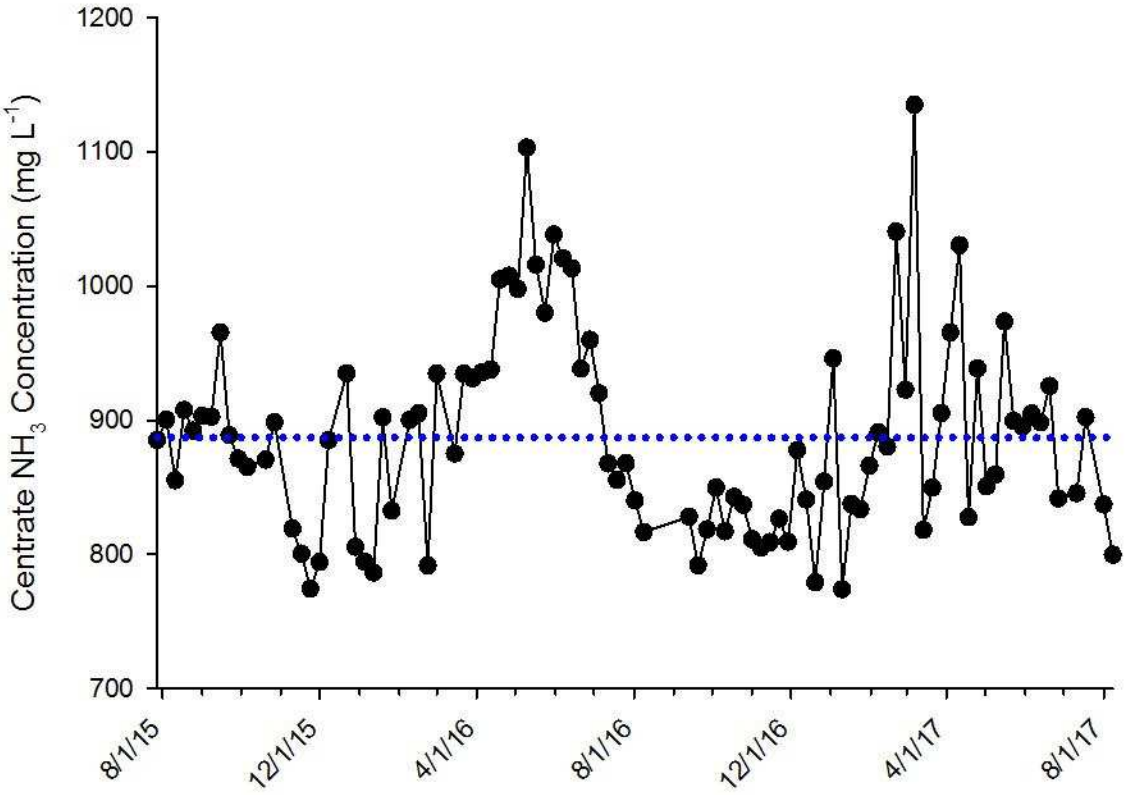

Figure 2.5: Drake Water Reclamation Facility centrate ammonia concentration between July 2015 and August 2017. Data provided by Link Mueller of the DWRF. The blue line represents average NH3 concentration over the time course shown (887.62 mg NH_3 L^{-1}).

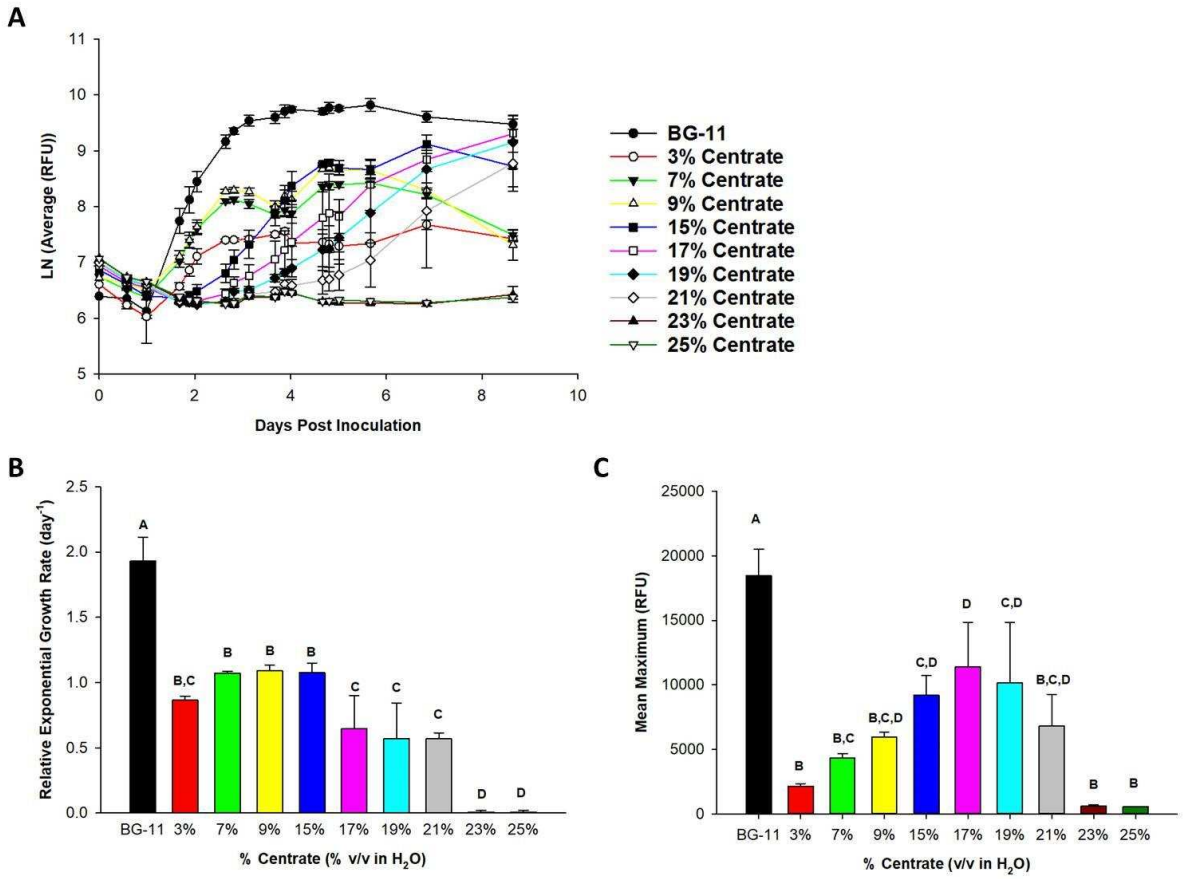

Figure 2.6: Filtered centrate as the sole nutrient source for PCC6803. A) Growth curves of PCC6803 by the natural log transformed relative fluorescence units (RFU) in increasing concentrations of centrate (n=3, error bars = 1 standard deviation). B) Relative exponential growth rates for PCC6803 in each centrate dilution. C) Maximum chlorophyll fluorescence measured during the experiment. Different letters represent statistically different groups calculated using a one-way ANOVA (n=3, error bars = 1 standard deviation) followed by a Tukey's HSD post-hoc test.

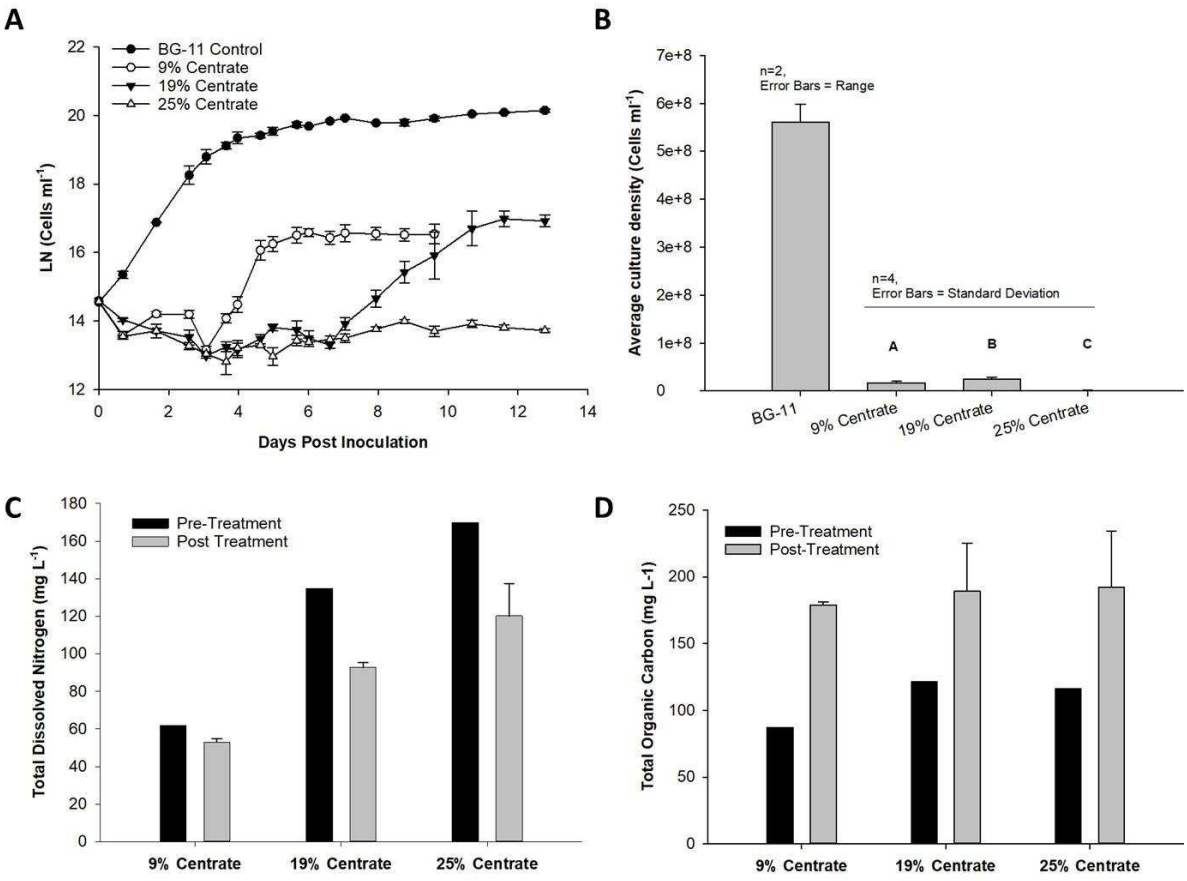

Figure 2.7 Bioremediation of nitrogen by PCC6803 grown in filtered centrate as the only nutrient source. A) Natural logarithmic transformed growth curves generated by hand counts of cells using a haemocytometer. B) Final culture densities (cells ml^{-1}) of PCC6803 in three centrate dilutions, and a BG-11 positive control. A one-way ANOVA was performed where different letters represent statistically different groups using a Tukey's HSD post-hoc test (n=4, error bars = 1 standard deviation; $p<0.05$, BG-11 control culture: n=2, error = range, not included in ANOVA). The raw total nitrogen and total organic carbon measurements are reported for pre- and post-treatment in figures C and D, respectively (Pre-treatment: n=1; Post-treatment: n=4, error bars = 1 standard deviation).

