Algal biomass production and nutrient removal from high strength anaerobic digestate

by

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Abstract

The integration of microalgal biomass production with nutrient removal from the liquid portion of anaerobic digestate holds the potential to close the loop on waste. However, algal growth inhibition in anaerobic digestate has greatly suppressed the development of growing microalgae in anaerobic digestate at scale. Typically, 10-50 fold dilution were used to overcome the inhibition in lab scale studies which tried to grow microalgae in anaerobic digestate, but it is not cost-effective using dilution as the primary approach for inhibition alleviation in large scale algae-digestate treatment systems when considering the expansion of reactor volume, the large amount of freshwater usage, and the increased land occupancy. This dissertation focuses on alleviating algal growth in anaerobic digestate by a non-dilution biological pretreatment process.

The algal inhibitory effects from anaerobic effluent were observed shortly after the attempts of growing microalgae in the digestate. The inhibition was severe and ubiquitous for a variety of microalgae in different types of anaerobic digestate based on our own work. The most common hypothesis for inhibition on digestate is the high total ammonia nitrogen (TAN) in the digestates. However, TAN inhibition did not fully explain the observations from algal growth in anaerobic digestate. High ammonium tolerating algae strain such as *Chlorella sorokiniana* can be robust in a chemical medium with 3500 mg L^{-1} ammonium. Moreover, the meta-analysis also revealed relationships between cultivation factors (e.g., light intensity, solid-separation, initial TAN, dilution factor, axenic condition etc.) and algal productivity in anaerobic digestate. Interestingly, neither TAN nor dilution were significant factors. In contrast, the use of chemical or biological pretreatment of digestate, solids removal, increased light intensity, and lower pH also resulted in significantly higher algal productivity. This analysis suggests that the

development of non-dilution pretreatment approaches is essential for scale-up of algae-digestate treatment systems.

Biological wastewater treatment such as activated sludge is a relatively mature technique in most wastewater treatment plants. The use of aerobic bacteria can be effective in removing organic and inorganic pollutants. First objective in this dissertation was to use aerobic bacteria as a pretreatment process for anerobic digestate before the inoculation of microalgae. A consortium of bacteria obtained from an activated sludge wastewater treatment plant was used to pretreat digestate prior to algae growth. No dilution of digestate was used. *C. sorokiniana* achieved very high biomass productivity of 250-500 mg L^{-1} day^{-1} in bacteria-pretreated municipal sludge digestate and food waste digestate whereas little to negative productivity was observed in the digestates without pretreatment. Pretreatment also led to significant increase in nutrient removal rate compared to the non-pretreated ones.

The second objective of this research was to understand what cultivation factors contribute to successful pretreatment of digestate prior to algae growth… The performance of aerobic bacteria pretreatment for alleviating algal growth inhibition in undiluted anaerobic digestate was tested with two different strains of algae (*C. sorokiniana* and *A. protothecoides*) in two different strengths of anaerobic digestate. Both digestate types were obtained from a sludge digester at a municipal wastewater treatment plant but were collected at two different times: one digestate contained 1372 mg/L NH4-N (high strength) and the other contained 433 mg/L NH4-N (low strength). In high strength municipal sludge digestate, both strains of algae benefited from pretreatment, but in low strength digestate, the growth of *C. sorokiniana* was suppressed due to nutrient limitations. The performance test also revealed that longer pretreatment period generally had positive effect on alleviating algal growth inhibition from the digestate. Interestingly, the

xenic (vs. axenic) condition was not a significant factor in this experimental result which is consistent with the result of multiple regression model from the meta-analysis study.

Up until this point, model strains of algae were used in all experiments. However, such strains are unlikely to be practical in real-world systems given concerns about introducing non-native organisms to the environment. Consequently, the third objective of this dissertation was to adapt locally obtained consortia of algae to pretreated digestate and test the adapted community's growth and nutrient removal performance. Local consortia were collected from local fishponds and the biofloc solids from Auburn University's aquaponics system. The consortia were initially inoculated in 10% aerobic bacteria pretreated dairy manure anaerobic digestate, and gradually increased to 100% pretreated digestate. A complete restructuring of the algal community was observed in which the initial eukaryotic community was 95% Euglena the final was 70% Coelastrum with complete die-out of Euglena. Although the adapted consortium had 75% of the growth productivity of *C. sorokiniana* in the pretreated digestate, it did not grow in the non-pretreated digestate. This result reinforced the importance of digestate pretreatment for this native consortium.

In summary, aerobic bacteria pretreatment is confirmed to be effective and critical for algal biomass production and nutrient removal in undiluted anaerobic digestate.

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List of Abbreviations

FAO	Food and Agricultural Organization
WHO	World Health Organization
WWTPs	Wastewater Treatment Plants
COD	Chemical Oxygen Demand
GHG	Greenhouse Gas
CI	Confidence Interval
VAs	Veterinary Antibiotics
LD	Liquid Digestate
MAD	Municipal Anaerobic Digestate
AS	Activated Sludge
FWAD	Food Waste Anaerobic Digestate
OD	Optical Density
ICP-OES	Inductively Coupled Plasma – Optical Emission Spectrometry
LC	Liquid Chromatography
VFAs	Volatile Fatty Acids
AD	Anaerobic Digestate
DMSO	Dimethyl Sulfoxide
FAME	Fatty Acid Methyl Esters
DNS	Dinitrosalicylic

Chapter 1: Introduction

1.1 Motivation

Living in the 21st century is really a combination of luck and challenge. On one hand, the access to information is historically convenient for everyone with modern technologies. On the other hand, the environmental issues are starting to impact everyone's daily life, mainly due to the ongoing development of industry. With the global population projected to pass 8 billion in the near future, human society is facing challenges from almost every aspect. Based on the Food and Agricultural Organization (FAO) at the United Nations, 770 million people were undernourished in 2020, and this number is an 18% increase from one year ago (FAO, 2021). Also, the World Health Organization (WHO) predicted that over half of the world's population will experience water stress by 2025. Other than food and water, energy and material demand is skyrocketing, but the concerns on greenhouse gas emissions and climate change conspire against increasing the use of fossil fuels. Therefore, the need for innovation in food, water, energy, and material supply has never been so urgent.

1.2 The role of algae in solving global problems

Algae account for 50% of the total photosynthesis on earth (Chapman, 2010). Similar to crop plants, algae are able to produce a variety of organic compounds such as protein, lipids and carbohydrates which are essential to heterotrophic organisms and therefore form the base of aquatic and marine food webs. They also are the fastest-growing photosynthetic organisms for biomass production (Jones & Mayfield, 2012) and are well-positioned for engineering applications.

The term of algae incorporates two major groups: macroalgae and microalgae.

Macroalgae are plant-like species which usually live in aquatic/marine environments. The major difference between macroalgae and plants is that macroalgae get their nutrients from the surrounding water instead of through a root system. Macroalgae are widely used in making products like agar, alginates, etc. because their cells contain up to 20% polysaccharides (Shackira et al., 2021).

Microalgae, on the other hand, have the features of both bacteria and plants. Similar to bacteria, microalgae have single cells and lack tissue structures which allow them to easily access surrounding nutrients. Moreover, many microalgae thrive under extreme environmental conditions which means that they can be cultivated on non-arable lands; so, growing microalgae does not compromise current food production (Geada et al., 2021). Microalgae also have the ability to reproduce by binary division asexually (Russell et al., 2022). The fast replication of microalgal cells gives them more advantage in biomass production compared to conventional crops. Similar to plants, microalgae dominantly contribute to the conversion of mineral compounds into organic biomass. There is increasing interest in using algal biomass for bioproducts. Depending on the algal strains and cultivation conditions, dry algal biomass can have up to 80% of lipid content (Chiappe et al., 2016). High lipid-containing algal biomass could potentially be used as a feedstock for biocrude production (Mathimani & Pugazhendhi, 2019). Additionally, some algal biomass is rich in proteins (50-70%) (Chew et al., 2017a). The potential to supplement algal protein into animal feeds could provide a great relief on the increasing protein demand from other sources such as marine fish mill and soybeans. Algal biomass also contains a variety of value-added products such as pigments (Trivedi et al., 2015), enzymes, and vitamins (Wells et al., 2017). Other than the products from algal biomass, microalgae facilitate the task of recycling “waste compounds” (ammonium, carbon dioxide etc.) from heterotrophic

organisms by photosynthesis. The role of microalgae is critically important for treating waste in natural systems.

1.3 Wastewater treatment

The increasing population is adding pressure not only to supply but also on the generation of wastewater. In highly populated urban areas, wastewater treatment usually consists of several energy-intensive processes. For municipal wastewater, primary wastewater treatment usually consists of initial solid-liquid separation. Basically, the inflow wastewater is screened and settled during this process. Secondary wastewater treatment usually involves biological approaches. In this step, removing organic compounds are the target. Fecal pellets, pharmaceutical residues, hormones, and pathogens are all contributing to the organic loading in wastewater. Activated sludge basins are the most common approach for removing organic compounds in wastewater treatment plants (WWTPs). In short, air is pumped intensively into the basin to maintain a minimum dissolved oxygen level for the growth of heterotrophic bacteria. Up to 88% of chemical oxygen demand (COD) is removed from the wastewater by activated sludge processes (Wang et al., 2018a), but the excess bacteria biomass produced in the activated sludge process (biosolids) often undergoes anaerobic digestion in big WWTPs. Besides organics, activated sludge basins also contribute to partial removal of nitrogen via nitrification and denitrification (Wang et al., 2018a). However, the limitations of the activated sludge approach are getting more attention as the strength of inflow wastewater increases. More WWTPs are now relying on tertiary treatments for further nitrogen (N) and phosphorus (P) removal.

1.4 Anaerobic digestion

Anaerobic digestion is getting more attention due to its capacity to reduce greenhouse gas (GHG) emissions. Typically, organic wastes such as manure begin fermentation automatically

when oxygen is limited (land fill, piling, lagoons etc.). This uncontrolled methane gas (CH_4) causes 28-36 times more global warming potential than carbon dioxide (CO_2) in 100 years. Anaerobic digestion collects this CH_4 in a controlled way and uses it as a renewable energy source. Moreover, the solid product of anaerobic digestion is widely used as biofertilizer for organic farming. Anaerobic digestion can be used for a variety of organic-containing wastes such as biosolids, animal manure, food waste, tillage waste etc. Since anaerobic digestion has big environmental advantages, there is an increasing trend in the construction of new anaerobic digestors in the United States. However, the liquid portion of anaerobic digestate is a low value waste compared to its gas and solid products. Since liquid digestate usually contains high nutrient concentrations (up to 5000 mg L^{-1} NH_4-N, and up to 2000 mg L^{-1} COD), direct discharge without further treatments can cause serious problems. Therefore, new technologies are needed to remove the excess nutrients from the liquid digestate.

1.5 Algae wastewater treatment system and research gaps

Growing algae in liquid digestate could potentially achieve algal biomass production and nutrient removal simultaneously. The use of liquid anaerobic digestate for algal cultivation has been well studied as documented in a recent review (Chuka-ogwude et al., 2020c). A variety of research provides evidence that liquid digestate contains all the essential nutrients for algal growth (Chuka-ogwude et al., 2020c; Xia & Murphy, 2016a). However, there are still obstacles before large scale algal production can happen using anaerobic digestate. 1. Algal growth inhibitors are widely observed in digestates. There is little information about which compounds and what mechanisms are causing algal growth inhibition in digestate. 2. Although 10-50 fold dilution was the most adopted approach for alleviating algal inhibition from digestate in lab scales, it is not cost-effective to use dilution as the primary pretreatment. Expanded infrastructure

construction, large amounts of freshwater, and more requirement for land and space makes the dilution approach less favorable for scaling up algal-digestate treatment systems. 3. The current technology lacks an effective non-dilution pretreatment approach for alleviating algal growth inhibition in the digestate.

1.6 Research Objectives

Objective 1: to provide quantitative insight into algal growth and nutrient removal in anaerobic digestate including the influence of cultivation factors and digestate characteristics. (**Chapter 2**)

Objective 2: (i) assess whether ammonium, turbidity, or heavy metals in digestate were the primary sources of inhibition for a highly nutrient tolerant strain of *Chlorella sorokiniana* and (ii) test the effectiveness of aerobic activated sludge pretreatment of digestate as a means of reducing inhibitor concentrations in full-strength anaerobic digestates. (**Chapter 3**)

Objective 3: to investigate the conditions under which digestate pretreatment is effective in promoting algal growth, nutrient removal, and favorable changes in algal biomass composition. (**Chapter 4**)

Objective 4: to investigate the growth, community composition, and digestate treatment performance of a local algae consortium that was adapted to bacteria-pretreated digestate (**Chapter 5**)

Chapter 3-5 (Objectives 2-4) have been published in Water Research, Algal Research, and Bioresource Technology, respectively. Chapter 2 (literature review) will be submitted for publication in Algal Research.