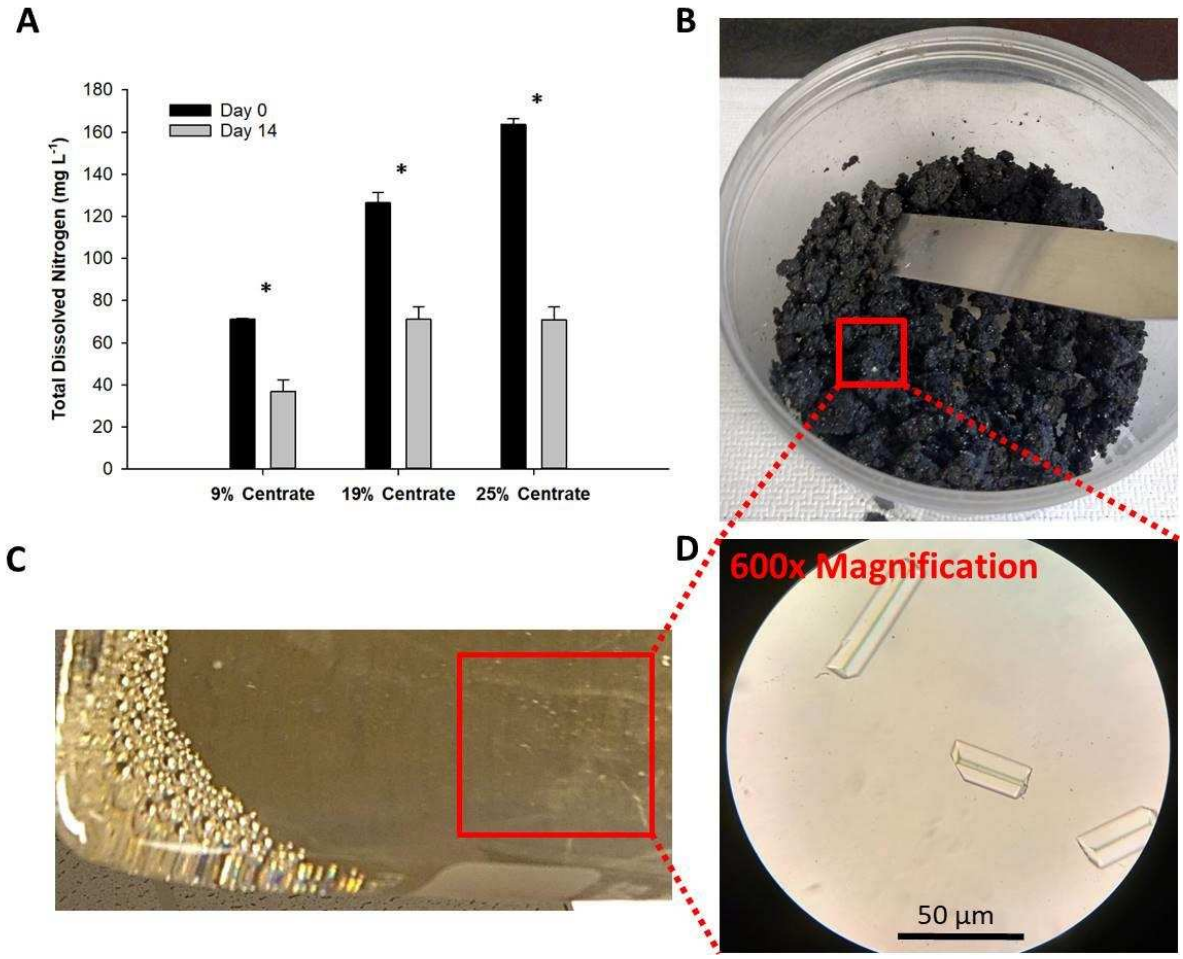

Figure 2.8: Struvite precipitation from centrate. A) presents the Total Dissolved Nitrogen at Day 0 (the day centrate was collected) and then after two weeks of incubation at 30°C under continuous light as previously described (n=3, error bars = 1 standard deviation, $p<0.005$ using unpaired t-test). Figures B-D provides visuals of struvite crystals that form in both the liquid and solid fractions of centrifuged sludge following anaerobic digestion. Figure B depicts large crystals found in the solids formed after sludge centrifugation. C) Crystal formation in filtered centrate after 24hrs. The crystals from the centrate in Figure C were collected and observed under 600x total magnification using a compound microscope (D).

3. CHAPTER **3**: DETERMINATION OF SULFATE LIMITATION IN CENTRATE FOR IMPROVED CULTIVATION OF *SYNECHOCYSTIS* SP. PCC6803

3.1 Introduction

Rising global temperatures from increased anthropogenic greenhouse gas emissions are developing a need for more sustainable alternatives to fossil fuels [3, 8, 14]. Fuels derived from biomass [174], or biofuels, are one of many options being considered and explored to replace fossil fuels [25, 175-177]. Biofuels can be produced to function as direct replacements or supplements into diesel and gasoline internal combustion engines, reducing the need to entirely replace the vast majority of petroleum driven vehicles [178-181]. Unfortunately, current production costs for biofuels are impeding large scale commercialization and immediate replacement of fossil fuels [182-185].

Exogenously added resources are required for high biomass production. The costs associated with these resources directly contribute to the high production price of biofuels [75]. To achieve high biomass of algal biofuel feedstocks water, carbon, nitrogen, and phosphorus must be available in excess [75, 80, 83]. Microalgae can use numerous water sources including sea, brackish, and waste waters [73, 186, 187]. Carbon is mostly supplied from the atmosphere [46, 65, 87, 188, 189], but can be supplemented through bicarbonate if required [190, 191]. Nitrogen and phosphorus are of particular interest as they are sourced from the unstainable processes of the Haber-Bosch Process [95] and as a mined mineral rock [94], respectively. To decrease the cost and increase the overall sustainability of algal biofuel production, N and P could be sourced from other sources.

Wastewaters are being explored as sustainable water and nutrient sources that do not compete for resources used in food crop production. Wastewaters are produced as a product of

industrial processes, as well as from human and animal activities [106, 154, 192, 193]. Municipal wastewaters – those resulting from residential and business waste – undergo extensive physical, chemical, and biological processing at municipal wastewater treatment facilities in order to separate and remove the organic solids and nutrients from wastewater [194, 195]. Biological nutrient removal (BNR) systems utilize bacterial communities to perform metabolic digestion of nutrients [196, 197]. A concentrated source of N and P can be found in a waste stream called centrate (the supernatant from centrifugation of solids formed after anaerobic digestion) at the end of the treatment process [110, 156, 194, 198]. In Colorado, Regulation 85 limits the discharge of nutrients to receiving bodies to less than 15 mg N L^1 and less than 1 mg P L^{-1} [107] in order to prevent eutrophication [97, 99, 101, 104, 107]. Due to these restrictions, centrate may not be released from wastewater treatment facilities. Therefore, utilization of centrate to supply nutrients to microbial autotrophs may not only increase the sustainability of the biofuel process, but also provide an additional bioremediation step for potentially polluting sources of N and P.

However, several challenges associated with centrate use in biofuel processes exist. Centrate composition varies dramatically by the type of BNR process a given treatment facility employs. The A^2O or Modified Bardenpho processes [153] are designed for high nitrogen and phosphorus removal [195], while older systems such as Plug-Flow or Ludzack-Ettinger processes would only be useful for phosphorus [199] or nitrogen removal [200], respectively. Additionally, the chemical composition of centrate is also prone to seasonal dynamics due to changes in the microbial communities of the BNR systems under temperature fluxes [115, 201, 202]. Differences among centrate source, and collection time, are clearly important factors to consider when implementing centrate as a nutrient media for the cultivation of photosynthetic microbes.

Cyanobacteria are promising organisms for advanced biofuel production. Cyanobacteria have simpler and more easily transformable genomes relative to eukaryotic microalgae – which

makes genetic modification of cyanobacteria with exogenous metabolic pathways more feasible [203]. Metabolic pathways for biofuel production have already been engineered in cyanobacteria to produce isobutanol [65] and 1-butanol [66], as well as the production and excretion of ethanol[64]. *Synechocystis* sp. PCC6803 is a model cyanobacterium with a small and fully sequenced genome [204], in which the physiology and biochemistry have been well characterized [166, 205-210]. Industrial cultivation of *Synechocystis* sp. PCC6803 for advanced biofuel production might be possible if wastewaters are used as the nutrient source. Cai *et al.* (2013) compared the lipid production of *Synechocystis* sp. PCC6803 to the eukaryote *Nannochloropsis salina* cultivated in artificial sea water amended with anaerobic digestion effluent; finding that *Synechocystis* sp. PCC6803 grew faster than *N. salina*, but had lower lipid productivities [142]. Lynch *et al.* (2015) evaluated seven different native cyanobacterial strains (*Synechococcus* sp., *Snowella litoralis, Microcystis* sp., *Scenedesmus* sp., two *Synechococcus* sp., and *Microcystis* sp.) and *Synechocystis* sp. PCC6803 for their ability to accumulate biomass, remediate nutrients, and produce neutral lipids on synthetic wastewater [140]. Neither of these studies used treated wastewaters (by the biological nutrient removal process), as the sole nutrient source for the cultivation of *Synechocystis* sp. PCC6803. Evaluation of the literature suggests that *Synechocystis* sp. PCC6803 has not been cultivated solely on municipal wastewater centrate, which presents an opportunity for further investigation.

In chapter 1, I demonstrated that the model cyanobacterium *Synechocystis* sp. PCC6803 (PCC6803, herein) could be grown on centrate was collected from the Drake Water Reclamation Facility (DWRF) in Fort Collins, CO. Our findings indicated that despite adequate concentrations of nitrogen and phosphorus in diluted centrate concentrations permitting higher biomass accumulation (centrate completely inhibited PCC6803 at dilutions greater than 20% v/v in sterile H_2O), PCC6803 cultures had a shorter exponential growth phase, resulting in lower biomass

accumulation compared to control cultures grown in normal media containing similar N and P concentrations. Our results suggest that centrate collected from DWRF is limited in a nutrient other than N or P.

The focus of the present study was to determine which nutrient limits biomass accumulation of PCC6803 in centrate collected from DWRF. A bioassay approach was taken to ascertain the limiting nutrient whereby change in biomass was monitored after supplementation of centrate with components of the standard culture media (BG-11) for PCC6803. To confirm the nutrient was limiting *in vivo*, the expression of genes relevant to nutrient uptake by RT-qPCR was analyzed. This study provides a simple approach to improve biomass production utilizing wastewater with minimal nutrient supplementation.

3.2 Materials and Methods

3.2.1 Culture conditions, standard growth media, and biomass measurements

3.2.1.1 Organism and normal growth media

The cyanobacterium *Synechocystis* sp. PCC6803, generously gifted from Dr. Jianping Yu of the National Renewable Energy Laboratory (NREL), was maintained axenically in BG-11 (pH 8) growth media modified from [148] as follows: 17.6mM $NaNO_3$, 304µM $MgSO_4$, 245µM $CaCl_2$, 31µM Citric Acid, 22.9µM $C_2H_8O_7 \cdot xFe_3^+ \cdot yNH_3$, 3.4µM EDTA, 1.05mM K_2HPO_4, 189µM Na_2CO_3, 46.3µM H_3BO_4, 14.4µM $MnCl_2$, 0.7µM $ZnSO_4 \cdot 7H_2O$, 1.9µM Na_2MoO_4, 0.3µM $CuSO_4 \cdot 5H_2O$, 0.2µM $Co(NO_3)_2 \cdot 6H_2O$; buffered with 10mM Tes-NaOH buffer (pH 8). All chemicals used for the BG-11 media were laboratory grade and purchased from either Thermo FisherScientific® or Millipore Sigma®. Unless otherwise noted, PCC6803 cultures were incubated in a Percival Model E30B (Perry, IA) Incubator under 165 µmol photons m^{-2} s^{-1} of continuous light at 30°C. 25ml cultures were cultivated in 125ml Erlenmeyer flasks on an orbital shaker (VWR, Model 3500).

3.2.1.2 Wastewater centrate collection and standardized use

The supernatant from the wastewater dewatering process of anaerobically digested sludge was collected directly from a decanter centrifuge (Alfa Laval/Sharples® Model DS 706) at the City of Fort Collins Drake Water Reclamation Facility (Fort Collins, CO) in 250ml plastic bottles provided by the dewatering facility. This is centrate. Immediately following collection from the centrifuge sampling port, the centrate was vacuum filtered through a 0.2µm filter (Thermo Scientific™ Nalgene™ Rapid-Flow™ 75mm Bottle Top Filter-500ml) to remove any remaining solids and native microorganisms. Filtered centrate was kept at 4°C until dilution and inoculation within 8 hours of collection. We found precipitates forming in the bottom of bottles after 24 hrs in preliminary experiments indicating changing chemical composition.

3.2.1.3 Cell counts

Experiments in which the density of the culture (cells ml^{-1}) was monitored, cell counts were done by either flow cytometry or via direct microscopic observation. Flow cytometry based cell counts were performed by first filtering diluted culture through a 30µm pre-separation filter (Miltenyi Biotec Inc.; Auburn, CA) and then running samples through a BD Accuri™ C6 Plus Personal Flow Cytometer (BD Life Sciences; San Jose, CA) at a flow rate of 14µl min^{-1}. The autofluorescence of chlorophyll *a*/phycobilisome (640 nm excitation, 675±25 nm emission detection) of pigment containing cells were gated from non-viable particles. Manual counts were performed using a Reichart Bright-Line Hemacytometer with Neubauer ruling (Hausser Scientific; Horsham, PA) under 400x total magnification with a bright field microscope.

3.2.1.4 In vivo fluorescence measurements

When cell counts were not required to measure biomass accumulation, *in-vivo* fluorometry (IVF) [149] was performed using a Turner Designs Trilogy® Fluorometer fitted with

a blue light module (460nm LED, 441-482nm excitation, 660-710nm emission). Chlorophyll a autofluorescence of a 500µl sample of culture was measured in a 2ml centrifuge tube

3.2.2 Determining nutrient in centrate limiting biomass accumulation

In the experiments described in chapter 1, I observed that cultures cultivated in 9% centrate had a shorter lag phase in comparison to 15% centrate and above, while still reaching roughly the same final cell density. This would reduce the time required for future experiments. In the following experiments, I rounded the dilution up to a concentration of 10% centrate (%v/v) for simplicity.

3.2.2.1 Nutrient supplemented centrate media

The four stock components of BG-11 media [Part A (1.76M $NaNO_3$, 30.4mM $MgSO_4$, 24.5mM $CaCl_2$, 3.12mM Citric Acid, 2.29mM Ammonium Ferric Citrate, 342µM EDTA), Part B (105mM K_2HPO_4), Part C (18.9mM Na_2CO_3), and Trace Minerals (46.3mM H_3BO_4, 14.4mM $MnCl_2$, 765µM $ZnSO_4 \cdot 7H_2O$, 1.9mM Na_2MoO_4, 320µM $CuSO_4 \cdot 5H_2O$, 171µM $Co(NO_3)_2 \cdot 6H_2O$)] were added individually to 10% centrate (% v/v centrate in sterile MQ water) to achieve the respective final stock concentrations found in BG-11 listed above (1% v/v for Parts A-C, 0.1% v/v for trace minerals).

Part A was then broken down into the individual components and supplemented into centrate. Part A compounds were used at the concentrations they were in complete BG-11 media. Additionally, all three components associated with iron (citric acid, ammonium ferric citrate, and EDTA) were placed together in normal stock concentrations for this first pass.

The individual components of $MgSO_4$ (Mg^{2+} and SO_4^{2-}) were then evaluated to determine which is limiting in centrate. The effects of magnesium and sulfate were tested using 304µM $MgCl_2 \cdot 7H_2O$ and 304 µM Na_2SO_4. Neither Na^+ nor Cl^- were expected to influence biomass accumulation given that neither $NaNO_3$ nor $CaCl_2$ had final cell densities near that of $MgSO_4$

supplemented cultures in the previous experiment. Controls included Centrate + BG-11 Part A only, Centrate + BG-11, just 10% Centrate and just BG-11 for a final evaluation of the limiting nutrient.

Each media variation was tested in biological triplicate inoculated from mid-exponential phase PCC6803 inoculum. The targeted starting density was $5.5x10^5$ cells ml^{-1}. The relative exponential growth rates were calculated for PCC6803 in different centrate medias by calculating the slopes from the natural log transformation of exponentially growing culture densities.

3.2.3 RT-qPCR for in vivo detection of sulfate transporter expression

3.2.3.1 Experimental design and sample preparation

PCC6803 cultivated in BG-11 media to mid-exponential phase was inoculated into 10% centrate media and 10% centrate amended with 304µM Na_2SO_4 media. Samples of culture for RT-qPCR was harvested as described below at day 2.1875 and 3.96 correlating to linear and stationary phase, respectively, for centrate only grown cultures; and mid-exponential and linear phase, respectively, for centrate + sulfate grown cultures.

Ten ml culture was collected in pre-chilled 15ml tubes and kept on ice until centrifugation at 3200 x g for 10 minutes at 4°C. The supernatant was poured off and the cells were re-suspended in the remaining media. The suspended cultures were then transferred to 2.0ml Eppendorf centrifuge tubes and centrifuged at 4500 x g for 2 minutes at 4°C. The supernatant was removed and 500µl of TRIZOL® Reagent was added to the tubes to re-suspend the cells. An additional 500µl of TRIZOL® was mixed into each tube. Tubes were stored at -80°C until the remainder of the protocol was performed. RNA was extracted using chloroform, followed by DNA removal-up using Turbo DNAse [211]. The reverse transcriptase reaction utilized the Superscript III 1^{st} strand synthesis system. Samples were then stored at -80°C until RT-qPCR.

3.2.3.2 Sulfate transporter primers

Primer sets were designed for the four genes of a sulfate transporter encoded by the *sbpA-cysTWA* operon (*slr1452-5*)[212]. Table 3.1 shows the primer pairs (including the forward and reverse DNA sequences) for the four primers generated.

3.2.3.3 RT-qPCR

The relative abundance of *sbpA, cysT, cysW,* and *cysA* transcripts were determined using *Power* SYBR™ Green (Applied Biosystems) real-time PCR on a Bio-Rad C1000 Touch™ Thermal Cycler. The cDNA was denatured at 95°C for 3 minutes, and then amplified using 45x cycles of 95°C for 10 seconds followed by 60°C for 30 seconds,

Following best practices, the ΔΔCt method was used to calculate the relative abundance of each the four transcripts (normalized to the housekeeping gene *RnpB*) with respect to the mean values of the centrate + Na_2SO_4 grown cultures [213]. Two tailed T-tests were performed between log2 transformed transcript abundance values of both treatments for each transcript

3.2.4 Nitrogen removal capacity of PCC6803 grown on centrate with sulfate

3.2.4.1 Culture conditions

Wastewater centrate was collected and filtered as described above. Eight hours post collection, centrate was diluted in sterile water to a final concentration of 5% v/v. Mid-exponential cells cultivated in standard BG-11 media were inoculated to an initial cell density of $2.5x10^6$ cells ml^{-1} in either 5% centrate or 5% centrate supplemented with 304μM Na_2SO_4. Four biological replicates were used for each treatment. As an additional control, three flasks containing just 5% centrate without culture were incubated to observe the change in nitrogen content due to abiotic processes.

3.2.4.2 Centrate media collection and sample dilutions

Pre-treatment (Day 0) samples of the centrate media were collected prior to inoculation with PCC6803 as follows. Ten ml each of 5% centrate and 5% centrate supplemented with 304μM Na2SO4 media was harvested into 15ml acid washed conical tubes and frozen at -80°C. Samples were kept at -80°C until analysis.

Once cells reached stationary phase (6 days) cultures were processed as follows. 15ml of culture was harvested into 15ml acid washed tubes with 150μl 1% v/v Tween added to each tube for a final concentration of 0.1% to aid in the pelleting of cells. Tubes were centrifuged at 3220 x g for 20 minutes at 4°C. 13ml of the supernatant was transferred to new 15ml acid washed conical tubes and frozen at -80°C until analysis. Day 6 samples were diluted 1:6 with DI H_2O in acid washed TOC glassware.

3.2.4.3 Total dissolved nitrogen analysis of centrate

Total dissolved nitrogen analysis was performed on pre- and post-treatment samples using a Shimadzu TOC-L Autoanalyzer with the Total Nitrogen Module (TNM-L). The method for analysis described in Caballero et al., 2016 [214] was used with the changes in media collection defined above. Prior to analysis, all samples were diluted with DI H_2O in acid washed TOC glassware in order to stay within the linear range of detection.

3.2.5 Quantitative elemental analysis of centrate

Elemental analysis of centrate was performed by digesting filtered centrate with concentrated nitric acid [215] followed by inductively coupled plasma – optical emission spectrometry (ICP-OES) . Digested centrate samples were submitted to the Soil, Water and Plant Testing Laboratory at Colorado State University for ICP-OES analysis, using the method described by Fassel (1978) [216].

3.2.6 Statistics

Statistical analysis was performed using Sigma Plot (v 1.3, Systat Software Inc.) on independent experimental replicates. A one-way ANOVA was used to compare relative exponential growth rates of PCC6803 in different centrate media – as well as the final culture densities. This was followed by a Tukey's post-hoc test. An unpaired t-test was used to compare the average TN values of Day0 and Day6 centrate media samples (with and without PCC6803). For both statistical tests, samples with $p<0.05$ were considered statistically different and data are presented as the means ±1 standard deviation (SD) throughout the chapter unless otherwise noted.

3.3 Results

3.3.1 Characterization of PCC6803 on centrate + BG-11 nutrients

We performed a series of bioassay experiments to determine the limiting nutrient for PCC6803 biomass accumulation on centrate. The first step was to observe the stimulatory effects of stock nutrient solutions used in standard BG-11 nutrient media when supplemented into centrate. We first compared the four stock components of BG-11 (Part A, B, C, and Trace Minerals, as described above) independently added to centrate media. As Figure 3.1A shows, centrate supplemented with the individual components of BG-11 had a two day lag phase, whereas the three cultures with complete BG-11 media did not exhibit a lag phase. Figure 3.1B is representative photographs of the cultures at stationary phase, showing that centrate + Part A, Centrate + BG-11, and the BG-11 control cultures were denser than centrate with the other BG-11 parts. As shown in Figure 3.1C, Centrate + Part A had the greatest final cell density with an average of $3.51\text{x}10^{8} \pm 2.66\text{x}10^{7}$ cells ml^{-1}. Centrate + Part B grown cultures averaged $3.22\text{x}10^{7} \pm 1.26\text{x}10^{6}$ cells ml^{-1}, centrate + Part C grown cultures averaged $3.46\text{x}10^{7} \pm 3.06\text{x}10^{6}$ cells ml^{-1}, centrate + Trace Minerals grown cultures averaged $3.34\text{x}10^{7} \pm 5.37\text{x}10^{6}$ cells ml^{-1}. The final cell

densities of centrate with Parts B, C, and Trace Minerals were significantly lower than centrate + Part A cultures ($p<0.05$), with all cultures averaging fewer than $3.5x10^7$ cells ml^{-1}. Relative exponential growth rate did not correlate with final culture density (Figure 3.1D). These results suggest that the major limiting nutrient from centrate is a compound found in BG-11 Part A (described in methods).