Chapter 2: Engineered algal systems for the treatment of anaerobic digestate: a meta-analysis

Abstract

The objective of this systematic review was to provide quantitative insight into algal growth and nutrient removal in anaerobic digestate. While there is great research interest in algal growth on digestate, a variety of challenges hinder industry adoption. To synthesize the relevant literature, a meta-analysis was conducted using data from 54 recent articles to elucidate key factors that impact algal biomass productivity and nutrient removal from anaerobic digestate. The analysis revealed that, on average, the difference between biomass productivity in anaerobic digestate vs in chemical media were not statistically significant ($p = 0.3876$). A multiple regression model of the raw data revealed that solids separation and biological or chemical pretreatment of digestate significantly increase productivity ($p < 0.001$) whereas the commonly-used practice of digestate dilution had no significant effect. Lower pH and higher light intensity also significantly promoted algal growth ($p < 0.0011$) whereas total ammonia nitrogen, xenic status, and temperature, and reactor volume were not statistically significant. Higher growth significantly increases NH_4-N and phosphorus removal with a linear relationship of 7.6 mg L^{-1} day^{-1} NH_4-N and 1.5 mg L^{-1} day^{-1} P removed per 100 mg L^{-1} day^{-1} increase in biomass productivity ($p < 0.001$). Algae showed signs of removing chemical oxygen demand from digestate and that this removal showed a significant relationship with growth in axenic cultures ($p = 0.01$) but not in xenic cultures ($p = 0.519$). The literature suggests that suboptimal algal growth in anaerobic digestate could be due to factors, such as turbidity, high free ammonia, and residual organic compounds. This analysis shows that non-dilution approaches for alleviating algal inhibition are recommended for engineered algal digestate treatment systems.

Keywords: microalgae, wastewater, nutrient recycling, meta-analysis

2.1 Introduction

Incorporating microalgae production into waste treatment holds great environmental and economic potential for facilities, such as wastewater treatment plants and confined livestock operations, to meet increasingly stringent nutrient discharge requirements. Nutrient reduction technologies such as nitrification/denitrification and phosphorus precipitation are costly processes require large inputs of energy, material, and specialized construction (Rout et al., 2021; Zubair et al., 2020). Technologies, like denitrification and the less-energy intensive denitritation, also dispose of fixed nitrogen via transformation into nitrogen gas. This is inherently wasteful given the large energy expenditures made to fix nitrogen gas into ammonia fertilizers (Vitosh et al., 2019). Given these challenges, innovative methods need to be developed to address the eutrophication, climate change, and water pollution associated with waste and wastewater disposal. Algal-based wastewater treatment technologies have the potential to recover nutrient pollutants from wastewater in their fixed form, while producing potentially valuable biomass. Doing so reduces environmental impacts while displacing the need for chemical fertilizer application in algal cultivation (Ahmad Ansari et al., 2020). Algal-based wastewater treatment has also been shown through technoeconomic and life cycle assessment to have lower cost and environmental impacts than conventional activated sludge technology (Garfí et al., 2017; Kohlheb et al., 2020).

Despite their promise, algal technologies have yet to achieve widespread adoption for wastewater treatment due to persistent technical challenges. Algae initially attracted attention for energy-efficient nutrient recovery from wastewater as part of a tertiary treatment process. Unfortunately, empirical studies to date show that treated wastewater is a poor growth medium

for algae, resulting in slow growth rates on the order of 8 g m^{-2}-d^{-1} (Craggs et al., 2011) or 0.022 g m^{-2}-d^{-1} (Ramanna et al., 2014). This is due primarily to the already-low nutrient levels in treated wastewater (typically <20 mg L^{-1} N and <5 mg L^{-1} P) that are roughly an order of magnitude lower than those in most algal media. Slow growth translates to larger and more expensive facilities for algae cultivation. In contrast, algal biomass production (and concomitant nutrient reduction) using high strength wastewater, like the liquid fraction of anaerobic digestate, has the potential to overcome this limitation, allowing for more rapid algal growth, smaller facility footprints, and the production of biomass whose value outweighs the cost of production.

Unlike the solid anaerobic digestate that is popularly used as a biofertilizer on crops, the application/treatment of the liquid portion is more difficult due to higher transportation cost (Duan et al., 2020), a complex chemical matrix, and higher environmental risks associated with disposal (Styles et al., 2018). This is largely because the high concentrations of nutrients contained in most liquid digestate (henceforth termed simply digestate) exceed the natural capacity of the surrounding environment for assimilation. Commercial scale municipal treatment plants generally redirect their high strength liquid digestate back into their treatment works for re-treatment by activated sludge (Pennington, 2018). This is inherently inefficient because it treats nutrients like a waste in need of disposal as noted above. Algae have great potential to treat anaerobic digestates while recovering nutrients in valuable biomass. Consequently, there has been a large increase in published studies on the topic of growing algae (and algae-bacteria consortia) on anaerobic digestate for nutrient recovery and biomass production over the past 5 years. These studies reveal wide variation in algal growth performance characteristics as well as some key barriers to progress in the field (Al-Mallahi & Ishii, 2022b). Several reviews have been written on this topic (Al-Mallahi & Ishii, 2022b; Chuka-ogwude et al., 2020c; Folino et al., 2020;

Xia & Murphy, 2016a) recently but none have approached the literature in a quantitative manner. The present study remedies this gap by synthesizing and evaluating data from the literature in both qualitative and quantitative (statistical) ways.

The objective of this review was to provide quantitative insight into algal growth and nutrient removal in anaerobic digestate including the influence of cultivation factors and digestate characteristics. It was hypothesized that a large number of factors, including digestate type, algae type, cultivation conditions, and digestate pretreatment, would influence cultivation success. It was also hypothesized that growth performance on digestate would in general be inferior to growth on chemical media given the latter's tailoring for optimized algal growth. This review reveals that there are still significant challenges to overcome to maximize the commercial viability of algal treatment of digestate. One of the biggest problems is that the production of microalgae biomass from high strength digestate is often found to be suppressed when comparing to the same algae in diluted digestate or in chemical media (Al-Mallahi & Ishii, 2022b). This review concludes with a review of research areas aimed at overcoming this growth inhibition.

2.2 Material and methods

2.2.1 Quantitative and qualitative approach

This literature review contains both a quantitative component in the form a meta-analysis and a qualitative component in which the results of the meta-analysis are placed in context. Together, these are used to identify promising management practices as well as areas of research that have a high likelihood of advancing algal treatment of digestate.

2.2.2 Literature search

The literature selection was conducted through a keyword search of the database, Web of Science, and search engine, Google Scholar. The selection process followed the PRISMA protocol (Page et al., 2021). Briefly, the literature search was first conducted with the keywords: "anaerobic digestate or biogas effluent" and "microalgae" that identified 172 articles in Web of Science and more than 10,000 search results were displayed in Google Scholar despite filtering for papers published after 2017. An initial screening process was then used to select the relevant studies by reading titles and abstracts. Two major classes of article emerged based on the searched keywords. The first class of articles focused on algal biomass as a feedstock for anaerobic digestion, which is not the topic of this analysis. These articles were therefore eliminated from further analysis. The other class of articles focused on use of anaerobic digestate as a nutrient source for algal cultivation and were retained. This latter group represented 20% of the initial search results and underwent additional screening processes. Studies that lacked either algal growth data or initial nutrient concentrations were eliminated from the analysis. The algal growth studies that were conducted only in artificial digestate were also excluded from this analysis. The result was a subset of 24 articles. a secondary search among paper published before 2017 on web of science resulted in 13 additional paper to the analysis with the same keywords. In total, the meta-analysis considered data from 37 published studies. Due to data availability constraints, only data from 12 studies were included in the meta-analysis, data from 29 studies were used in the multiple linear regression analyses, and data from 15 studies were used for regression analysis on nutrient removal rate against biomass productivity.

2.2.3 Statistical analysis

2.2.3.1 Descriptive statistics and hypothesis testing

The effect size represented the standard mean difference (Cohen's d):

$\frac{Digestate - Chemical\ mean\ difference}{pooled\ standard\ deviation}$ between the algal biomass productivity in anaerobic digestate versus its algal productivity in chemical growth medium. Weighted analysis was used to calculate 26 effect sizes and 95% confidence interval among 12 of the 37 studies because only these articles reported algal productivity both in anaerobic digestate and in chemical medium. Meta-analysis was conducted based on a random effect model with a random factor which was used to control multiple effects from one study.Effect sizes were calculated and meta-analysis was conducted in R using the *metafor* package (Viechtbauer, 2010), and the box plots were prepared in R using the *ggplot2* package (Wickham, 2016).

2.2.3.2 Multiple regression analysis

To test the hypothesis that specific digestate treatment and cultivation conditions impact algal growth on digestate, a multiple regression analysis was carried out in R using the '*plm*' package. A subset of 29 articles were included in this analysis because they contained data on parameters that were hypothesized to have significant impacts on algal growth. These parameters included: 1) reactor volume (L), 2) use of solids separation, 3) use of biological or chemical pretreatment of digestate, 4) digestate dilution (fraction basis such that 1 means full strength and 0.5 means half strength), 5) pH, 6) temperature (°C), 7) axenic versus non-axenic culture status, 8) light intensity (μmol m^{-2} s^{-1}), and 9) total ammonia nitrogen concentration (TAN in mg L^{-1}). Given that TAN, turbidity, and other chemicals are known to inhibit many types of algae, several of these factors directly account for these sources of inhibition. This set of 29 studies encompassed

188 observations of algae cultivation performance on digestates across a range of conditions and treatments. Each of these 188 observations was actually a mean of three or more biological replicates but here the means were treated as single observations. Because multiple measurements were taken from each study, a mixed model was constructed in which study was treated as a random factor. The same model was also constructed with study as a fixed factor and the significance of fixed effects was tested with an F-test, resulting in a p-value of 0.391. Therefore study was treated as a random factor in the model.

Most of the factors investigated are quantitative in nature (i.e., volume, dilution level, pH, temperature, light intensity, and TAN) and were treated as continuous variables in the multiple regression. The other factors were categorical in nature and were treated as binary variables. Binary variables included whether or not intentional solids separations were carried out on the digestate (i.e., filtration, centrifugation, flocculation) in a given treatment. Solids separation has the potential to lower turbidity and improve light penetration. Biological and chemical pretreatments of digestate are increasingly being investigated but the variation in method was so diverse across studies that binary variables could not be created for each without losing all but a few studies. Pretreatments used in the 19 studies included aeration stripping, precipitation, adsorption, aerobic bacteria, and ozonation. The final binary variable was axenic versus non-axenic culture status and, in nearly all studies, axenic culture status was achieved through autoclaving the digestate or via filtration through membranes of < 0.45 μm. In this case, axenic is in reference to a sterilized digestate medium. Of course, autoclaving likely changes digestate chemistry but separating the two effects is impossible and autoclaving was therefore not included in the pretreatment category. The response variable for the multiple regression was biomass productivity averaged over the first five days of batch growth (mg L^{-1} d^{-1}). A preliminary

regression analysis revealed that culture time was a significant factor (i.e., the first few days of culture growth were significantly faster than growth after, for example, 7 days where most cultures enter logarithmic and stationary phases). Hence, averaging productivity over the full time-span of longer-duration studies would have placed such cultures at an unfair disadvantage versus those in shorter-timeframe studies. The decision to use only the first five days of growth data allowed for the inclusion of studies of varying batch length. Both culture volume and TAN variables were log transformed to improve normality. A backward stepwise regression approach was used such that the least significant factor was dropped after each model run until the adjusted R^2 value was maximized. With an established optimal model, bootstrapping was used to determine the model's robustness. Two bootstrapping scenarios were used: one in which 75% of the data points were used in each round and one in which 50% of the data points were used in each round. In both cases, the model was bootstrapped 1000 times to determine the mean and standard deviation for coefficients.

2.2.3.3 Regression analysis on nutrient removal rate against biomass productivity

To find the relationship between growth and removal of total ammonium nitrogen (TAN), total phosphorus (TP) and chemical oxygen demand (COD), single regression models were constructed (Figures 3). These regressions were based observations across 15 studies for TAN and TP removal rate. The regressions for COD versus growth were based on the observations across 12 papers. Similar to the multiple regression, to represent the nutrient removal rate versus the biomass productivity during exponential phase, only the data from first five days was included in the single regression models. The single regression was conducted in R with *ggplot2* package. Results and Discussions.

2.3 Results and Discussions

2.3.1 Comparison of algal productivity in the liquid fraction of anaerobic digestate versus chemical media

Many studies included experimental designs focused on maximizing algal growth on anaerobic digestate versus control cultures grown in chemical medium. Comparing algal biomass productivity in digestate versus chemical medium can reveal the overall effectiveness of digestate as an algal growth medium while also demonstrating potential shortcomings that could be addressed through additional research and development. The comparison between algal biomass productivity in anaerobic digestate and in chemical media was conducted using Cohen's d as the effect size (Figure 1). There was no statistical significance ($p = 0.3876$) between the same strain of algae grew in digestate vs in chemical media which suggested that anaerobic digestates could potentially be good algal growth media. However, most studies reported that pretreatments such as solid-liquid separation, dilution, or other biological/chemical pretreatment were required before algae inoculation (Table 2). The negative average standardized mean difference (-0.49) supported that algal growth inhibition was still common in anaerobic digestate even with some pretreatments. Inhibition in digestate was expected that chemical media are often used as a positive control for algal cultivation given its well-balanced nutrient composition, and the liquid fraction of anaerobic digestates have complex compositions resulting in potential overabundance or insufficiency of certain nutrients. Moreover, digestate constituents can exhibit significant ecotoxicity to some types of algae (Tigini et al., 2016). Therefore, it is important to understand the characteristics of anaerobic digestates as well as the mechanisms of pretreatments for advancing this technology.

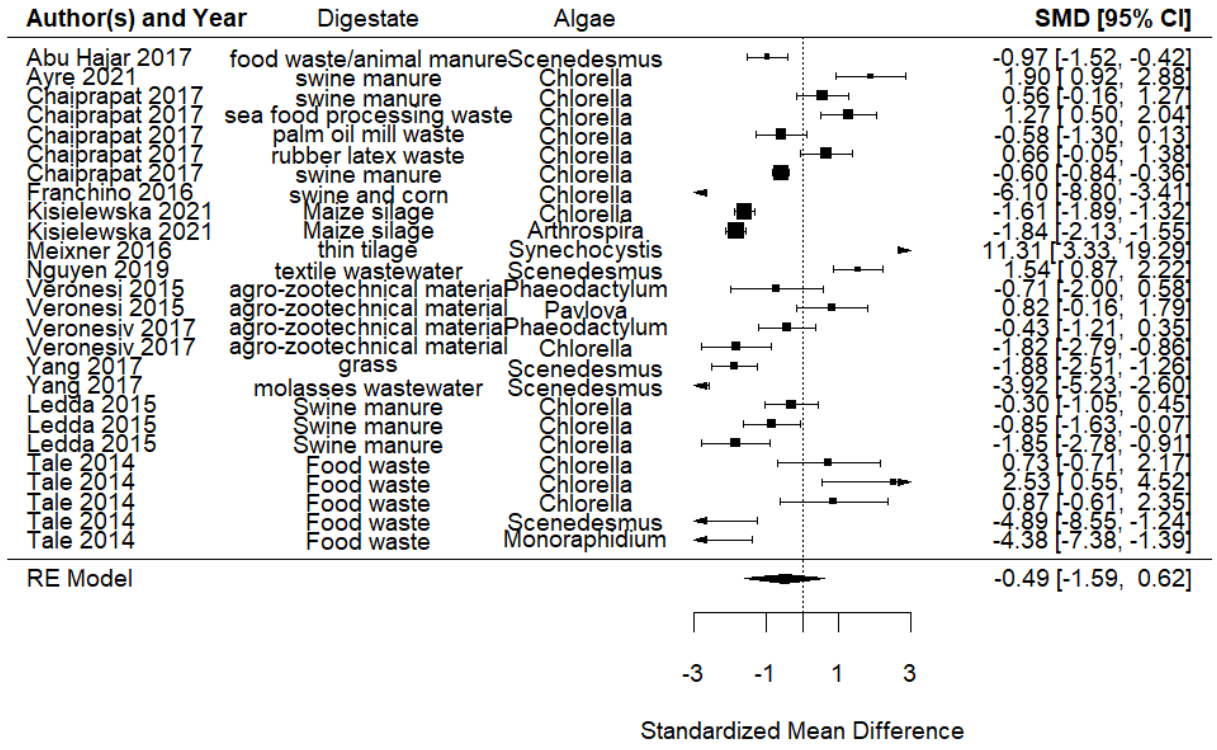


Figure 2.1 Forest plot of standard mean difference of biomass productivity between algae grown in chemical media versus in anaerobic digestate.
Digestate represents the feedstocks for anaerobic digestion. Algae is classified at genus level. Error bars represented the 95% confidence interval.

Significant differences ($p < 0.05$) were observed when comparing algal productivity based on the feedstocks of the digestates (Figure 2.A). Anaerobic digestion is generally used for carbon-recycling from a variety of organic wastes (feedstocks) to generate biogas. The diversity of feedstocks contributes to large variation in digestate composition (Table 1). For example, high protein feedstocks (food waste) can lead to high concentrations of ammonium (up to 5 g L^{-1}) in liquid effluent (Chuka-ogwude et al., 2020a; Wang et al., 2019f). Digestates of municipal wastewater sludge can contain derivatives of pharmaceutical compounds (Azizan et al., 2021; Gonzalez-Salgado et al., 2020). Animal waste feedstocks have been found to have elevated hydrogen sulfide in their digestates (Choudhury et al., 2019; Yan et al., 2018). Overloading of

the digestor with high amounts of organic compounds can result in excess volatile fatty acids (VFA) in the digestate (Wainaina et al., 2020). Furthermore, the status and the scale of digestors also impact the composition of their digestates. For instance, in a well-operating commercial scale thermophilic digestor, the rate of methanogenesis is compatible with the rate of acidogenesis that means organic compounds, such as lipids, proteins and carbohydrates, are converted to methane gas (Eryildiz et al., 2020). On the other hand, the breakdown of organic compounds is easily suppressed after acidogenesis in a low-rate anaerobic system. Such systems that are often found in small and poorly managed digesters result in higher concentrations of volatile fatty acids (up to 10,000 mg L^{-1}) observed in digestate (Franke-Whittle et al., 2014a). All of these variations in the composition of anaerobic digestate increase the complexity of algal-bacterial treatment and biomass growth.

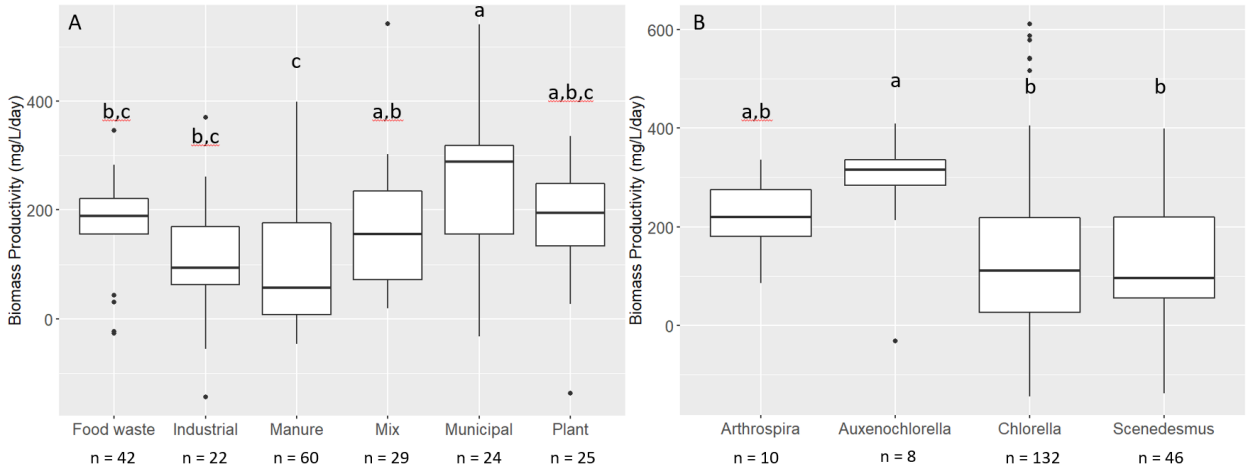


Figure 2.2 Box plots of biomass productivity from anaerobic digestate based on different feedstocks of anaerobic digestate. B. Box plots of biomass productivity from anaerobic digestate based on different algal genus. The n numbers represent the number of independent observations. Different letter above each box represents significant differences at the 0.05 level based on Tukey's HSD test.

Although the strains of algae seem to contribute a significant difference in biomass productivity (Figure 2.B), this result could be due to an unbalanced sample size. *Auxenochlorella* and *Arthrospira* accounted for only 8 or 10 measurements, respectively. The relatively low

sample size could increase the risk of type I error. Alternatively, there was no statistical significance when only the two mostly used algal genre (*Chlorella* and *Scenedesmus*) were compared. The dominant use of *Chlorella* (n = 132) and *Scenedesmus* (n = 46) is likely due to their potential for robust growth in a variety of high strength wastewater as well as their potential for value-added products including high protein or lipid content (Carneiro et al., 2021). These two genera have also been found to self-select in algal consortia when grown on digestate (Ayre et al., 2017; Wang et al., 2021a). However, both *Chlorella* and *Scenedesmus* had a wide variation in productivity (-100 to +500 mg L^{-1} d^{-1}) when they were cultivated in anaerobic digestate. This observation suggests that even with productive/tolerant algal strains, there are other cultivation factors (such as temperature, pH, light intensity, initial ammonium concentration) that impact the productivity of algae in digestate. Therefore, a multiple regression model was constructed to elucidate the contributing factors to algal growth in digestate.

Table 2.1 Anaerobic digestate information

Feedstocks	*Digestor type*	*Digestor scale*	*TN**	*NH_4-N**	*PO_4-P**	*COD**	*References*
Food and animal manure	-	Commercial	4,000	2,920	480	20,480	(Abu Hajar et al., 2017)
Municipal	Mesophilic	Lab	-	389	-	-	(Arias et al., 2017)
Swine manure	-	Farm	-	890	-	-	(Ayre et al., 2021)
Vegetable waste Cow manure Swine manure Marine algae	-	Lab	-	-	-	-	(Bjornsson et al., 2013)
Poultry litter	Mesophilic	Lab	-	688	99.5	1,080	(Bankston et al., 2020a)
Swine manure Sea food processing waste Palm oil mill waste Rubber latex waste	-	Full	315-689.2	257-486.7	15.9-61.2	1,689.0-3,158.2	(Chaiprapat et al., 2017)
Food waste	-	Commercial	-	3,800	290.3	40,604	(Chuka-ogwude et al., 2020a)
Swine manure corn	-	Farm	3,355	2,050	318.5	17,600	(Franchino et al., 2016b)

Maize silage Cattle slurry	Mesophilic	Lab	1,290-1,750	980-1,280	68-104	7,400-9,200	(Kisielewska et al., 2020)
Maize silage	Mesophilic	Pilot	3,710	2,540	350	8,190	(Kisielewska et al., 2021)
Maize silage	-	-	-	-	-	9,140	(Krzeminska et al., 2019)
Thin tillage	-	Pilot	3,530	-	574	7,910	(Meixner et al., 2016)
Textile wastewater	Mesophilic	Lab	-	-	-	13,000	(Nguyen et al., 2019)
Food waste	-	Commercial	-	5,226	216.3	-	(Nwoba et al., 2019)
Agro-zootechnical material	-	-	-	1,435	31.3	-	(Veronesi et al., 2015)
Agro-zootechnical material	-	Commercial	-	1,155	28	1,487	(Veronesiv et al., 2017)
Swine manure	-	Farm	138.8		185.4	3,402.5	(Xu et al., 2015)
Swine manure	Lagoon	Farm	1,316	1,300	28	-	(Dinnebier et al., 2021)
Dairy manure	-	Commercial	648	542	80.92	1,560	(Feng et al., 2020)
Swine manure	-	Farm	826	334	20.8	3,034	(Gu et al., 2021)
Swine manure	-	Farm	-	1,317	20.2	-	(Li et al., 2018)
Swine manure	-	Farm	411.5	255.5	14.5	1,316.6	(Lu et al., 2020)
Food waste Municipal sludge	Mesophilic	Commercial					(Wang et al., 2019f)
Hydrothermal liquefaction effluent	Mesophilic	Full	505	298	14.2	5,700	(Yang et al., 2018)
Swine manure	Thermophilic	-	1,600	610	961	84,900	(Wang et al., 2019a)
Municipal sludge	Mesophilic	Commercial					(Wang et al., 2021c)
Starch wastewater	Mesophilic	Commercial	265.1	240.9	28.3	926.3	(Yang et al., 2015)
Starch wastewater	Mesophilic	Commercial	240.3-382.7	217.6-334.7	19.3-32.9	702-1,026	(Tan et al., 2014)
Swine manure sewage	-	Farm	1,135	1,093	24.5	2,000-4,000	(Cheng et al., 2015b)
Food waste	-	Commercial	1,280	1,172	11.7	6,090	(Zhang et al., 2018)
Food waste	-	Commercial	2,200	2,120	44	3,108	(Cheng et al., 2016)
Swine manure	-	Commercial	-	2055	620	37,643	(Ledda et al., 2015)
manure	-	-	457.3	-	15.6	1358.5	(Ouyang et al., 2015)
Poultry litter	-	Lab	2473	1787	214	-	(Singh et al., 2011)
Food waste	-	Commercial	950	-	8.7	852	(Tal et al., 2014)

* Units in mg L^{-1}

2.3.2 Multiple regression analysis reveals factors that significantly influence algal productivity

Great variation in growth performance was observed among individual algae cultures, with growth in digestate occasionally exceeding that of chemical control cultures (Figure 1). Multiple linear regression was used to probe the underlying factors that govern growth performance across study, digestate, and algal types.