To further resolve the limiting compound in centrate from BG-11 Part A, we broke Part A down into the individual compounds. Additionally, all three components associated with iron (Citric acid, Ammonium Ferric Citrate, and EDTA) were placed together in normal stock concentrations for the first set of experiments. As Figure 3.2A shows, PCC6803 cultures in centrate supplemented with magnesium sulfate ($MgSO_4$) had a longer exponential growth phase than any of the other Part A compounds. As shown in Figure 3.2B, Centrate + $MgSO_4$ had the greatest final cell density with an average of $3.26x10^8 \pm 9.08x10^7$ cells ml^{-1}. Centrate + iron complex grown cultures averaged $7.11x10^7 \pm 8.96x10^5$ cells ml^{-1}, centrate + $NaNO_3$ grown cultures averaged $5.40x10^7 \pm 1.23x10^7$ cells ml^{-1}, centrate + $CaCl_2$ grown cultures averaged $5.56x10^7 \pm 1.10x10^7$ cells ml^{-1}. The final cell densities of centrate with the iron complex, $NaNO_3$, and $CaCl_2$ were significantly lower than centrate + $MgSO_4$ cultures ($p<0.05$). Centrate supplemented with $MgSO_4$ yielded 5.46 times more cells ml^{-1} than the average culture densities of cultures supplemented with the iron complexes, $NaNO_3$, or $CaCl_2$. The final culture density of the BG-11 control was $5.76x10^8$ cells ml^{-1} ($n=1$), and that of the centrate + BG-11 control was $5.61x10^8$ cells ml^{-1}($n=1$), which is 1.8 and 1.7 times greater (respectively) than the average centrate + $MgSO_4$ final cell density.

We then observed PCC6803 biomass accumulation in centrate amended with either sodium sulfate (Na_2SO_4) or magnesium chloride ($MgCl_2$). We would not expect either Na^+ or Cl^- to influence the final culture densities given the results described in Figure 3.2A&B. As Figure

3.2C demonstrates, Na_2SO_4 supplemented centrate media (open circles) yielded a longer exponential growth phase over $MgCl_2$ supplemented centrate media (closed circles) and centrate only media (closed squares). The longer exponential growth phase of centrate + Na_2SO_4 cultures yielded an average of $3.18x10^8 \pm 2.24x10^7$ cells ml^{-1}; while centrate + $MgCl_2$ cultures yielded an average of $4.98x10^7 \pm 7.52x10^5$ cells ml^{-1}. Centrate only grown cultures yielded an average of $4.67x10^7 \pm 7.31x10^5$ cells ml^{-1}, while BG-11 grown cultures yielded an average of $3.32x10^8 \pm 4.63x10^7$ cells ml^{-1}. Na_2SO_4 supplementation yielded significantly higher biomass accumulation than cultures grown in centrate + $MgCl_2$ and just centrate alone (Figure 3.2D) ($p<0.05$ following a one-way ANOVA).

3.3.2 RT-qPCR to confirm sulfate limitation in centrate

In order to confirm that cells experience sulfate limitation, we performed experiments to observe the response of gene transcripts associated with sulfate transporters during growth in centrate. We targeted four transcripts encoded by the high affinity sulfur transport system encoded by the *sbpA-cysTWA* operon [212].

The expression of the first three genes on the operon were significantly increased in centrate only grown cultures compared to cultures grown in centrate supplemented with sulfate (n=3, un-paired two tailed t-test $p<0.05$) (Figure 3.3 A & B). Centrate only grown cultures had an increase in transcript abundance of the sulfate binding protein (*spbA*, *slr 1452*) by 57.85 ± 10.49 fold compared to centrate + Na_2SO_4 grown cultures ($p<0.01$). Centrate only grown cultures had an increase in transcript abundance of the in *cysT* (*slr 1453*) by 33.84 ± 17.00 fold compared to centrate + Na_2SO_4 grown cultures ($p<0.01$). The *cysW* (*slr 1454*) transcript was increased by 44.29 ± 7.43fold compared to centrate + Na_2SO_4 grown cultures ($p<0.01$). The ATP binding protein transcript (*cysA*, *slr 1455*) had a 2.8 fold increase (n=3, standard deviation = 1.05) compared to centrate + Na_2SO_4 grown cultures ($p=0.07$).

3.3.3 Nitrogen removal capacity of PCC6803 grown in centrate with sulfate

Following a 6 day incubation period, PCC6803 grown in centrate supplemented with Na_2SO_4 yielded an average final culture density of $1.96x10^8 \pm 4.70x10^7$ cells ml^{-1} compared to just centrate grown cultures with an average final density of $2.63x10^7 \pm 3.19x10^4$ cells ml^{-1} (Figure 3.4A). PCC6803 grown in centrate with Na_2SO_4 yielded 7.46 times more cells ml^{-1} than cultures grown in just centrate. Na_2SO_4 supplemented PCC6803 cultures in centrate removed over 2.7 times more nitrogen from the media compared to centrate only grown cultures (Figure 3.4B). As shown in Chapter 1, the nitrogen content of centrate without PCC6803 was reduced by over 50%, which was twice as much as we observed from PCC6803 cultures grown in just centrate (average of 24.28% nitrogen removed). The fraction of nitrogen reduced in each of the treatments and the control were found to be significantly different from one another following a one-way ANOVA (Figure 3.4B).

3.3.4 Elemental analysis of centrate by ICP-OES

ICP-OES analysis was performed to determine the relative abundance of elements in centrate. Table 3.2 shows the concentrations of elements in undiluted centrate in mg L^{-1}. The element concentrations were converted to molarity for direct molar comparison to BG-11 nutrient composition (also presented in Table 3.2). The molar concentration of sulfur in un-diluted centrate was found to be 27% less than that of BG-11. This translates to 93% less sulfur in 10% centrate compared to BG-11. The molar concentrations were then normalized to the phosphorus molarity in centrate and BG-11, respectively; and showed that all nutrients in centrate were in lower relative concentrations than in BG-11. The relative differences of nutrient concentrations in centrate compared to BG-11 are as follows: 27.36 times less boron, 3.94 times less calcium, 7.10 times less cobalt, 69.18 times less copper, 67.87 times less iron, 106.86 times less manganese,

20.07 times less molybdenum, and 18.41 times less sodium in undiluted centrate than in BG-11; with zinc being below the limit of detection in centrate.

3.4 Discussion

3.4.1 Determination of limiting nutrient in centrate

By breaking down the components of BG-11 and independently supplementing them into centrate, we were able to deduce that sulfate was a principal macronutrient limiting biomass accumulation. When sulfate was supplemented into diluted centrate, PCC6803 reached final culture densities that were only ~35% lower than that of centrate diluted in complete BG-11 media. High biomass accumulation is a desirable trait for biofuel feedstocks [217]. Sulfate supplementation provided a significant improvement for PCC6803 biomass accumulation compared to cultures grown in just centrate which had final culture densities that were 91% lower than cultures grown in media containing centrate supplemented with complete BG-11 media. While we were able to significantly increase PCC6803 biomass in centrate by adding sulfate, our result suggests that additional nutrients may need to be supplemented in order to achieve equal cell concentrations to cultures grown optimally in BG-11 media. Mass cultivation of PCC6803 at an industrial scale aims to achieve high biomass yields [218], which would require additional exogenous nutrient supplementation into centrate and therefore increase production costs for producing PCC6803 feedstocks.

Sulfur is an essential element for all living organisms. Sulfur is needed for the production of the amino acids cysteine and methionine and for the production of redox active Fe:S clusters in electron transfer mediating enzymes such as ferredoxin [219]. Ferredoxins play several cellular roles such as electron transfer, redox sensing, and gene regulation [212]. In cyanobacteria and microalgae, sulfur is generally assimilated as exogenous sulfate and is reduced in the plastids to form cysteine and methionine and Fe:S clusters [220].

3.4.2 Sulfate limitation increase transcription of sulfate transporters

Diluted centrate media induced significantly higher expression levels of sulfate binding and transport genes (encoded by the *sbpA-cysTWA* operon) in PCC6803 than in sulfate supplemented centrate media (Figure 3.3B). Zhang et al. (2008) showed that this operon is known to be activated by sulfate limitation, resulting in the up-regulation and expression of sulfur transport systems [212]. Transcriptomic profiling of the cyanobacterium *Arthrospira platensis* grown under sulfate deplete conditions by Kumaresan et al. (2017) also showed that sulfate transport proteins – including CysA – were upregulated in S-limitation compared to replete conditions [221]. Given the significantly higher transcript abundance of sulfate transporter genes in PCC6803 cultures grown in just centrate compared to centrate + Na_2SO_4, we have *in vivo* confirmation that PCC6803 experiences sulfur limitation in centrate.

3.4.3 Sulfate supplementation improves N removal by PCC6803

The increased final cell yields observed in centrate + Na_2SO_4 cultures of PCC6803 compared to centrate alone (Figure 3.4A) suggests that Na_2SO_4 amendment may improve the ability of cyanobacteria to drawdown dissolved nitrogen from centrate. PCC6803 has a nitrogen content of 0.11 pg N $cell^{-1}$ (previously established in the lab). Therefore, we can estimate the amount of N removal that could be achieved by adding S to reach maximal densities. W estimated that a 5% centrate solution should contain 35mg N L^{-1} based on the experiments described in Chapter 1. We had thus hypothesized that PCC6803 grown in 5% centrate (containing ~35mg N L^{-1}) and supplemented with 304μM Na_2SO_4 would be able to reach a final culture density of 3.18 $x10^8$ cells ml^{-1} and completely remove the nitrogen. Contrary to our estimates, we observed centrate + Na_2SO_4 grown cultures only accumulated 1.96$x10^8$ cells ml^{-1} and subsequently the TN was only reduced by 69% ± 0.02%.

Bioremediation of nitrogen from artificial centrate by *Synechocystis* sp. PCC6803 has been demonstrated previously by Cai *et al.* (2013). Artificial seawater (f/2 Guillard solution supplemented with Instant Ocean® sea salts) was diluted with various concentrations of centrate and used as the culture medium to compare the remediation of nutrients by PCC6803 and *Nannochloropsis salina.* The authors showed PC6803 could remove 100% of the nitrogen in 3% centrate + artificial seawater media (80 mg N L^{-1}), and 93.4% ± 5.5 of the 160 mg N L^{-1} present in 6% centrate + artificial seawater media [142]. In the experiments described by Cai *et al.*, the centrate media used was enriched with f/2 solution containing major nutrients, vitamins, and trace metals; indicating that the media was nutrient replete. In our studies, we used 5% centrate diluted in water and supplemented only with Na_2SO_4, measuring a 69% reduction in the total nitrogen content of the diluted centrate (30.62 mg N L^{-1}). The nitrogen removal differences between our experiment and that of Cai *et al.*, might by indicative that our cultures were still nutrient limited or that inhibitory substances present in acutal centrate vs synthetic analogues reduce productivity. The lower cell density and the lower TN reduction would suggest either that not all of the nitrogen is bioavailable, or that sulfate is not the only limiting nutrient in centrate.

Consistent with our TN analysis performed in chapter 1, PCC6803 inoculum reduced the amount of nitrogen removed from centrate by chemical precipitation. In sterile centrate, over 50% of the nitrogen was removed following incubation; whereas only ~25% of the nitrogen was removed from centrate media inoculated with PCC6803. Nitrogen precipitates out of centrate as magnesium ammonium phosphate hexahydrate in a molar ration of 1:1:1 magnesium : nitrogen: phosphorus to produce struvite [170] under alkaline conditions [169]. PCC6803 could be inhibiting the formation of struvite by utilizing any bioavailable phosphate or magnesium which would in turn affect the availability of magnesium or phosphate for struvite precipitation.

3.4.4 Sulfate removal in the wastewater treatment process

3.4.4.1 Reduction of sulfate to sulfide

Sulfur is removed from wastewater during anaerobic digestion. Influent containing high concentrations of sulfate promotes bacterial community shifts towards sulfate-reducing bacteria [196, 222]. Sulfate-reducers use organic carbon sources (e.g. acetate, proprionate, butyrate, lactate, and ethanol) or hydrogen to reduce sulfate to hydrogen sulfide – contributing to the foul odors associated with municipal wastes [223]. Sarti et al. (2005) showed that the sulfate concentration of the influent to anaerobic digestion was reduced by over 70%, while the sulfide concentration of the effluent increased by over an order of magnitude [224]. Sulfate-reduction to hydrogen sulfide is corrosive, toxic, and directly interferes with methane production – a more favorable byproduct of anaerobic digestion that can be used for heating and transportation [225]. Thus it must be removed [226] via venting into a biofilter [227]. At the DWRF, the sulfide gas produced during anaerobic digestion is vented into a biofilter comprised of compost and wood chips where sulfide oxidizing microbes convert sulfide into elemental sulfur.