The backward stepwise multiple regression led to a final model in which the log of culture volume, the use of solids separation, use of chemical or biological pretreatment of digestate, temperature, pH, light intensity, and the log of TAN concentration were retained (Table 3). Dilution factor and axenic versus non-axenic culture conditions were dropped from the model due to very low significance levels. Doing so improved the adjusted R^2 value to a maximum of 0.296. In other words, this final model could explain about 30% of the algal-bacterial growth rates observed on a variety of anaerobic digestate types using different types of algae across 29 studies. Notably, this model does not include digestate type and algal strain which gives it some universal insight into factors that can largely be controlled through engineering.

Table 2.2 Multiple linear regression to predict 5-day average algae productivity (mg L-1 d-1)

Model Coefficient	Estimate	p-value
Intercept	289	0.083
ln(Volume, L)	-8.10	0.300
Solids separation (binary)	62.8	0.049
Biological or chemical pretreatment (binary)	120	5.22E-11
Temperature (C)	10.9	0.0026
pH	-65.0	8.96E-05
Light intensity (μmol m^{-2} s^{-1})	0.179	0.2580
ln(TAN, mg L^{-1})	-5.28	0.5530
R-squared	0.323	
Adjusted R-squared	0.296	
Model p-value	3.18E-15	
Observations	188	

Examination of the coefficients for each factor in the final model reveals both the direction and magnitude of its effect on algal productivity. Use of solids separation was a significant factor ($p = 0.049$). Although this analysis did not (due to insufficient sub-sample size) differentiate among the modes of solids separation (e.g., centrifugation, filtration), employing solids separation is expected to increase algal productivity by 63 mg L^{-1} d^{-1} compared to not doing so, all else held constant. Reducing turbidity through filtration is expected to improve light penetration in otherwise dark-colored digestates. This aligns with literature suggesting that turbidity in digestate inhibits algal growth (Marcilhac et al., 2014). Another way to improve light penetration into turbid digestates is with higher-intensity light radiation. Although light intensity was retained in the model and had a positive coefficient, it was not a significant factor ($p = 0.26$).

Perhaps the most commonly-cited inhibitor of algal growth in digestate is unionized ammonia (Al-Mallahi & Ishii, 2022a; Al-Mallahi & Ishii, 2022b). However, the results here only partially support this theory. Total ammonia nitrogen (which includes unionized ammonia and ionized ammonium) was retained in the model but was not statistically significant ($p = 0.55$). The ambivalence of the model toward this parameter may stem from the fact that TAN is both an algal nutrient and a potential inhibitor (in the free ammonia form). Total ammonia nitrogen only becomes problematic at high pH (>8) where free ammonia levels increase under elevated temperature. To this point, the model appears supportive showing that raising pH by just one point is expected to decrease algal productivity by 65 mg L^{-1} d^{-1}, all else held constant. The pH in the 29 studies ranged from 6.25 to 9.4 indicating that neutral to slightly acidic pH was preferable. Free ammonia is a negligible (<0.5%) component of TAN ≤ pH 7. With regard to temperature, increasing the culture temperature by 1 °C is expected to increase algal growth by 10.9 mg L^{-1} d^{-1} on average, all else held constant. Although higher temperatures can increase stress from free

ammonia, it can also stimulate faster biological reactions and therefore growth. From this analysis, it appears that temperature results in a net benefit toward growth.

One of the most-commonly used methods for overcoming turbidity and growth inhibition in general is to dilute digestate. All but a few of the studies in the regression analysis used dilution in some form, but dilution factor was the first factor to be dropped from the model. When it was initially included, it had a p-value of 0.87. That said, growing algae on full-strength digestate is often impossible without some type of pretreatment. The regression results show that employing chemical or biological pretreatment of digestate is very helpful toward increasing algal growth rates. Use of chemical or biological pretreatment increased algal productivity by 120 mg L^{-1} d^{-1} versus not doing so, all else held constant. Taken together, the above results suggest that pretreatment rather than dilution is the preferred route to promoting algal growth on digestate.

Because regression results can be biased by the inclusion of outlier data points that have high leverage over the fit, bootstrapping was used to understand the robustness of this model. Bootstrapping with inclusion of 75% of data points and with 50% of data points showed little difference in mean coefficient values compared to the full model (Table 4). This is expected. More importantly, the standard deviations around those means indicate a robust model fit, even with the 50% bootstrap. The one exception was the coefficient associated with TAN whose standard deviation bar crossed above and below zero. This aligns with the relatively low significance of this factor in the model.

Table 2.3 Multiple linear regression bootstrapping

	75% bootstrap		50% bootstrap	
Model Coefficient	Mean	SD	Mean	SD
Intercept	296	139	706	142
ln(Volume, L)	-8.54	3.42	65.5	25.4
Solids separation (binary)	63.6	19.6	114	33.4
Biological or chemical pretreatment (binary)	115	32.9	38.6	40
Temperature (C)	10.5	3.48	-90.6	17.6
pH	-65.8	13.1	0.382	0.178

The bootstrap percentage indicates the percentage of datapoints used in each round of bootstrapping. SD is the standard deviation based on 1000 rounds of bootstrapping (resampling with replacement).

2.3.3 Nutrient removal from liquid digestate by engineered algal digestate treatment system

In natural aquatic systems, microalgae carry out primary production by harnessing C, N, and P from their surrounding environment to make organic molecules. This ability will not only assimilate inorganic nutrient ions such, as NH_4^+, NO_3^-, PO_4^{3-}, into their cells but also enable production of essential materials, such as enzymes (Pal et al., 2011), photosynthate, vitamins (Markou & Nerantzis, 2013b), protein, nucleic acids, and chlorophyll. Algae also engage in photosynthesis, generating dissolved oxygen *in-situ*. The latter can support heterotrophic and nitrifying organisms that are also important for wastewater treatment processes (Bankston et al., 2020c; Holmes et al., 2019). In this manner, microalgae contribute to both nutrient assimilation and bacteria-mediated oxidative processes.

A wide range of algae have been used to successfully produce biomass while also removing nutrients from a variety of anaerobic digestates in engineered systems as reviewed by (Chuka-ogwude et al., 2020b; Xia & Murphy, 2016a). Figure 3A and 3B indicated a positive linear correlation ($p < 0.001$) between biomass productivity and nutrient removal rate. Assuming a linear relationship, increasing algal growth by 100 mg L^{-1} day^{-1} is expected to increase removal

of ammonium nitrogen by 6.4 mg L^{-1} day^{-1} and increase removal of phosphorus by 1.4 mg L^{-1} day^{-1} from the digestate. The nitrogen value suggests marginal biomass production contains 6.4% nitrogen on average which is remarkably close to nitrogen content predicted by commonly used empirical formulas for microalgae that predict anywhere from 6.3-8.6% N content (Picardo et al., 2013; Randrianarison & Ashraf, 2017). Phosphorus content of marginal biomass is predicted to be 1.4% which is slightly higher than the 0.89-1.2% predicted by empirical formulas for green algae like *Chlorella* (Picardo et al., 2013; Randrianarison & Ashraf, 2017). In other words, efforts to improve algal growth should also improve algal assimilation of NH_4-N and TP from digestate.

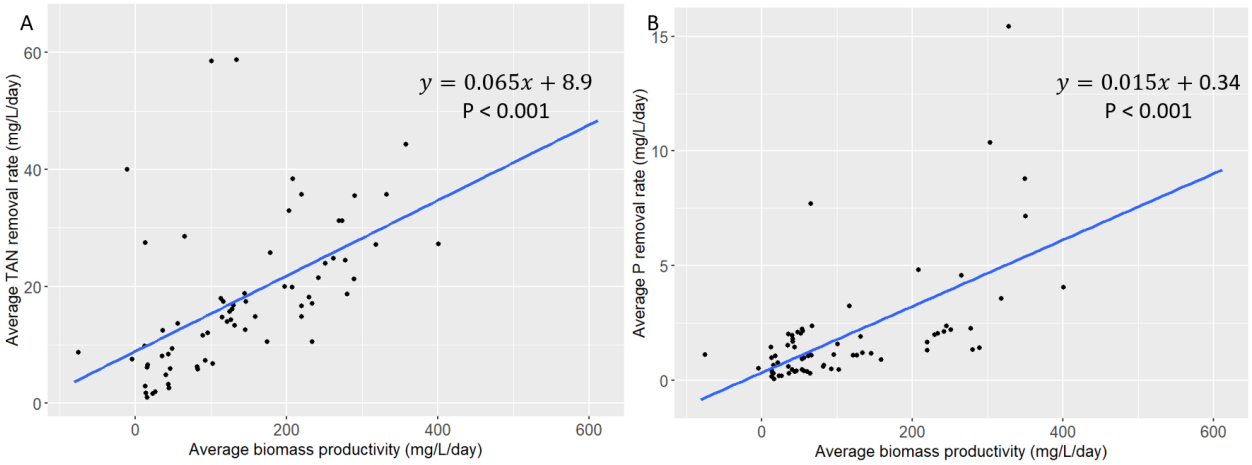


Figure 2.3 Ammonium nitrogen removal rate against biomass productivity. B. Phosphorus removal rate against biomass productivity

The N:P removal ratio (~10.2:1) was lower compared to the classic Redfield N:P ratio (16:1) in algal biomass (Redfield, 1958). This is because the removal rate of ammonium nitrogen and total phosphorus are affected by other factors besides nitrogen and phosphorus assimilation to biomass. For example, there was a big difference between total nitrogen and ammonium nitrogen in manure based anaerobic digestates (Table 1). In one case, ammonium nitrogen accounted for only 40% of the total nitrogen in the digestate (Gu et al., 2021). Farms usually

adopt low cost sealed lagoon systems as anaerobic digestors. The drawback of such systems is that the digestion was much less complete compared to a commercial municipal sludge digestion system. Although ammonium is the preferred nitrogen source for a wide range of algal growth, they are still able to consume other forms of nitrogen (organic nitrogen, nitrite, and nitrate). The consumption of other forms of nitrogen in the digestate could potentially lower the NH_4-N: P removal ratio. Likewise, further mineralization of nitrogen-containing compounds can generate additional NH_4-N at the same time it is consumed by algae. Finally, there are alternative P removal mechanisms, like precipitation, that coincide with algal assimilation.

Chemical oxygen demand (COD) removal is another important parameter in the wastewater treatment processes. The effluent of anaerobic digestate usually consists of a high concentration of COD (up to more than 10 g/L, table 1). Many studies cite the ability of algae to utilize organic substances in wastewaters via heterotrophic growth. This is plausible in anaerobic digestate where volatile fatty acids (VFAs) can be prevalent (Zhu et al., 2020). Many green algae, like *Chlorella*, are known to consume VFAs (Patel et al., 2021). However, unlike the N and P removal, the regression on biomass productivity against COD removal rate was not significant ($p = 0.21$). This result indicated that COD removal pathways were more complicated when grew mixotrophic algae in anaerobic digestate. Moreover, the COD measurement does not present carbon quality change in the processes. Therefore, more research is needed to fully understand the COD conversion in engineered algal-wastewater treatments.

2.3.4 Algal growth inhibitors in anaerobic digestate

Raw undiluted digestate is generally not recommended for algal cultivation/treatment due to its severe algal growth inhibition (Chuka-ogwude et al., 2020b; Xia & Murphy, 2016a). Most included studies adopted some form of pretreatment processes (e.g., solid-liquid separation,

dilution, chemical/biological treatments). The regression models support the idea that engineering approaches that improve light penetration and remove chemical inhibitors in digestate can be effective at promoting algal growth and nutrient removal. However, current pretreatments of anaerobic digestate for algal cultivation, such as dilution, may not be optimal. Optimizing such strategies requires a deeper understanding of the underlying causes of algal growth inhibition on digestate. Some research has already been conducted in this area, but knowledge gaps remain.

2.3.4.1 Turbidity

High turbidity limits light access and is one of the most obvious sources of inhibition when trying to use anaerobic digestate to grow algae. Undigested feedstocks and the cells of anaerobic organisms are contributing to the turbidity of anaerobic digestate. The presence of these solid particles could reduce the light penetration for algal photosynthesis. Although anaerobic digestate contains organic compounds that can potentially support algal heterotrophic growth, not all algae prefer to grow heterotrophically and most of the organic compounds in anaerobic digestate are not assimilable by algae (Scarponi et al., 2021). Therefore, photosynthesis is critically important for the growth of algae as well as the removal of nutrients. The multiple regression results support the importance of overcoming turbidity effects through the use of solids separation techniques and ensuring good light penetration into cultures.

2.3.4.2 Chemical inhibition

High ammonium/ammonia concentration was one of the most studied algal inhibitors. Organic nitrogen in feedstocks is converted to ammonium/ammonia due to lack of oxygen during anaerobic digestions. Normally, a full strength anaerobic digestate contains ammonium concentration ranging from 200 mg/L to 5000 mg/L (Table 1). Although ammonium is

considered as a preferred nitrogen source for the growth of many algal strains (Uggetti et al., 2014), the high TAN concentration in the digestate is considered toxic depending on the strain of algae, the cultivation pH, and the cultivation temperature (Akerstrom et al., 2014; Xia & Murphy, 2016b). The multiple regression model in section 3.2 also supported the observation of algal biomass productivity suppression in high TAN plus basic pH condition. Since ammonium/ammonia was the most addressed problem when cultivating algae in anaerobic digestate, a variety of studies adopted dilution as a pretreatment and the dilution factors were mainly based on achieving optimum TAN concentrations (Chaiprapat et al., 2017; Prandini et al., 2016). However, the fact that using only dilution as a pretreatment method was not sufficient to alleviate all algal growth inhibition from the digestate (Wang et al., 2019f) suggested that ammonium was not the only algal inhibitor (Al-Mallahi & Ishii, 2022b).

Besides the popular focus on TAN inhibition, the algal inhibition from residue organic compounds were highly underestimated. High concentration of volatile/medium chain fatty acids was known to inhibit algal growth (Lacroux et al., 2021; Wu et al., 2006b). There was evidence showing anaerobic digestate could potentially have inhibitory concentration of VFAs. For example, Frank-Whittle *et al.* (2014) observed approximately 9000 mg/L of propionic acid and 2000 mg/L of butyric acid in their anaerobic digestate samples (Franke-Whittle et al., 2014a).

Volatile Fatty acids only show algal toxicity at high concentrations, but other organic compounds, such as residue pharmaceuticals, are inhibitory at low concentrations. Lehmann and Bloem (2021) detected residue antibiotics (tetracycline, oxytetracycline, chlortetracycline, and sulfametazine etc.) in 83% of the anaerobic digestate that they tested. Gaballah et al. (2021), also indicated that the removal of veterinary antibiotics (VAs) by anaerobic digestion was around 73%, which means 27% VAs remained in the digestate. They also indicated that only

thermophilic anaerobic digestion can perform the removal of VAs effectively. Thermophilic digestors were generally not adopted by farms which means more antibiotics could potentially be detected in their digestate. Most antibiotics are showing toxic effects on green algae at a relatively low concentration (< 5mg/L) (Xu et al., 2019). Therefore, more research needs to be conducted on the removal of residue antibiotics for future efficient algal digestate treatment systems.

Addition to the algal inhibition from single chemicals, recent increasing concern about nano-scale polymer pollution revealed the inhibitory impact of these microplastic to aquatic organisms (including algae). Generally, microplastics have strong resistance to biological enzymes. Due to lack of breaking down pathways, microplastics could potentially impair anaerobic digestion and accumulate in the digestate (Mohammad Mirsoleimani Azizi et al., 2021). Moreover, microplastics were found to increase the reactive oxidative stress (ROS) in algae which could inhibit algal productivity by more than 55% (Wu et al., 2019). Treatments specially focused on microplastic removal are urgently required in many wastewater treatment plants.

2.3.4.3 Non-dilution pretreatment approaches for overcoming inhibition

Although dilution was used as a pretreatment in 90% of the surveyed studies for overcoming growth inhibition when growing algae in anaerobic digestate, the consumption of freshwater and the expanded operation volume makes it less favorable to be used as a primary pretreatment in industrial scale algal biomass production from anaerobic digestate (Nwoba et al., 2019). The multiple regression model in section 3.2 also indicated that dilution factor was not a significant variable towards algal biomass productivity. Therefore, non-dilution pretreatment approaches are preferred for future engineered algal-digestate treatment systems.

The existence of solid-liquid separation showed a significant impact on algal growth based on the multiple regression model. Settling, filtration and centrifugation are the most commonly used methods. Even though space/energy is required to achieve an effective suspended solids removal, the increase of light penetration is essential in an engineered algal digestate system (Xia & Murphy, 2016a). Adsorption approaches (e.g., adding activated carbon) could also help removing some suspended solids, but the major contribution of adsorptive materials is reducing dissolved compounds such as ammonium, heavy metals, and organic compounds. Adsorption approaches are suitable as a pretreatment process for digestate which were from industrial waste as feedstocks. Chemical approaches, such as struvite precipitation (Jiang et al., 2018a; Wang et al., 2019a) and ammonia stripping (Folino et al., 2020), are designed to reduce TAN concentration in the digestate by forming and removing precipitations. Chemical approaches are generally better at treating high TAN containing digestates (food waste digestates). Biological approaches, such as aerobic bacterial pretreatment (Jiang et al., 2018c; Wang et al., 2021c), are also effective in partially removing ammonium and organic compounds. The adoption of biological pretreatments is generally better for long-term processes with relatively lower cost. In sum, there are different pretreatment approaches, but none of them can achieve an optimum result solely. Future studies need to focus on the combination of different pretreatment processes for the best algal treatment of a particular digestate.

Table 2.4 Algal reactor information

Pretreatment	*PBR scale (L)*	*Temp ℃*	*pH*	*Light intensity (µmol m^{-2} s^{-1})*	*Final Biomass productivity (mg L^{-1} day^{-1})*	*Nutrient removal (mg L^{-1} day^{-1})*	*References*
Dilution (secondary effluent)	30	22-29	7.5-8.9	200–400	84	96% NH4N 100% TP	(Arias et al., 2017)
Centrifugation, dilution (DI)	1	24.5	-	500	-	-	(Ayre et al., 2021)
Centrifugation, filtration, dilution (DI)	0.2	25	7.2	140	400	-	(Bankston et al., 2020a)
Dilution (DI), filtration	0.25	22	-	85-90	45	100% NH4N 99.8% TP	(Bjornsson et al., 2013)
Filtration Autoclave Dilution	0.5	25	-	39	80	93.7% TN	(Chaiprapat et al., 2017)
Dilution Algal acclimation	0.025	21	-	150	-	-	(Chuka-ogwude et al., 2020a)
Dilution	0.5	25	-	300	229	>90%TN 100%TP	(Franchino et al., 2016b)
Dilution	2.5	22	-	9.45	225.4	>96%TN 100%TP	(Kisielewska et al., 2020)
Dilution Distillation Centrifugation	2.4	23	7	9.45	230	81.1%TN 94.2%TP	(Kisielewska et al., 2021)
Dilution	0.2	-	-	80	-	79.45%TN 78.4%TP	(Krzeminska et al., 2019)
Dilution Precipitation Autoclave	200	25	-	277	150	100%TN 100%TP	(Meixner et al., 2016)
Dilution	0.9	25	7.3	196	180	-	(Nguyen et al., 2019)
Dilution	0.15	25	8	220	200	22.1%NH4N	(Nwoba et al., 2019)
Dilution Filtration	3	22	8.68	120	24	-	(Veronesi et al., 2015)
Dilution Filtration	3	22	8.25	90	49.2	92% TN	(Veronesiv et al., 2017)
Dilution Autoclave	48	25	6.43	200	263.45	74.63% TN 88.79% TP	(Xu et al., 2015)
Dilution	1	25	8	100	198	70% TN 90% TP	(Dinnebier et al., 2021)
Dilution	-	-	5.5-7	200	340	100% NH4N 100% TP	(Feng et al., 2020)
Autoclave	0.3	26	9.5	33.75	55.3	43.27% TP	(Gu et al., 2021)

Dilution Autoclave	0.8	25	7.4	80	31	80% NH4N 85% TP	(Li et al., 2018)
Dilution Autoclave	0.2	27	7	40.5	14.02	98% NH4N 97.14% TP	(Lu et al., 2020)
Flocculation Struvite precipitation Dilution	0.1	25	-	150	332	99.9% NH4N 99.7% TP	(Wang et al., 2019a)
Dilution Ozone	-	25	7.61	95	180	100% NH4N 100 TP	(Yang et al., 2018)
Activated sludge Filtration	0.2	25	7.5	170	500		(Wang et al., 2019f)
Activated sludge Filtration	0.2	25	7.5	170			(Wang et al., 2021c)
Settling Filtration Sterilization Dilution	2	25	6.5	127	333.3	91.64% TN 90.74% TP	(Yang et al., 2015)
Precipitation Filtration	890	10-37	7.4	-	370	83.06% TN 96.97% TP	(Tan et al., 2014)
Centrifugation P supplement	0.3	27	6.25	80.96	601.2	73% NH4N 95% TP	(Cheng et al., 2015b)
Dilution	0.8	25	8.31	81	29.8	-	(Zhang et al., 2018)
Centrifugation	0.8	25	8.3	60	-	-	Yu 2017
-	0.3	27	6.25	80.96	456	99% NH4N 99% TP	(Cheng et al., 2016)
Centrifugation Filtration	0.3	25	8.0-8.6	150	100	98% NH_4N Up to 99% TP	(Ledda et al., 2015)
Filtration UV sterilize	4	25	7.14	230	76.12	84.12% TN 86.76% TP	(Ouyang et al., 2015)
Centrifugation	0.06	25	-	77.5	66.2	49% TN 100% TP	(Singh et al., 2011)
Cloth filter Autoclave	0.1	25	7.58	13.5	32.2	-	(Tal et al., 2014)

2.4 Application of algal biomass

The produced microalgal biomass from cultivating in anaerobic digestate have applications in various sectors, including biofuels, animal food and feed, soil additive, and biomaterial (Ansari et al., 2021). Microalgal biomass are consisted of proteins with essential amino acids, lipids with polyunsaturated fatty acids (PUFA), carbohydrates, pigments, and other bioactive compounds.

Much of these algal-produced products are already commercially available in the nutraceutical market, however, such a market is an unlikely destination for digestate-grown algae. Hence, industries that are able to use lower-quality algae from wastewater processes should be sought out.

The aquaculture industry is a growing food sector and needs a huge amounts of fish feed. Moreover, aquaculture feed represents 40–75% of aquaculture production cost (Ansari et al., 2021). It has been shown that digestate-grown microalgae can be a good candidate to be used as a crucial, feasible, and alternative feed ingredient in aquaculture feed (Hyman et al., 2021b). Also, algal biomass could play a significant role as supplements in animal feed as they constitute valuable nutrient source, thanks to their nutritional composition and abundance in polysaccharides, polyphenols, and fatty and amino acids (Coudert et al., 2020).

Microalgal biomass have been considered a promising source for biofuel production since they are a clean, natural friendly, and reliable sources. The several ways for conversion of microalgal biomass to bioenergy sources categorized into chemical reaction (biodiesel), combustion (heat), biochemical conversion (bioethanol, methane), and hydrothermal liquefaction (bio-oil and biochar) (Karthik et al., 2020). However, there are still some obstacles in utilization of microalgal biomass as biofuels feedstock among which economic issues are the most important. Finally, wastewater-grown algae have been used in the synthesis of biopolymers (Kartik et al., 2021). Limitations for large-scale microalgae production include: 1-nutrient supply in this process could be expensive, 2- contamination with bacteria may shut down the process, 3-microalgal biomass harvesting is still a challenge and need a huge amount of investment, and 4-a large amount of water is needed for microalgae biomass production (Fallahi et al., 2021).

2.5 Future research needs

It is clear from analysis of the literature that algal growth is naturally inhibited on anaerobic digestate but that this can largely be overcome through digestate pretreatments. However, understanding of the sources of this inhibition is limited and the methods used to overcome it are varied. Approaches to date have relied primarily on dilution and the assumption that ammonia is the predominant inhibitor. While important, the results of this analysis suggest a more nuanced situation where dilution may not be the best approach and TAN itself is not necessarily problematic. In-depth understanding of chemical inhibitors in digestate and the rational design of pretreatments targeting such inhibitors are needed. This will improve both algal growth and nutrient assimilation in anaerobic digestate. Finally, continued progress in developing value-added uses for the resulting algal biomass is needed.

Chapter 3: Aerobic bacterial pretreatment to overcome algal growth inhibition on high-strength anaerobic digestates

Abstract

Coupling anaerobic digestion and algae cultivation has attracted attention as a sustainable means of treating high-strength wastewaters. In such a scenario, nutrients from the liquid anaerobic digestate are used by algae to produce biomass. However, use of full-strength digestate results in poor algal growth and nutrient removal. Most researchers have overcome this challenge by diluting digestate 10-30 fold prior to algae growth but such dilution rates demand large amounts of fresh water, posing challenges for scale-up. The objectives of this study were to 1) assess whether ammonium, turbidity, and heavy metals in digestate were the primary sources of inhibition for a highly-nutrient tolerant strain of *Chlorella sorokiniana,* and, 2) develop a biological pretreatment strategy to overcome algal growth inhibition on full strength digestate. Ammonia toxicity, turbidity, and heavy metals have been commonly hypothesized as the source of algal growth inhibition, but our results showed that these factors were not critical inhibitors of *C. sorokiniana*. Dose response studies showed that *C. sorokiniana* could grow robustly on 3,500 mg/L ammonium. Regardless, full strength digestates of wastewater sludge and food waste were very inhibitory to *C. sorokiniana*. We utilized a pretreatment approach using activated sludge which led to robust algal growth on full-strength digestate. High growth rates of 250-500 mg/L/d were achievable on pretreated digestates despite very high ammonium levels of ~2,000 mg/L. Pretreating digestate also led to significantly faster algal nutrient uptake compared to untreated digestate ($p < 0.001$).