3.4.4.2 Opportunity to recycle sulfide for cyanobacteria production in centrate

The requirement of sulfur supplementation for improved PCC6803 biomass accumulation in centrate could be accommodated by hydrogen sulfide. Nagy et al. (2014) provided evidence that PCC6803 can oxidize hydrogen sulfide during anoxygenic photosynthesis. When sulfide is present, an ArsR-type repressor responding to both arsenic and sulfide exposure induces expression of the *suoRSCT* operon found on the pSYSM plasmid in PCC6803, which encodes for a light dependent, type I sulfide:quinone oxidoreductase (SQR, sll5036) [228]. The authors confirmed functionality of the SQR enzyme comparing WT PCC6803 with a *suoRSCT* knockout grown anaerobically with sulfide under light and dark conditions. Only WT cultures grown in the

light were able to oxidize hydrogen sulfide, despite sulfide inhibition of photosystem II[228]. Therefore, H_2S produced from anaerobic digestion at the DWRF may be able to be supplemented directly into centrate grown cultures. H_2S from off-gassing at the DWRF would be a more sustainable sulfur source compared pyrite and gypsum which must be mined[223]. Future studies evaluating hydrogen sulfide supplemented centrate as a nutrient source for *Synechocystis* sp. PCC6803 will need to be performed.

3.4.5 Quantitative elemental analysis of centrate

Elemental analysis by ICP-OES demonstrated that several nutrients were in low abundance in centrate compared to BG-11 (Table 2.2). Comparing the molar ratio of nitrogen to phosphorus to sulfur (N:P:S; normalized to nitrogen concentration, nitrogen content determined by the TN analysis described in 3.3 above), BG-11 has an N:P:S of 50:3:1 whereas centrate has an N:P:S of 50:3.5:0.25. We would therefore anticipate sulfur to be limiting following Leibig's law of the minimum[79]. Extending the nutrient ratio to include micronutrients, we see that calcium, iron, and manganese may also become limiting for increased biomass accumulation (Table 2.2). Calcium and iron are both constituents of BG-11 Part A; which yielded significantly higher culture densities in stationary phase than BG-11 Parts B, C, and trace minerals. Additionally, cultures grown in centrate + complete Part A yielded higher culture densities than cultures grown with centrate + the individual components of Part A. The elemental analysis of centrate provided additional evidence that PCC6803 might be under multiple nutrient limitations – such as calcium and iron – as the concentrations are reduced in centrate compared to BG-11, yet exogenous supplementation into centrate improves the final cell density of the culture.

Our analysis also suggests that the concentrations of many metals found in DWRF centrate may not be toxic to PCC6803. Undiluted centrate contains less than 1 mg Fe L^{-1}, which is significantly less than the experimental EC_{50} values (concentration of toxin that inhibits half

the population [229]) of 5-10 mg L^{-1} Demirel *et al* (2009) provided for iron in five cyanobacterial strains (four *Synechocystis* sp. isolates, and one *Microcystis* sp. isolate) [230]. The nickel, cadmium, cobalt, chromium, and arsenic concentrations in centrate were all lower than the toxic concentrations of nickel above 27μM, cadmium above 10μM, cobalt above 32μM, chromium above 40μM, and arsenic concentrations above 3.5mM for PCC6803 as reported by Peca, et al. (2007) [231]. Zinc was below the detectable limit in centrate; and therefore is below zinc toxicity range of 32-39μM reported by Xu and Juneau (2015) [232]. The concentration of copper in centrate was 0.02μM, which is well below the toxic concentration of 3μM determined by Giner-Lamia *et al.*(2014) for *Synechocystis* sp. PCC6803 [233]. Elemental analysis of centrate showed that the concentrations of all of these metals were below toxic levels, with zinc being below the detectable limit. This suggests that the metal toxicity proposed in Chapter 1 may not occur during cultivation in diluted centrate. This analysis provides additional support for the hypothesis that there are inhibitory organic substances present in centrate.

3.5 Conclusions

Our work has shown that *Synechocystis* sp. PCC6803 biomass accumulation is significantly limited by the relatively low concentrations of sulfate found in wastewater centrate. Supplementing centrate with sulfate yielded higher culture densities and increased dissolved nitrogen drawdown compared to non-amended cultures. This suggests that cultivation of cyanobacteria on centrate can achieve a dual purpose: the production of biomass for uses such as biofuels and assistance in the remediation of polluting nutrients from wastewater. We propose that S could be directly supplied upstream in the wastewater treatment processes by redirecting the H_2S gas produced by anaerobic digestion to a cyanobacterial culture, increasing the overall sustainability of biofuel production and sewage treatment.

3.6 Figures

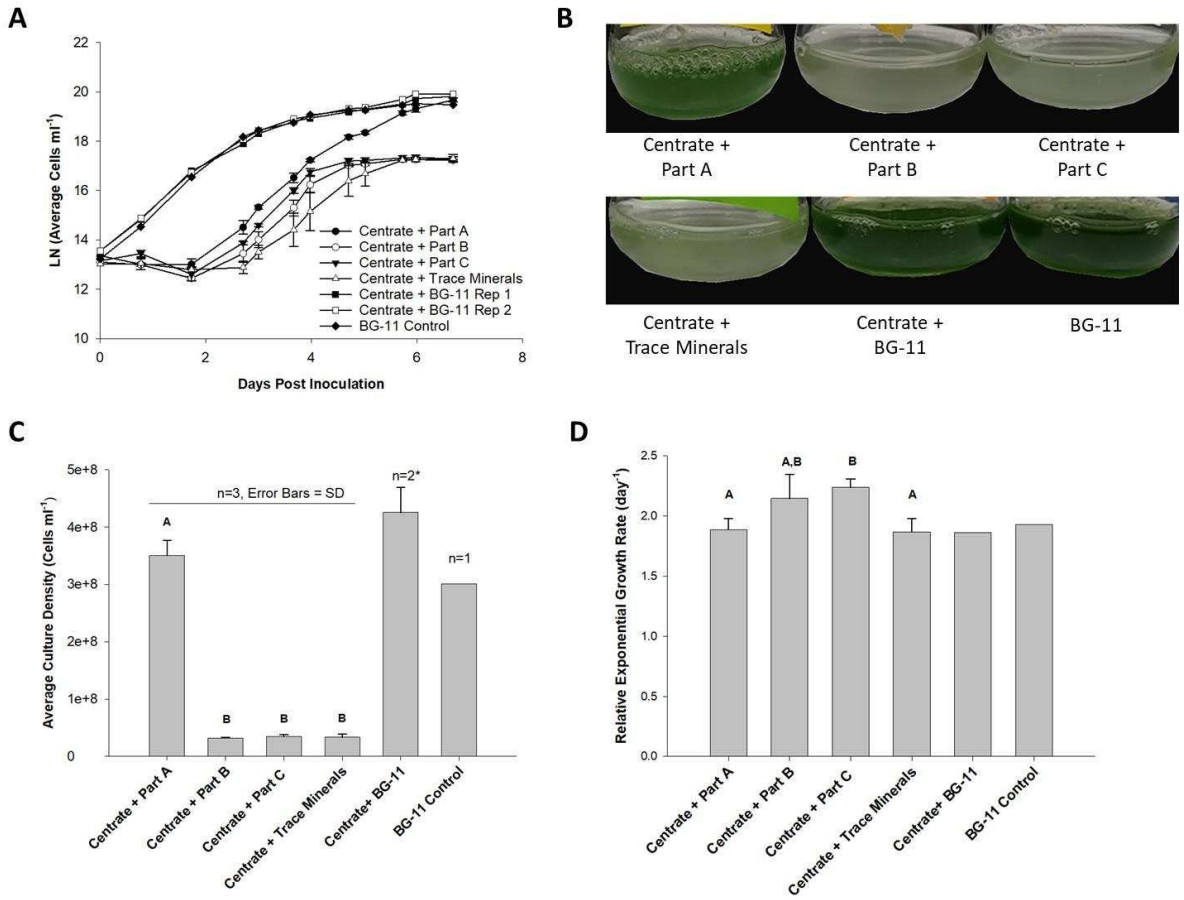

Figure 3.1: PCC6803 growth in centrate supplemented with BG-11 components. A) PCC6803 growth curves using the natural log transformed average cells ml^{-1} (n=3, error bars = 1 standard deviation; controls n=1). B) Representative photograph of cultures grown with different nutrient supplements. C) Final average cells ml^{-1} in stationary phase and (D) relative growth rates where different letters indicate significant differences in final culture densities and growth rates as determined by a one-way ANOVA followed by Tukey's post-hoc test ($p<0.05$, n=3, error bars = standard deviation; *n=2, error bars = range; n=1 for BG-11 only, no error bars).