Keywords: Anaerobic digestate, inhibition, algae, activated sludge, pretreatment

3.1. Introduction

With increasingly stringent nutrient discharge standards, municipal wastewater treatment plants (WWTPs) and industrial wastewater generators are seeking innovative nutrient removal technologies. Utilization of algae in wastewater treatment has gained attention for its ability to remove and recover nutrients in their fixed form, mostly as amino acids (Cai et al., 2013). Use of algae also reduces greenhouse gas emissions through CO_2 sequestration and the resulting algal biomass has a variety of beneficial uses (Spolaore et al., 2006) .

There are increasing numbers of municipal and industrial treatment systems that employ anaerobic digestion to convert organic matter and bacteria biomass (e.g. excess sludge from aeration tanks) into biogas and digestate. The liquid digestate (LD) fraction is rich in nutrients which can lead to environmental nutrient pollution if not adequately treated. Municipal WWTPs typically reintroduce the LD back into the headworks of the treatment plant (personal communication, William Kent, Manager of Environmental Services, Columbus Water Works), creating a parasitic load on the system. The elevated nutrient concentration not only puts a burden on downstream tertiary treatment but also potentially impacts the efficiency of downstream secondary treatment and anaerobic digestion (Chen et al., 2008).

A variety of algal species are known to quickly assimilate inorganic nutrients (Franchino et al., 2016a), and algae have been studied for nutrient recovery from a variety of anaerobic digestates (Ruiz-Martinez et al., 2012; Wang et al., 2010). In fact, digestates are rich in the nitrogen and phosphorus nutrients that typically limit algal growth in nature (Stanley et al., 1990). However, most researchers have found that full strength digestates severely inhibit algal growth (Cho et al., 2013; Franchino et al., 2016a), a finding that was common across a wide range of digestate types. In these cases, dilution rates of 10–30 fold were typically used to alleviate inhibition of LD in lab-scale experiments (Cho et al., 2013; Franchino et al., 2016a).

However, diluting LD with freshwater is a non-starter in water-scarce regions and may even be suboptimal given the simultaneous dilution of nutrients needed for algal growth. We hypothesize that removal or transformation of inhibitory compounds in LD will lead to rapid algal growth rates without the need for dilution water. Knowledge regarding specific inhibitors in LD is limited. Most studies cite ammonia (Cho et al., 2013), turbidity (Wang et al., 2010), and heavy metals (Wong et al., 1994) as the primary sources of algal inhibition on LD. A few others have mentioned unknown organic constituents and COD as potential inhibitors of algae (Franchino et al., 2016a; Tigini et al., 2016).

The objectives of this research were to 1) assess whether ammonium, turbidity, and heavy metals in digestate were the primary sources of inhibition for a highly-nutrient tolerant strain of *Chlorella sorokiniana* and 2) test the effectiveness of aerobic activated sludge pretreatment of digestate as a means of reducing inhibitor concentrations in full-strength anaerobic digestates.

3.2. Material and methods

3.2.1 Anaerobic digestate and activated sludge collection

Municipal anaerobic digestate (MAD) was collected from a mesophilic anaerobic digestor at the South Columbus Water Resources Facility (Columbus GA, USA) which is used to treat excess wastewater sludge and waste cooking oil. Activated sludge (AS) was collected from an aerated activated sludge tank used for secondary wastewater treatment at the same facility. Both MAD and AS were immediately transported back to the lab and stored in a cold room (4 °C) until use. Food waste anaerobic digestate (FWAD) was collected from a commercial-scale high-solids anaerobic digester at UC Davis (Davis, CA, USA), and was shipped to the lab overnight on ice and was stored in a freezer (-80°C) until use. LD was prepared by a combination

of centrifugation and filtration to remove solid components from the digestate as follows. The upper liquid portion of the anaerobic digestate (both MAD and FWAD) was centrifuged at 4696 x g for 30 minutes. The supernatant was then passed through a series of filters of the following sizes using a vacuum filtration apparatus: Whatman No.4 filter paper (20-25µm), No.1 (11µm), No.2 (8µm), No.5 (2.5µm), Advantec GA-55 glass fiber (1.6µm), GC-50 glass fiber (1.2µm), Advantec mixed cellulose ester membrane (0.8µm), and Whatman GF-F glass fiber (0.7µm). The resulting liquid was termed "clarified" digestate. Sterile filtered digestate was later prepared by passing clarified digestate through Advantec mixed cellulose ester membranes (0.45 µm and 0.2 µm), and a 0.2 µm sterile filtration apparatus (VWR PES filter). Filtration was used to control turbidity and to isolate treatment effects of algae without the assistance of wastewater bacteria.

3.2.2 Algae culture experimental plan

The first experiments tested *Chlorella sorokiniana* (UTEX 2805) on different dilutions of MAD and FWAD to determine the extent of inhibition. This strain of *C. sorokiniana* was originally isolated from a wastewater treatment plant (de-Bashan et al., 2008a) and has successfully been used in treatment of winery wastewater (Higgins et al., 2017). Given the frequently-cited hypothesis that ammonia is the most important inhibitor in digestate, we next cultured *C. sorokiniana* on different concentrations of ammonium chloride in chemical N8-NH_4 medium (Higgins & VanderGheynst, 2014). The pH of this medium was adjusted to 7.5. Next, *C. sorokiniana* was cultivated in AS-pretreated LD (treatment 1) and non-treated LD (treatment 2) in bubble column bioreactors (Wang et al., 2019d) to study algal growth and inhibition. This experiment was carried out for both types of anaerobic digestate (MAD and FWAD). Specific culture methods for AS-pretreatment and algae cultivation are described in subsequent sections. Control cultures were cultivated in defined chemical N8 medium (Tanadul et al., 2014b).

Because AS-pretreatment resulted in decreases in the ammonium concentration, a third treatment group was tested with addition of ammonium chloride to restore the ammonium level to that of the untreated LD. The purpose of this third treatment was to confirm if ammonium removal during AS pretreatment had a meaningful impact on algal growth inhibition. All experimental treatments and controls were tested in biological triplicate. All LD was sterile filtered and supplemented with micronutrients and magnesium (same final concentration as in the control chemical medium) to ensure trace metals were not limiting growth.

3.2.3 Activated sludge pretreatment of anaerobic digestate

Clarified digestate (passed through a 0.7 µm filter) was treated with activated sludge by adding 2% (v/v) activated sludge slurry (0.67% solids content) to digestate. pH of the digestate was adjusted to 7.5 with 3M HCl and aerated with 1 vvm air for 4-5 days. The AS-treated anaerobic digestate was then sterile filtered through a VWR 0.2 µm sterile PES vacuum filtration unit for use in the algae cultivation test.

3.2.4 Algae cultivation method

The algae cultivation method has been described previously (Higgins & VanderGheynst, 2014; Higgins et al., 2018a; Higgins et al., 2018b; Wang et al., 2019d). Briefly, *C. sorokiniana* was initially cultured on a modified Bold 3N agar plate for 5-7 days to isolate single colonies. Colonies were selected and used to grow 1L stock cultures in N8 medium under a fluorescent light bank and aeration (0.5 vvm, 2% CO_2) until the optical density (550 nm) reached 0.2-0.3. Stock cultures were then settled for 24-48 hours at room temperature. After removal of 90% of the supernatant, the concentrated algae slurry was evenly transferred into each bioreactor to inoculate the experiment. Algae were grown in bubble column bioreactors over 5 days with light (170 µmol photons/m^2/s on a 14h:10h light-dark cycle) at 25 °C. Bioreactors were aerated at 0.5

vvm and air was supplemented with 2% CO_2. pH was controlled at 7.5 for all cultures using either 3M HCl or 3M NaOH. Daily samples (2 ml) were taken from each bioreactor for optical density (OD) measurement at 550nm and 680nm. The samples were then centrifuged, and the supernatant filtered through 0.2 µm syringe filters and stored at -80℃ until further analysis.

3.2.5 Heavy metal analysis

Inductively coupled plasma – optical emission spectrometry (ICP-OES) (Spectro Ciros ICP, SPECTRO Analytical Instruments, Kleve, Germany) was used to analyze the metal concentrations in the anaerobic digestate. The digestate was first filtered through Whatman No. 42 filters, and then 5 ml of filtered samples were digested with 1:1 (v/v) nitric acid in a microwave digestion system as described previously (Chaump et al., 2018b). Digested samples were analyzed via ICP-OES for metals (Cu, Mn, Al, Ca, Zn and Fe).

3.2.6 Chemical oxygen demand (COD) and total nitrogen tests

A HACH DR900 was used to measure the soluble COD concentration in the sterile-filtered digestates (5x diluted in DI water). A HACH total nitrogen assay was also used to measure the nitrogen content of harvested algae cells following a previously published procedure (Higgins et al., 2015a). Nitrogen content was multiplied by growth rate to determine the rate of nitrogen assimilation into algal cells.

3.2.7 Optical Density and spectrum absorbance

A SpectraMax M2 Plate reader was used for OD and spectrum absorbance measurements. OD was measured in triplicates for each sample at 550 nm and 680 nm. Spectrum absorbance was conducted on membrane-filtered LD (0.2 µm) from 200 nm to 1000 nm in 10 nm increments in order to assess interference of LD absorbance with chlorophyll absorbance.

Pigment and lipid extracts from *C. sorokiniana* were analyzed as a point of reference when assessing absorption interference by digestate. *C. sorokiniana* extracts were obtained using a previously-published modified Folch method (Folch et al., 1956; Wang et al., 2019d).

3.2.8 Ion chromatography for nutrients analysis

A Prominence Liquid Chromatography (LC) system coupled with a conductivity detector (Shimadzu, Japan) was used to analyze ion concentrations (sodium, potassium, ammonium, calcium, magnesium, chloride, nitrite, nitrate, phosphate, and sulfate) in digestate samples based on a previously published method (Chaump et al., 2018b). Briefly, A Dionex IonPac CS12 column (4x250mm, Thermo science) and a Dionex IonPac AS22 column (4x250mm) with suppression (Dionex CERS 500 4mm and Dionex AERS 500 4mm, respectively) were used for ion separation. Acidic eluent (20mM methanesulfonic acid) was used on the CS12 column, and basic eluent (4.5 mM sodium carbonate and 1.4mM sodium bicarbonate solution) was used on the AS22 column.

3.2.9 Data analysis and statistics

Experiments were all conducted in biological triplicate except where noted. Statistical analyses (ANOVA and Turkey's HSD test) were carried out in R with the 'car' package and 'agricolae' package. Standard deviations were calculated in Microsoft Excel.

3.3. Results

3.3.1 Algal inhibition in anaerobic digestate

The growth of *C. sorokiniana* was severely inhibited in both municipal sludge and food waste anaerobic digestates (Fig. 3.1). Diluting digestates with deionized water helped alleviate some inhibition, with a 16x dilution of MAD yielding ~1.5 g/L dry algal mass. However, the

defined algal growth medium (N8) yielded the highest overall growth at ~2.1 g/L dry mass after the 5-day cultivation period. In full strength MAD, *C. sorokiniana* did not have a detectable biomass increase until the last day of cultivation. Algal growth in diluted MAD had a relatively faster growth rate than the chemical medium in the first 48-72 hours, but they reached an early growth "ceiling," suggesting potential nutrient limitation at high dilutions despite supplementation with micronutrients. The most diluted MAD (16x) had the highest algal growth rate in this experiment. Similar trends were observed when cultivating *C. sorokiniana* in FWAD except algae cells experienced complete inhibition in either full strength or 2x diluted FWAD. The growth "ceiling" was also higher in dilutions of FWAD compared to MAD.