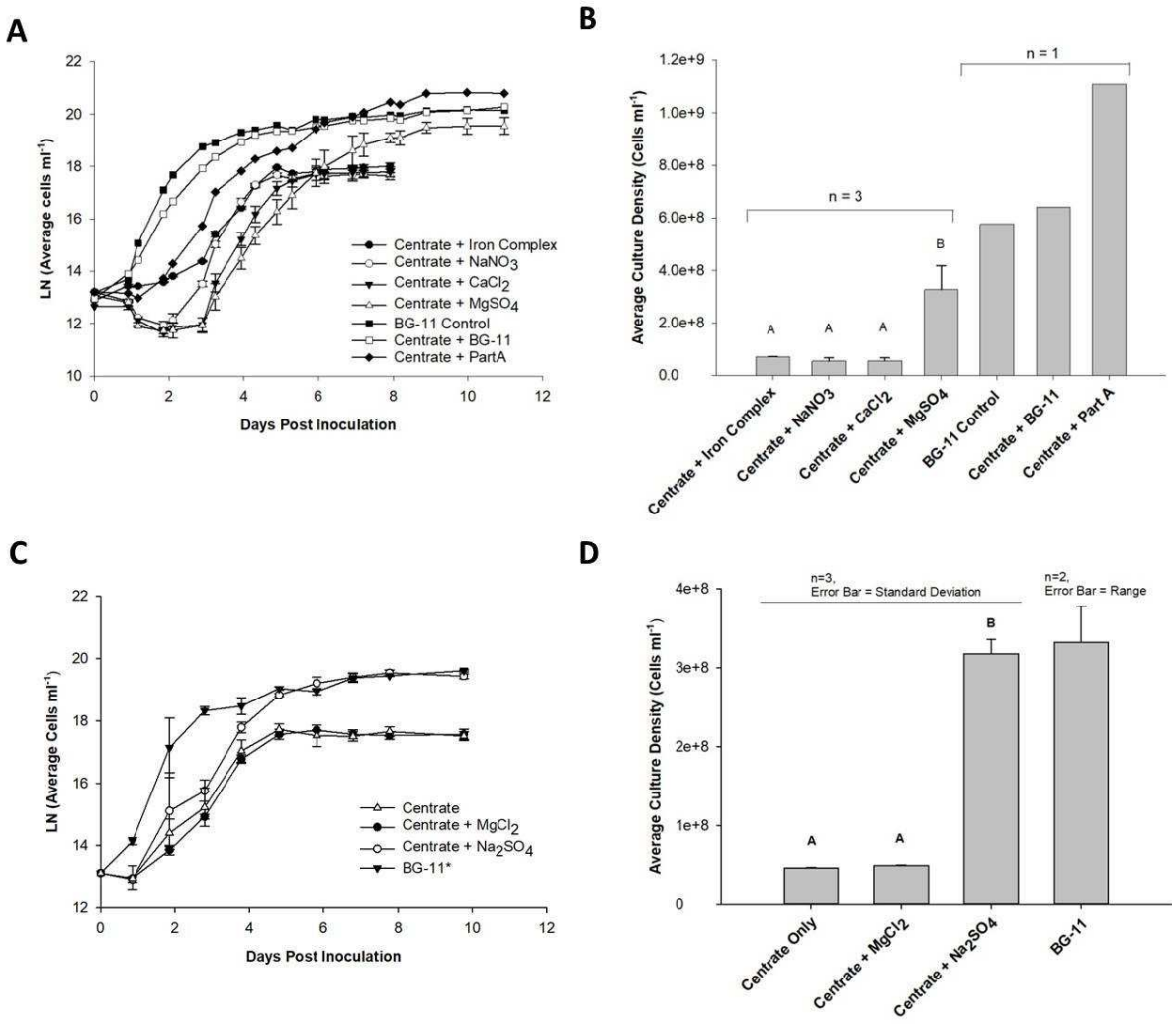

Figure 3.2 PCC6803 growth in centrate supplemented with BG-11 Part A compounds. A) PCC6803 growth curves using the natural log transformed average cells ml^{-1} (n=3, error bars = 1 standard deviation; n=1 for BG-11 control, Centrate+BG-11, and Centrate+PartA) for cultures grown in the supplied compounds of BG-11 Part A. C) PCC6803 growth curves using the natural log transformed average cells ml^{-1} for cultures grown with 304µM Na_2SO_4 or 304µM $MgCl_2$ (n=3, error bars = 1 standard deviation, BG-11* n=2, error bars = range). B and D) Final average cells ml^{-1} in stationary phase. Significant differences in final culture densities among treatments were calculated using a one-way ANOVA, where different letters represent statistically different groups using a Tukey's HSD post-hoc test (n=3, error = standard deviation, $p<0.05$).

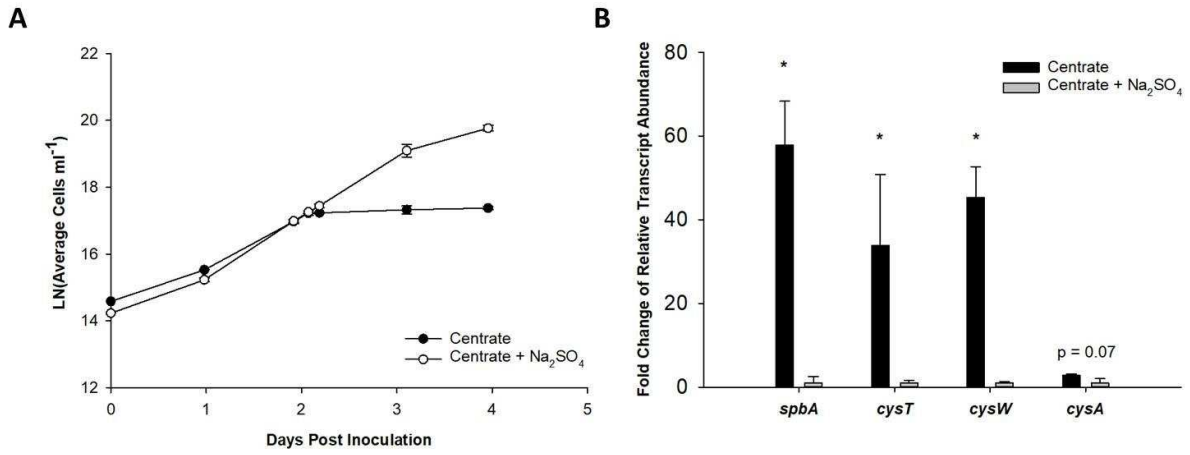

Figure 3.3 Sulfate transporter expression analysis by RT-qPCR. A) Growth curves of PCC6803, where the last time point indicates the extraction point for centrate only grown cultures (closed circles); and centrate + 304µM sulfate grown cultures (open circles) (n=3, error bars = standard deviation). B) Linear fold change of transcript abundance for the four genes of the *spbA-cysTWA* operon. Two tailed T-tests were performed between log_2 transformed transcript abundance values of both treatments for each transcript (n=3; error bars = standard deviation; asterisks indicate significant differences with $p<0.05$).

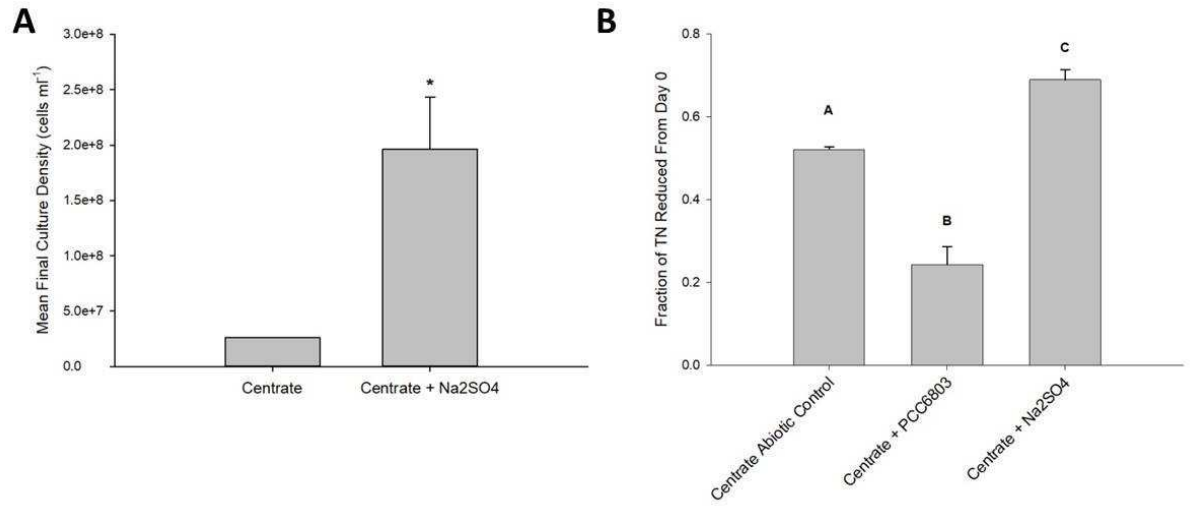

Figure 3.4 Nitrogen reduction of centrate by PCC6803 grown in centrate and centrate + 304µM Na_2SO_4. A) Final culture densities of PCC6803 on just centrate 5% centrate and cultures grown in centrate supplemented with 304µM (n=4, error bars = standard deviation; T-test $p<0.001$). B) The fraction of nitrogen reduced in each of the treatments and the control where significance was determined by a one-way ANOVA with letters showing the significant relationships determined by a Tukey's post-hoc test.

3.7 Tables

Table 3.1: Sulfate Transporter Primer Pairs for RT-qPCR

Locus	GeneID	Product Length (bp)	Primer Direction	Primer Sequence 5' to 3'
slr1452	sbpA	158	Forward	CTTGGTCAGCTACGCCGTTA
slr1452	sbpA	158	Reverse	CCGCTTCTAGACCATCCACC
slr1453	cysT	237	Forward	GTGCGAACTCTACAGCCAGT
slr1453	cysT	237	Reverse	AAACACTAACACCGGGGCAA
slr1454	cysW	159	Forward	TCTGTGCCGCTTGGGTTTTA
slr1454	cysW	159	Reverse	AGCTAGCAAACCAGCTACCG
slr1455	cysA	197	Forward	CCCTGCCAGTCCTTTTGTGA
slr1455	cysA	197	Reverse	CTCTTTTGATGGTCCCCGCT

Table 3.2: Elemental Analysis of Centrate by ICP-OES. The average elemental concentration (μg L-1) of centrate is presented (n=4 technical replicates). The molarity of each nutrient in undiluted centrate was calculated using the atomic mass for each element and is presented next to the molarity of nutrients in standard BG-11 media. Below Detection Limit indicates measurements <0.00. N/A = nutrient not added

Element	Raw element concentration ($\mu g\ L^{-1}$)	Element Molarity in 100% Centrate (μM)	Ratio Of Elements In Centrate Relative to P	Element Molarity in BG-11 (μM)	Ratio Of Elements In BG-11 Relative to P
Ag	Below Detection	0.00		N/A	
Al	6.58	0.24	7E-05	N/A	
As	176.10	2.35	7E-04	N/A	
Au	125.90	0.64	2E-04	N/A	
B	60.55	5.60	2E-03	46.26	4E-02
Ba	7.23	0.05	2E-05	N/A	
Be	Below Detection	0.00		N/A	
Ca	8253.63	205.93	6E-02	244.88	2E-01
Cd	11.25	0.10	3E-05	N/A	
Co	4.73	0.08	2E-05	0.17	2E-04
Cr	17.98	0.35	1E-04	N/A	
Cu	0.98	0.02	4E-06	0.32	3E-04
Fe	62.65	1.12	3E-04	22.98	2E-02
Hg	12.75	0.06	2E-05	N/A	
K	130545.00	3338.58	1E+00	2101.27	2E+00
Li	38.18	5.50	2E-03	N/A	
Mg	11931.75	490.78	1E-01	304.30	3E-01
Mn	24.50	0.45	1E-04	14.38	1E-02
Mo	30.00	0.31	9E-05	1.89	2E-03
Na	74597.00	3244.79	9E-01	18028.42	2E+01
Ni	Below Detection	0.00		N/A	
P	107819.00	3480.97	1E+00	1050.64	1E+00
Pb	Below Detection	0.00		N/A	
S	7124.05	222.18	6E-02	305.38	3E-01
Sb	84.40	0.69	2E-04	N/A	
Se	482.65	6.11	2E-03	N/A	
Si	7616.58	271.19	8E-02	N/A	
Sn	257.38	2.17	6E-04	N/A	
Sr	130.38	1.49	4E-04	N/A	
Ti	1.38	0.03	8E-06	N/A	
Tl	57.63	0.28	8E-05	N/A	
U	Below Detection	0.00		N/A	
V	112.88	2.22	6E-04	N/A	
W	Below Detection	0.00		N/A	
Zn	Below Detection	0.00		0.77	7E-04

SUMMARIZING DISCUSSION

The literature provided very inconsistent insight on how to use centrate for microalgal cultivation. The arrays of methods were confounded due to the numerous sources and types of wastewaters, as well as the microalgal and cyanobacterial diversity studied in the experiments. Due to these factors, I had to develop a method for using anaerobic digested sludge centrate for axenic culturing of the model cyanobacterium *Synechocystis* sp. PCC6803.

I observed native microorganisms in the centrate that were able to grow on BG-11 liquid and agar mediums. I found that acidifying centrate with HCl and heat treating at 40°C for 24 hrs alkalizing to pH 8.0 with NaOH successfully sterilized the centrate, and had modified the composition of centrate such that the toxicity was alleviated in undiluted centrate. I hypothesized that the toxicity was removed during the acidification step; in which HCl would have hydrolyzed – and thus chemically altered – organic micro molecules present in the centrate. I would not expect that the use of concentrated acids and bases to perform this sterilization would be an economically or environmentally sustainable treatment process. I therefore only used a 0.2µm vacuum filter to sterilize centrate in proceeding experiments. This size filter is significantly smaller than the 100µm filter used by Gómez-Serrano *et al.* (2015) to remove solids [127]. Filtration is an important consideration for using actual centrate, as opposed to artificial or synthetic, as real centrate still contains solid wastes when removed directly from the decanter centrifuges.