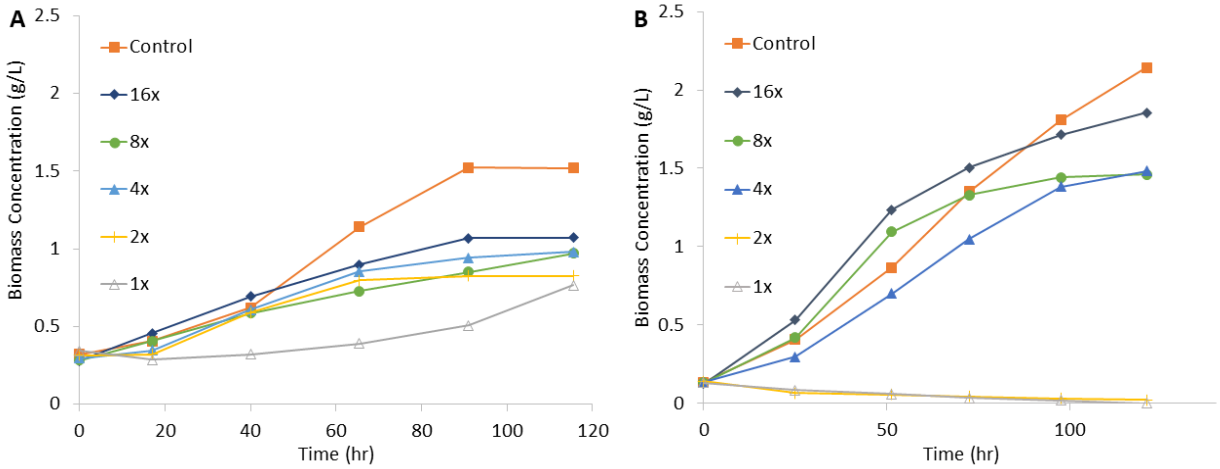


Figure 3.1 Growth of C. sorokinianaon varing concentrations of digestate
A) Response to municipal anaerobic digestate and B) food waste anaerobic digestate. Control cultures were grown on chemical N8 medium. Each data point represents the average of biological replicates (n = 2). Biomass concentration is reported on a dry-weight basis.

3.3.2 Ammonium tolerance test on *C. sorokiniana*.

Anoxic conditions and nitrogen-rich organic feed material provide a suitable environment for ammonium production in anaerobic digestors. Most anaerobic digestate contains a large amount of ammonium ranging from 100-3,000 mg/L in the liquid fraction (Xia & Murphy, 2016a). The syringe-filtered MAD contained approximately 2,000 mg/L of ammonium and the

syringe filtered FWAD had approximately 3,000mg/L. of ammonium. Although ammonium is an important nitrogen source for algal growth, excess ammonium combined with high pH can lead to high free ammonia concentrations. Free ammonia is typically harmful for algal growth (Gutierrez et al., 2016) but can be partly controlled through pH. We therefore tested the tolerance of *C. sorokiniana* to high ammonium concentrations while controlling pH at 7.5. An ammonium dose-response test on *C. sorokiniana* revealed it to be highly tolerant to extreme ammonium concentrations (up to 3,500 mg/L) (Fig 3.2). Little difference in algal growth was observed on media containing ammonium concentrations ranging from 1,000 mg/L to 3,500 mg/L. Below 1,000 mg/L ammonium, algal growth decreased (Fig 3.2A).

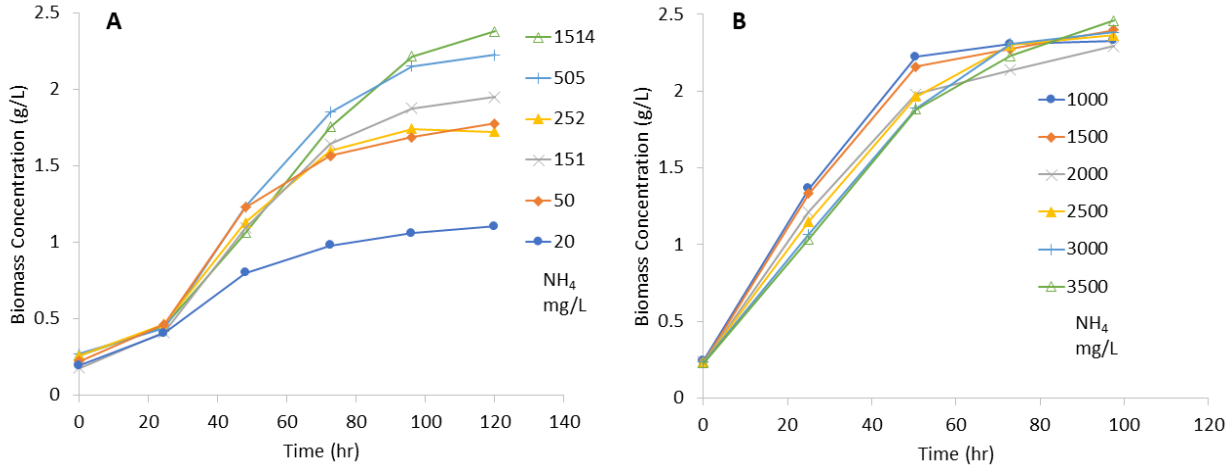


Figure 3.2 Growth of *C.sorokiniana* in different ammonium concentrations.
A) lower range (20 mg/L to 1500 mg/L) ammonium dose response and B) higher range (1000 mg/L to 3500 mg/L) ammonium dose response. Each data point represents the average of biological replicates (n = 2). Biomass concentration is reported on a dry-weight basis.

3.3.3 Heavy metals and turbidity

The inhibitory effects of certain metals, such as copper, aluminum, and manganese, have been known for several decades (Wong et al., 1994). These metals could have resulted in algal growth inhibition on digestates. However, the ICP measurement of metals in MAD and FWAD (Table 3.1) showed that concentrations of copper, aluminum, and manganese in the digestates

were lower than those in the defined chemical algal medium (N8). In addition, the high turbidity of raw MAD and raw FWAD should inhibit algal photosynthesis by reducing light penetration. However, filtration through 0.2 μm membranes greatly alleviated the turbidity for both MAD and FWAD (Fig. 3.3). The spectrum absorption (Fig. 3.4) also indicated that the filtered MAD did not have strong absorbance at 350-500 and 630-680 nm which are key bands of chlorophyll *a* absorbance. The filtered FWAD had elevated absorbance below 400 nm, but it only partially blocked the useful spectrum for photosynthesis.

Table 3.1 ICP-OES metal concentrations in raw digestate

	Aluminum	Copper	Manganese	Zinc	Calcium	Iron
Raw MAD[a] (mg/L)	ND[d]	0.23	0.00	0.18	15.01	0.25
FWAD[b] (mg/L)	ND	0.10	ND	0.10	5.09	0.41
N8 medium[c] (mg/L)	0.29	0.47	3.60	0.73	3.55	1.52

[a] Raw municipal liquid anaerobic digestate. [b] Raw food waste liquid anaerobic digestate. [c] N8 medium is the defined chemical algal growth medium. [d] ND = not detected.

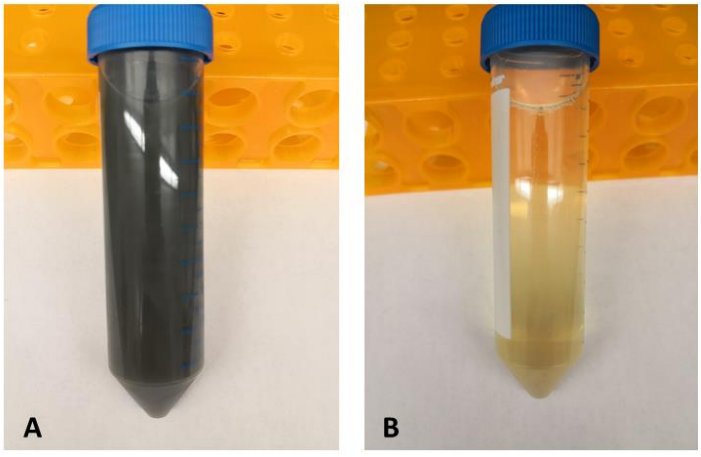


Figure 3.3 Picture of municipal anaerobic digestate before (A) and after (B) particle removal
A combination of centrifugation and a series of filtrations were used to remove solid particles in municipal anaerobic digestate.

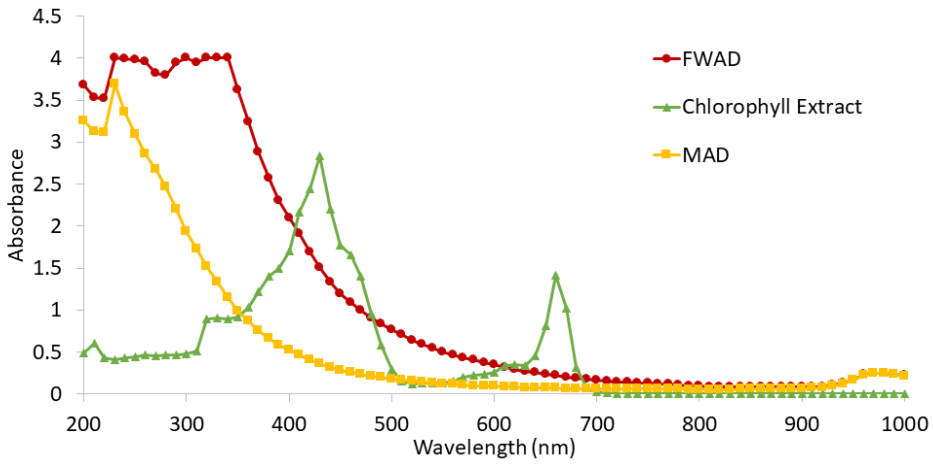


Figure 3.4 Comparison of absorption spectrum of filtered liquid anaerobic digestate (full strength) versus C. sorokiniana chlorophyll extract
Solid particles were removed by a combination of centrifuge and filtration down to 0.2µm. Algal pigments and lipids were extracted using a modified Folch method. All spectrum absorption measurements were conducted in quartz cuvettes.

3.3.4 Pretreating anaerobic digestate with activated sludge (AS)

3.3.4.1Anaerobic digestate nutrient composition

The change in anaerobic digestate nutrient composition before and after AS pretreatment is shown in Table 3.2. There was approximately 1,300 mg/L COD in MAD before and after AS treatment. However, a significant decrease ($p = 0.01$) followed by an increase in soluble COD was observed during the AS treatment process (Fig. 3.5). This suggests removal of organics followed by degradation of recalcitrant material and release of soluble metabolites by AS bacteria. Ammonium concentration in MAD decreased from approximately 2,000 mg/L to 1,100 mg/L. Increases in soluble phosphate and sulfate were observed during the AS treatment process for MAD, indicating solubilization under the aerobic conditions. Nitrate and nitrite were not detected during the process. Similar changes were observed in FWAD during the AS treatment process: COD and ammonium decreased whereas chloride increased due to pH adjustment. All other ions were relatively constant. The ammonium concentration was originally 3,200 mg/L, and it decreased to roughly 2,000 mg/L after AS treatment.

Table 3.2 COD and Ion composition in LD before and after AS treatment

	MAD AS Pretreatment		FWAD AS Pretreatment	
	Before (mg/L)	After (mg/L)	Before (mg/L)	After (mg/L)
COD	1278 ± 37.5[a]	1236 ± 30.4[a]	1521.2	1304.1
pH	7.5[b]	7.5[b]	7.5[b]	7.5[b]
NH_4^+	2052 ± 7.1[a]	1166 ± 9.9[a]	3177.5	1953.1
Na^+	75.8 ± 1.3[a]	76.8 ± 3.8[a]	971.8	974.6
K^+	199.8 ± 3.7[a]	175.7 ± 0.8[a]	1844.4	1817.2
PO_4^{3-}	585.6 ± 3.1[a]	607.4 ± 1.2[a]	8.9	9.1
Cl^-	625.1 ± 1.8[a]	665.2 ± 3.2[a]	1160.8	1197.3
NO_3^-	ND[c]	ND[c]	ND[c]	ND[c]
NO_2^-	ND[c]	ND[c]	ND[c]	ND[c]
SO_4^{2-}	35.5 ± 0.06[a]	49.5 ± 0.4[a]	4.6	5.2

[a] Error represents SD, n = 3; [b] pH was maintained at 7.5 by adding NaOH or HCl; [c] ND = no detection. Standard deviations for FWAD were not calculated because AS pretreatment was carried out in duplicate reactors.

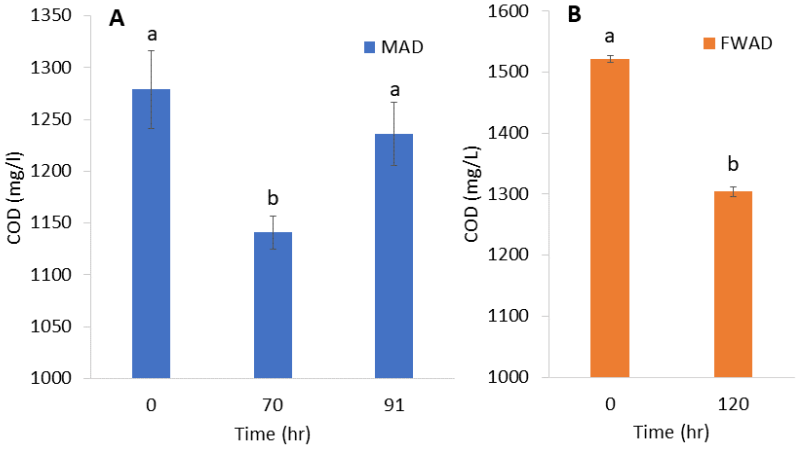


Figure 3.5 Change in COD concentration in municipal anaerobic digestate (A) and food waste anaerobic digestate (B) during AS treatment process
The COD concentration was measured using a HACH colorimeter. Error bars are SD, n = 3 biological replicates.