Additionally, I established for the first time that centrate should be used within 24hrs of collection. After 24 hours, samples of centrate stored in 4°C began to show struvite precipitation as Figure 2.8 depicts in chapter 1. As struvite crystalizes and precipitates out of centrate, the concentrations of ammonia, magnesium, and phosphate are reduced from the media [169]. The

precipitation of struvite occurs under alkaline conditions [170]. I would therefore hypothesize that reducing the pH to 7.0 or just below might stabilize centrate and thus prolong the time frame for centrate use, without catalyzing hydrolysis reactions.

The literature consistently reports that microalgae and cyanobacteria can only survive in diluted centrate. It has previously been hypothesized that heavy metals and ammonia concentrations are toxic to microalgae in less dilute centrate. I evaluated the useable concentration range of centrate from the Drake Water Reclamation Facility (Fort Collins, CO) for the cultivation of *Synechocystis* sp. PCC6803. I performed a serial dilution of centrate in normal cyanobacterial growth media (BG-11) from 10% centrate (v/v) up to 100% centrate (v/v); which was then used to grow PCC6803. The *in vivo* fluorescence provided a means to monitor the biomass accumulation of PCC6803 cultures. My results showed that PCC6803 could be cultivated in centrate concentrations up 18% centrate for the experimental data shown. I observed that the lag phase increased as the centrate concentration increased, until the maximum tolerance was reached at 18% centrate. My results for the highest tolerated concentration of centrate in growth media was similar to that reported previously by Cai *et al.* (2013) of 24% centrate v/v in normal growth media [142]. I would hypothesize that the differences in centrate composition, starting inoculum, and lab strain of PCC6803 may contribute to the difference in observed maximum tolerance.

The literature describes the use of centrate as the sole nutrient source for microalgal cultivation, but not for cyanobacteria. By limiting the addition of exogenous nutrients to centrate, the feasibility of producing microalgae and cyanobacteria for wastewater remediation and biofuel production ought to be improved [117, 121, 131, 182, 234]. I therefore evaluated centrate for use as the only nutrient source for *Synechocystis* sp. PCC6803 cultivation. In the first experiment, dilutions of centrate from 3% up to 25% v/v in sterile water were used as the culture media for

PCC6803. I observed an increase in *in vivo* fluorescence as the centrate concentration increased up to 17% centrate, but a decrease in fluorescence in centrate concentrations above 17%. This would suggest that centrate cultures are nutrient limited in low concentrations of centrate (below 15%) but are under a toxic stress at concentrations above 19%, with complete inhibition at 23 and 25% centrate (based on growth curves in Figure 2.6 from chapter 1).

In the next experiment, I re-evaluated PCC6803 in 9, 19, and 25% centrate, but with the goal of testing the nitrogen drawdown from centrate by PCC6803. I performed cell counts on these cultures using flow cytometry, allowing me to compare the cell densities of the cultures in stationary phase. The final cell densities of the cultures in 9, 19, and 25% centrate at the time of harvest were $1.56x10^7$ cells ml^{-1} (n=4, SD = $3.86x10^6$ cells ml^{-1}), $2.25x10^7$ cells ml^{-1} (n=4, SD = $3.51x10^6$ cells ml^{-1}), and $9.13x10^5$ cells ml^{-1} (n=4, SD = $3.86x10^4$ cells ml^{-1}), respectively. PCC6803 grown in BG-11 grew to $5.57x10^8$ cells ml^{-1} (n=2, range = $3.7x10^7$ cells ml^{-1}). These results were in agreement with my *in vivo* fluorescence measurements, which I hypothesized was due to nutrient limitation. Following the incubation period, I performed a total dissolved nitrogen analysis of the cell free media and compared it to samples of diluted centrate collected before inoculation. I found that nitrogen was reduced from all cultures, with the greatest reduction in nitrogen from the 25% centrate media – despite no growth from these cultures. By calculating the expected nitrogen drawdown using the nitrogen content per cell and the density of the culture at the time of sample collection, I recognized that significantly more nitrogen was removed from all cultures than expected. An additional control experiment confirmed that the excess nitrogen removal was from nitrogen precipitation out of the media as struvite without the influence of PCC6803. The literature does not report similar analysis for expected vs observed nitrogen removal in experiments measuring the change in TN following incubation of microalgae in centrate, and therefore do not take into account the effects of chemical, in addition to biological,

removal of nitrogen. The poor biomass accumulation of PCC6803 in diluted centrate led to minimal nitrogen removal from centrate; and therefore suggests that PCC6803 does not remediate nutrients well when centrate is the sole nutrient source.

In order to improve the PCC6803 biomass accumulation on non-toxic concentrations of centrate, I performed a series of bioassays to elucidate which essential nutrient was limiting in centrate, with the assistance of Abby Sulesky and Bjoern Andersson, from the lab (see Acknowledgements). As chapter 2 describes, we supplemented centrate with the four different stock solutions of normal BG-11 growth media and assessed the biomass accumulation of PCC6803 on these medias. We continued by supplementing centrate with the individual compounds that make up BG-11 stock solution Part A, finding that centrate supplemented with $MgSO_4$ yielded the highest cell densities of cultures grown to stationary in comparison to the other Part A components. We tested the compounds $MgCl_2$ and Na_2SO_4 to determine if Mg^{2+} or SO_4^{2-} was the limiting nutrient in centrate; finding that Na_2SO_4 supplementation results in final cell densities of cultures in stationary phase ($3.18x10^8$ cells ml^{-1}, SD = $2.24x10^7$ cells ml^{-1}) that were closer to those observed in BG-11 control cultures ($3.32x10^8$ cells ml^{-1}, range = $4.63x10^7$ cells ml^{-1}). This result suggested that sulfate was limiting in centrate. To confirm that centrate induced a sulfate stress response *in vivo*, we performed RT-qPCR analysis of the sulfate transporter genes encoded by the *sbpA-cysTWA* operon, and found that the fold changes of *sbpA, cysT,* and *cysW* were all significantly higher in centrate grown cultures compared to centrate + Na_2SO_4 grown cultures, with the fold change of *cysA* being non-significant (p=0.07). The general trend that the genes of the *sbpA-cysTWA* operon are upregulated under sulfur starvation are consistent with those reported by Zhang *et al.* who performed a very comprehensive analysis of the gene expression changes associated with sulfur starvation in PCC6803 [212]. We then performed an elemental analysis of centrate using inductively coupled plasma – optical emission

spectrometry (ICP-OES) to evaluate the sulfur concentration. By combining the data collected from ICP-OES for phosphorus and sulfur, and combining it with TN analysis for dissolved nitrogen, we can generate N:P:S ratios in centrate. Centrate has an N:P:S of 50:3.5:0.25 whereas BG-11 has an N:P:S of 50:3:1, confirming that sulfur is limiting in centrate. We had hypothesized in chapter 1 that the concentrations of metals could be causing the observed centrate toxicity. Elemental analysis of centrate also provided the concentrations of several present in the sample. Iron, nickel, cadmium, cobalt, chromium, arsenic, and zinc were all below the toxic concentrations reported in the literature for cyanobacteria [230-233].

Sulfate supplementation improved PCC6803 biomass accumulation in centrate, and subsequently improved the measured nitrogen drawdown. Improved nitrogen remediation from centrate supplemented with exogenous sulfate by PCC6803 makes the model cyanobacterium a more industrially feasible candidate for combined wastewater treatment and biofuel production. Additionally, this study presents the novel finding that a single macronutrient can be significantly limiting in centrate for microalgal and cyanobacterial cultivation.

In order to further integrate wastewater treatment with biofuel production, I proposed redirecting the H_2S gas produced by sulfur reducing bacteria during anaerobic digestion towards the cultivation of PCC6803 in centrate. H_2S is an un-desirable product of the biological nutrient removal process due to its pungent odor. Currently, H_2S produced at the DWRF is vented to biofilters consisting of wood chips and compost where sulfide oxidizing microbes convert sulfide into elemental sulfur. H_2S from anaerobic digestion would be an economical and sustainable sulfur source compared pyrite and gypsum which must be mined [223]. PCC6803 has already been shown to oxidize hydrogen sulfide using the light dependent, type I sulfide:quinone oxidoreductase (SQR, sll5036) [228]. This study further supports the feasibility of using H_2S as the sulfur source for PCC6803 cultivation on centrate for further nutrient removal during

wastewater treatment for biofuel production applications. Future studies will need to evaluate the interactions of H_2S gas in centrate, and then characterize biomass accumulation of Synechocystis sp. PCC6803 in this media.

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APPENDIX: METHODS DEVELOPMENT FOR MUTAGENESIS AND DIRECTED EVOLUTION FOR *SYNECHOCYSTIS* SP. PCC6803 FOR IMPROVED CENTRATE TOLERANCE

Introduction

The following represents a significant portion of work dedicated at the beginning of the project aimed at increasing the tolerance of *Synechocystis* sp. PCC6803 to centrate. The experiments leading to the proposed methods were conducted prior to establishing that sulfur limited biomass accumulation of PCC6803 grown in centrate. It was hypothesized that using random chemical mutagenesis followed by selection on agar plates containing high concentrations of centrate would yield novel centrate tolerant mutant strains of PCC6803. Upon characterizing the growth rates, biomass accumulation, and centrate tolerance of the mutant strains, the most productive strains (highest biomass accumulation, greatest growth rate, highest centrate tolerance) would then sent for genomic sequencing using next generation sequencing to elucidate the genetic mutations that provided centrate tolerance.

Materials and Methods

Culture conditions, standard growth media, and growth measurements

Organism and normal growth media

The cyanobacterium *Synechocystis* sp. PCC6803, generously gifted from Dr. Jianping Yu of the National Renewable Energy Laboratory (NREL), was maintained axenically in modified BG-11 pH8 growth media (17.6mM $NaNO_3$, 304µM $MgSO_4$, 245µM $CaCl_2$, 31µM Citric Acid, 22.9µM $C_2H_8O_7$•xFe_3^+•yNH_3, 3.4µM EDTA, 1.05mM K_2HPO_4, 189µM Na_2CO_3, 46.3µM H_3BO_4, 14.4µM $MnCl_2$, 0.7µM $ZnSO_4$•$7H_2O$, 1.9µM Na_2MoO_4, 0.3µM $CuSO_4$•$5H_2O$, 0.2µM $Co(NO_3)_2$•$6H_2O$; buffered with 10mM Tes-NaOH buffer (pH 8)) (Peebles, need

reference). All chemicals used for the BG-11media were laboratory grade and purchased from either Thermo FisherScientific® or Millipore Sigma®. Unless otherwise noted, PCC6803 cultures were incubated in a Percival Model E30B (Perry, IA) Incubator under 165 µmol photons m^{-2} s^{-1} of continuous light at 30°C. Liquid cultures were grown in 125ml Erlenmeyer flasks on an orbital shaker (VWR, Model 3500).

Wastewater centrate collection and standardized use

The supernatant (centrate henceforth) from the wastewater dewatering process of anaerobically digested sludge was collected directly from the north decanter centrifuge (make and model) at the City of Fort Collins Drake Water Reclamation Facility (Fort Collins, CO) in 250ml plastic bottles provided by the dewatering facility. Immediately following collection from the centrifuge sampling port, centrate was vacuum filtered through a 0.2µm filter (Thermo Scientific™ Nalgene™ Rapid-Flow™ 75mm Bottle Top Filter-500ml) to remove any remaining solids and native microorganisms. Filtered centrate was kept at 4°C until use.

Centrate + BG-11 media used for mutant selection and the directed evolution experiments described below was made by diluting filtered centrate with 2x BG-11 and autoclaved MilliQ H_2O (e.g. to make 1L of 20% centrate + BG-11 media; combine 200mls of filtered centrate with 300mls of MilliQ H_2O and 500mls of 2x BG-11). Centrate was collected once a week from DWRF, filter sterilized, and stored at 4°C until use. Centrate + BG-11 media was made fresh just before use.

Cell counts

Cell counts were done by either flow cytometry or by hand. Flow cytometry based cell counts were performed by first filtering diluted culture through a 30µm pre-separation filter (Miltenyi Biotec Inc.; Auburn, CA) and then running samples through a BD Accuri™ C6 Plus Personal Flow Cytometer (BD Life Sciences; San Jose, CA). Hand counts were accomplished

using a Reichart Bright-Line Hemacytometer with Neubauer ruling (Hausser Scientific; Horsham, PA) under 40x magnification with a bright field microscope. As an additional proxy for culture density, a Cary 60 UV-Vis Spectrophotometer was used to measure the *in vivo* absorption at 750 nm (OD_{750}).

Glycerol stocks

Aliquots of *Synechocystis* cultures (10-15mls) grown to mid/late-exponential were spun down in 15ml conical tubes at 3220g for 15 minutes at 4°C. 500µl of supernatant was pulled and used to re-suspend the pellet after the remainder of the supernatant was discarded. 500µl of sterile 50% Glycerol (v/v in MilliQ H_2O) solution was then added to the suspension. The 1ml of cells was transferred to a 2ml Nalgene® Cryogenic Vial and flash frozen in liquid nitrogen before being stored at -80°C.

Mutagenesis and plate based mutant selection

Mutation induction methods for cyanobacteria – previously described by [235] and [236] – were adapted using methods described by [237] for *Escherichia coli* and *Salmonella typhimurium.*

Culture conditions

A wild type population of *Synechocystis* sp. PCC6803 grown to exponential phase in liquid BG-11 media was streaked out onto BG-11 agar medium and incubated as defined above. Individual colonies were plucked at random and grown to mid-exponential phase in fresh BG-11 media. Glycerol stocks were made of the monoclonal WT culture prior to subjection to the proceeding mutagenesis protocol.

The cell density of a monoclonal PCC6803 WT culture was measured using flow cytometry. The culture was then either concentrated by centrifugation or diluted with fresh BG-11 media as necessary to obtain a concentration of $5x10^7$ cells ml^{-1}. In order to determine the

volume of culture necessary to mutagenize, the number of plates to be inoculated was multiplied by the final inoculum of $5x10^6$ cells (the number of cells in 100µl of $5x10^7$ cells ml^{-1}). This calculation was performed for both the expected number of centrate plates (described below) to be plated by mutagenized PCC6803 as well as non-mutagenized PCC6803 WT culture.

Chemical mutagenesis by MMS

Cells to be mutagenized were pelleted by centrifugation at 3220g for 15 minutes at room temperature in 50ml conical tubes. 5ml of supernatant was used to re-suspend the pellet after the remaining supernatant was discarded. In a chemical fume hood, 50µl of methylmethanesulfonate (MMS) was added to the 5ml suspension for a final concentration of 1%. The suspension was mixed by shaking and incubated at room temperature for 1 minute. Following the incubation period, the suspension was immediately diluted 1:10 with 45 mls of BG-11. Cells were pelleted again by centrifugation at 3220g for 15 minutes at room temperature. The supernatant was discarded into hazardous waste and the cells were washed with fresh BG-11. Cells were again pelleted by centrifugation, the supernatant removed, and the cells re-suspended in fresh BG-11 for a second wash step. Cells were then concentrated or diluted (as necessary) to achieve a final cell concentration of $5x10^7$ cells ml^{-1} before plating on selection plates.

Treatment lethality

In order to establish the dosage affect and lethality of MMS on *Synechocystis*, mutagenesis was performed using different concentrations of MMS ranging from 0.1% up to 10%. Cell counts of two monoclonal wild type cultures in mid-exponential were performed using flow cytometry. Cultures were then concentrated or diluted as necessary in order to obtain $2x10^5$ cells ml^{-1} in a total of 8mls for each of the two biological replicates. The 8ml aliquots of culture were then divided amongst 8x 15ml conical tubes (1ml each) for each biological replicate.

Mutagenesis was performed by adding 0, 1, 5, 10, 15, 20, 25, and 100µl of MMS directly to the 8 conical tubes for each biological replicate to make final dilutions of roughly 0, 0.1, 0.5, 1, 1.5, 2, 2.5, and 10% MMS (a non-mutagenized control was also included for each biological replicate). Following the 1 minute incubation and 1:10 dilution using 9mls of BG-11, the two washing steps were performed with a final re-suspension of the pellets in 400µl of BG-11 media. 100µl of the mutagenized cultures were immediately plated onto each of four BG-11 agar plates (technical replicates) per set of biological replicates for each mutagen dose. Plates were incubated under continuous light of ~65 µmol photons m^{-2} s^{-1} at room temperature for 12 days before colony counts were performed.

Putative mutant selection on centrate agar medium

Selection of putative centrate tolerant PCC6803 strains occurred on diluted centrate + BG-11 + 1.5% agar plates (centrate plates henceforth). All centrate plates were poured at the DWRF Pollution Control lab. The process of making centrate plates differed from that of making BG-11 agar plates as follows. Dried agar was added to twice concentrated (2x) BG-11 to a final concentration of 3% agar, and then autoclaved to melt and sterilize the 2x BG-11 + 3% Agar medium. Centrate (collected and filtered as described above) was diluted to 2x the final desired concentration with MilliQ H_2O (e.g. to make 15% centrate plates, full strength filtered centrate was first diluted to 30% in MilliQ H_2O), and added 1:1 directly to the melted BG-11 + agar. Plates ranged in centrate concentration from 10% to 30% centrate. Sodium thiosulfate pentahydrate ($Na_2S_2O_3 \cdot 5H_2O$) was added to a final concentration of 1mM to the melted centrate + BG-11 agar medium prior to pouring plates. Plates were allowed to cool at room temperature for at least 3 hrs, and were then stored at 4°C. Plates were brought back to room temperature just prior to use.

100µl of mutagenized PCC6803 ($5x10^7$ cells ml^{-1}) were plated out onto centrate plates. Inoculated centrate plates were incubated under ~65 µmol photons m^{-2} s^{-1} continuous white light at room temperature for up to one month. Plates were checked twice weekly for colony growth. Surviving colonies were then subjected to the putative mutant triage described below.

Directed evolution

An alternative approach to generating high centrate concentration tolerant mutants utilized successive passaging of *Synechocystis* culture into increasing concentrations of centrate over long periods of time. The directed evolution method described by [238] was used with the modifications described below.

Mutant population generation and selection in centrate media

Monoclonal wildtype *Synechocystis* culture were grown to mid-exponential under continuous light. Culture was mutagenized with 1% MMS as previously described in order to generate random mutations within a monoclonal population. The mutagenized culture was then used as inoculum (inoculum contained $1x10^8$ cells) into 20% and 25% centrate in BG-11 media; instead of being plated as described above. Non-mutagenized culture was also used to inoculate 20% and 25% centrate + BG-11 media as controls. All cultures were incubated under 165 µmol photons m^{-2} s^{-1} of continuous light at 30°C in 250ml Erlenmeyer flasks on an orbital shaker for 18 days.

Increasing population tolerance to centrate

Following the incubation period, surviving cultures were allowed to recover in BG-11 media to promote cell division and growth of the tolerant cells within the population. Glycerol stocks were then made of the tolerant populations that survived mutagenesis and the preliminary centrate selection in liquid media. 5 ml of each of the populations of mutagenized and non-

mutagenized cultures that survived the initial selection were then used to inoculate fresh 22% centrate + BG-11 media (just at the maximum centrate tolerance of WT cultures as described in Chapter 1) by pelleting the cells by centrifugation at 3220g for 15 minutes at 25°C and re-suspending in 25mls of 22% centrate + BG-11 media. Cultures were then incubated for up to one week under 165 µmol photons m^{-2} s^{-1} of continuous light at 30°C in 125ml Erlenmeyer flasks on an orbital shaker, completing the first round of directed evolution.

From each of the cultures surviving the first round of directed evolution in 22% centrate, 10 mls was used to make glycerol stocks and 5 mls was used as the inoculum for the next round of directed evolution in 24% centrate (set up as previously described for 22% centrate + BG-11 media). The remaining ~10mls of each culture, BG-11 media was added to allow recovery and to preserve the culture as a back-up to return to should the population not survive in 24% centrate BG-11 media. An additional control was included of one wild type culture (that had not previously been exposed to centrate) as inoculum into 24% centrate + BG-11 media. Cultures were then incubated for up to one week under 165 µmol photons m^{-2} s^{-1} of continuous light at 30°C in 125ml Erlenmeyer flasks on an orbital shaker, completing the second round of directed evolution. This process continued until cultures were no longer able to tolerate a centrate concentration after three attempts.

Isolation of putative tolerant strains

In order to select single isolates of a tolerant monoclonal strain to subject to a triage for further strain characterization, each of the tolerant populations were streaked out for isolation on both BG-11 agar plates and 27.5% Centrate + BG-11 agar plates. Plates were incubated as previously described for up to 2 weeks.

Results and Discussion

In total, I performed over 30 mutagenesis experiments for the development of the described methods (Data not included in this thesis). With the methods established, I proceeded to increase the scale of which I was attempting to select for mutants. Through 14 large scale experiments, I was able to identify and isolate 94 putative mutant colonies from 812 centrate + BG-11 agar plates ranging in centrate concentration from 20-27.5% centrate. Of the 94 putative mutant strains, 91 came from one experiment in which I plated out 200 centrate + BG-11 agar plates. Colonies were observed on centrate plates only after at least three weeks of incubation. Despite performing well over 40 experiments in total, no centrate tolerant mutants could be confirmed. When putative mutants were re-plated onto new centrate + BG-11 agar plates of equal and greater centrate concentrations, I did not observe growth for any of the putative centrate tolerant mutants. Putative centrate tolerant mutants were further confirmed to be false positives as they did not show improved growth rates, biomass accumulation, or tolerance to centrate compared to wild type PCC6803. Experiments were discontinued after one year to focus on the work presented at the end of Chapter 1 through Chapter 2.

Important to note is that these mutagenesis and directed evolution experiments were performed prior to our characterization of PCC6803 grown on centrate supplemented with sulfate. Integrated into the initial hypotheses for these experiments was the prediction that high concentrations of centrate would provide industrially relevant biomass for cyanobacterial cultivation. As shown in Chapter 2, high biomass could be achieved when PCC6803 was grown on only 10% centrate supplemented with sulfate; and attempting to achieve higher biomass would result in cultures becoming light limited as described by Andersson (2017) [211]. These results suggest that there is not a demand for generating high centrate tolerant PCC6803 for improved biomass accumulation. This is especially true considering that PCC6803 can tolerate

up to 20% centrate which would account for any substantial fluxes in centrate nutrient composition during PCC6803 cultivation on 10% centrate.

Generating high centrate tolerant mutants could still be of benefit for the mass cultivation of PCC6803. A one to two day longer lag phase was consistently observed for PCC6803 cultures grown on centrate compared to BG-11 grown cultures. If cultures were more tolerant to centrate, then perhaps this could reduce the initial lag phase for mass cultivation. Reducing the lag phase of PCC6803 grown on centrate would improve the turn-over of cultures, such that instead of taking 7 days for a culture to reach high biomass, only 5 days would be necessary. Assuming no set-backs; reducing the lag phase by two days would increase the number of cultures grown annually from 52 (assuming one culture/week) to 73; which is a 40% increase in the annual biomass yield. Additionally, more centrate could be remediated annually by increasing the number cultivation cycles; and thus both biomass production on- and bioremediation from centrate would become increasingly economical and sustainable. Therefore, attempting to generate high centrate tolerant mutants with selection for centrate tolerance using the methods described in this appendix could be of significant value for improving the simultaneous production of cyanobacterial biomass and nutrient remediation of centrate.