3.3.4.2 Suppression of nitrification in full-strength municipal anaerobic digestate

Nitrification carried out by aerobic bacteria during wastewater treatment is well-established (Ge et al., 2015). As activated sludge is known to harbor nitrifying organisms, it was

surprising that AS pretreatment did not lead to any detectable increase in the nitrate concentration. This led us to hypothesize that nitrifying organisms were also inhibited in the full strength digestates. We carried out a test on full strength and 10x-diluted MAD (Fig. 3.6). Nitrification was suppressed in full strength MAD with both nitrate and nitrite concentrations remaining undetectable during the AS pretreatment process. However, significant nitrification was observed during AS-treatment of 10x-diluted MAD ($p < 0.001$). The nitrate concentration increased linearly over time. Moreover, the nitrite concentration began increasing 48 hours after the inoculation of activated sludge.

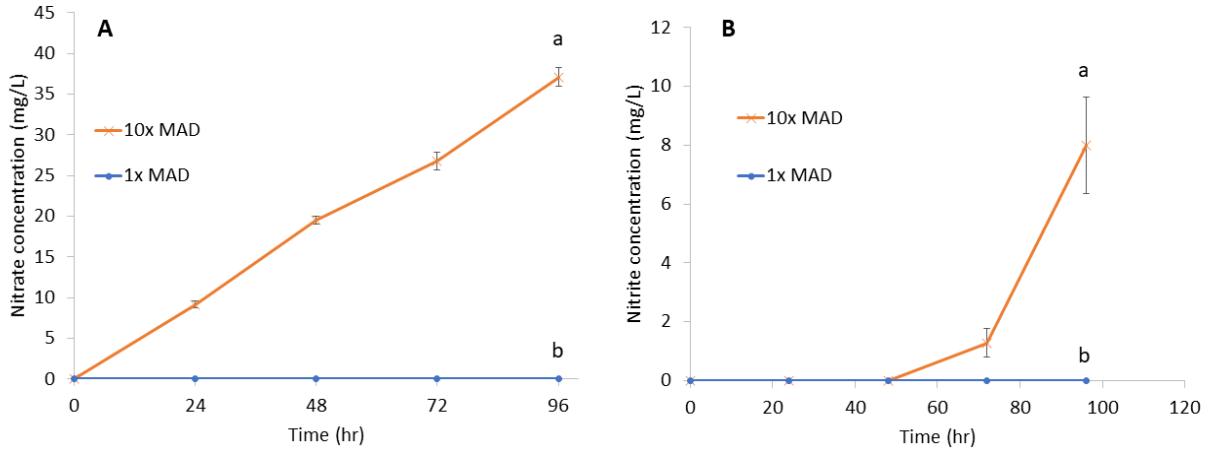


Figure 3.6 Nitrification during AS treatment in different dilutions of MAD.
Nitrification during the AS treatment process was determined by monitoring the nitrate (A) and nitrite (B) concentration in the spent medium. Error bars are SD, n = 3 biological replicates. Bars with the same letter are not significantly different at the 0.05 level.

3.3.5 Algae cultivation in AS-pretreated anaerobic digestate

3.3.5.1 Algal growth

AS pretreatment greatly alleviated the inhibitory effects of full-strength MAD on algae (Fig. 3.8.A). Culturing *C. sorokiniana* on AS-pretreated MAD resulted in 3.5 times faster growth (532 mg/L/day over a 5 day average) than the culture in untreated MAD (150 mg/L/day) and 1.4 times faster than the control culture. The addition of ammonium to AS pretreated MAD (to

compensate for ammonium lost during the pretreatment process) only had a negligible impact on algal growth (516 mg/L/day) compared to the AS-pretreated MAD. We also experimented with simultaneous "co-treatment" of digestate using AS and algae. The result was continued inhibition of algal growth (Figure 3.7).

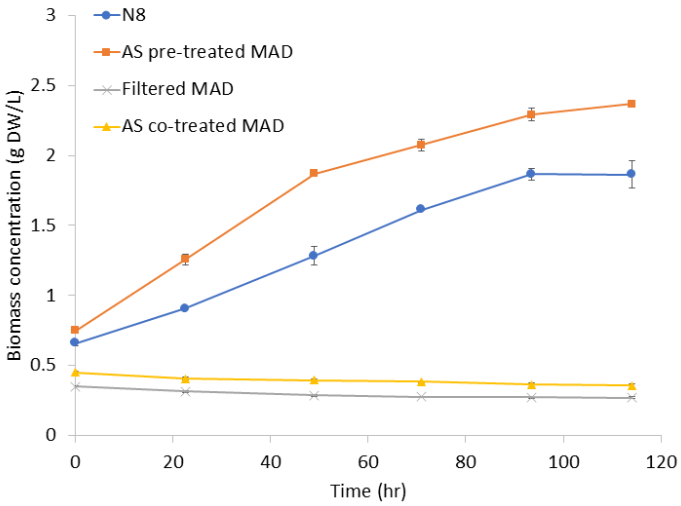


Figure 3.7 Growth of *C. sorokiniana* in AS-pretreated municipal anaerobic digestate and digestate that was co-treated with AS and *C. sorokiniana*.
Bacteria were removed in sterile MAD before algal inoculation. Clarification (0.7 µm filtration) was used to remove the solid particles in non-sterile MAD, but bacteria were not completely removed. This clarified digestate was either pretreated with AS or co-treated with AS. Error bars are SD, n = 3 biological replicates. Biomass concentration is reported on a dry-weight basis.

AS pretreatment of FWAD resulted in partial alleviation of algal growth inhibition (Fig. 3.8.B). Pretreatment of FWAD with AS resulted in a decline in ammonium content from 3,100 mg/L to 2,000 mg/L. Out of concern that such a high ammonium level could have a negative interactive effect with other inhibitors, we diluted untreated FWAD by a factor of 1.4 to reduce the ammonium concentration to the same level as AS-pretreated FWAD (2,000 mg/L ammonium). We also added a third group of reactors in which we diluted pretreated digestate by 1.4 fold and then supplemented it with ammonium chloride to restore the ammonium level to 2,000 mg/L. This treatment was included to control for the dilution benefit afforded to the untreated digestate. Although the highest average growth rate (318.5 mg/L/day) was still

observed in the control N8 medium, there was strong cell growth in the diluted AS pretreated FWAD and moderate growth in the full-strength AS-pretreated FWAD. The untreated FWAD completely inhibited algal growth even with the 1.4-fold dilution, consistent with the previous dose-response experiment.

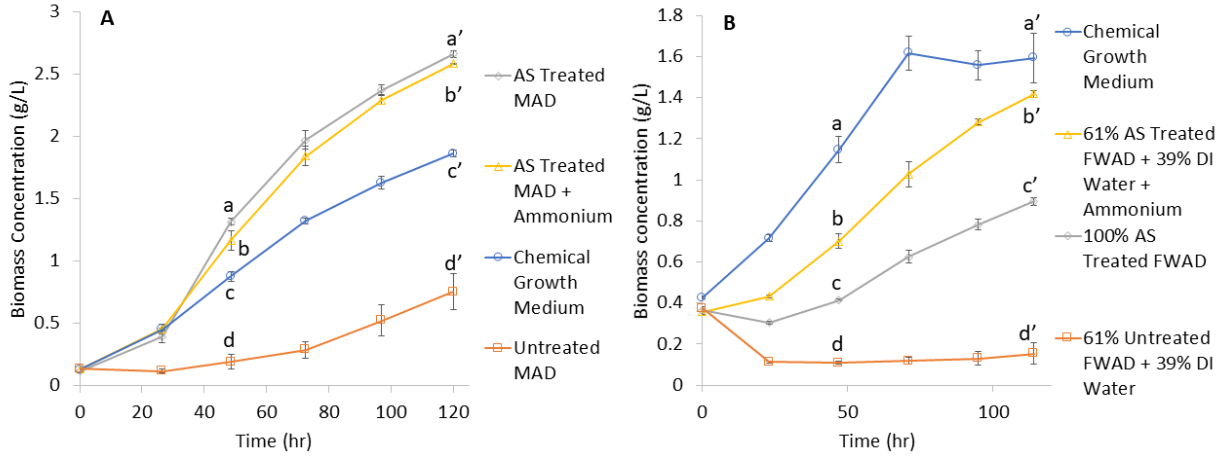


Figure 3.8 Growth of *C. sorokiniana* on municipal anaerobic digestate (A) and food waste digestate (B) with and without pretreatment with activated sludge (AS).
Note bacteria were removed before algae cultivation so growth is only due to algae. Control cultures were grown on chemical medium (N8). Because activated sludge treatment resulted in removal of some ammonium in the food waste digestate, the untreated digestate was diluted 1.4 fold to equalize ammonium concentrations (~2 g/L) in all digestates. Because of the dilution advantage afforded to the untreated digestate, an additional set of reactors was prepared with 1.4-fold-diluted AS-pretreated digestate. Ammonium was added to this last set of reactors to equalize to other cultures at 2 g/L. Errors bars are SD, n = 3 biological replicates. Letters above data points at 48 hours and 120 hours of growth indicate statistical significance where data points with the same letter are not significantly different at the 0.05 level.

3.3.5.2 Nutrient removal

With the alleviation of algal growth inhibition by AS pretreatment of anaerobic digestate, there was also a significant increase in nitrogen assimilation into algal biomass. Increased assimilation of nitrogen was observed in both MAD and FWAD (Fig. 3.9) after pretreatment. Measurements of nitrogen assimilation were used rather than measurements of nitrogen removal in order to understand algae's contribution to removal as opposed to other means, such as volatilization. Over 40 mg/L/day nitrogen removal was observed when culturing algae in AS-pretreated MAD with or without exogenous ammonium addition. This was significantly higher

than the algal nitrogen assimilation rate in untreated MAD which was ~10 mg/L/day ($p < 0.001$). Significantly higher nitrogen assimilation ($p < 0.001$) was also observed in AS-pretreated FWAD (~10 mg/L/day) compared to nitrogen assimilation in untreated FWAD (-3 mg/L/day). The untreated FWAD ended with a negative nitrogen assimilation due to net cell death in these cultures.

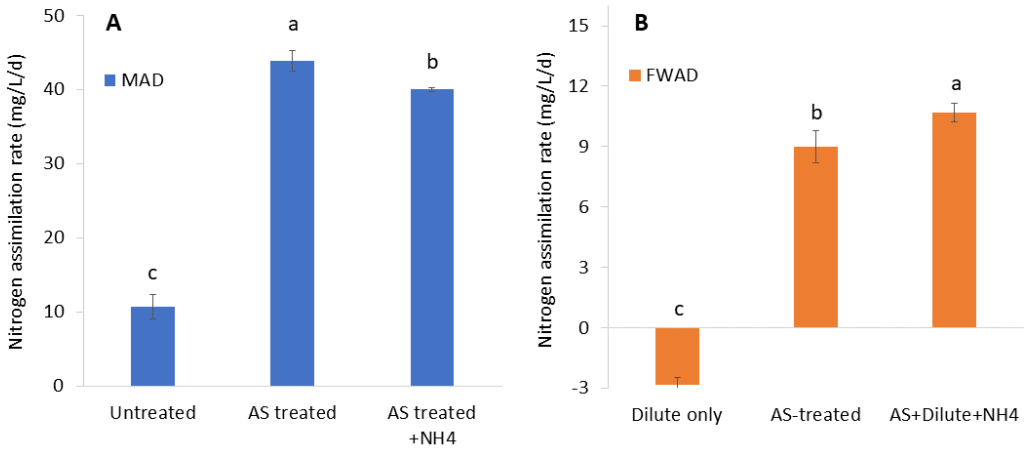


Figure 3.9 Nitrogen uptake rates by algae grown on municipal anaerobic digestate (A) and food waste digestate (B). Nitrogen uptake was determined through digestion of algae and analysis of nitrogen content. Negative values indicate net nitrogen release due to net loss of algal biomass over the culture period. Error bars are SD, n = 3 biological replicates. Bars with the same letter are not significantly different at the 0.05 level.

Phosphate removal was likewise faster in the AS-pretreated anaerobic digestates compared to the untreated digestate (Fig. 3.10). Around 15 mg/L/day phosphate removal was observed in MAD compared to a net negative phosphate removal (release of phosphate into the media) in untreated MAD. Similar observations were found when growing algae in FWAD: algae did not remove a significant amount of phosphate from the untreated FWAD ($p = 0.718$). On the other hand, algae removed all of the phosphate in the 1.4-fold diluted, AS-pretreated FWAD, averaging a phosphate removal rate of 10 mg/L/day. Moreover, positive phosphate removal (5 mg/L/day) was also observed in full strength AS-pretreated FWAD.

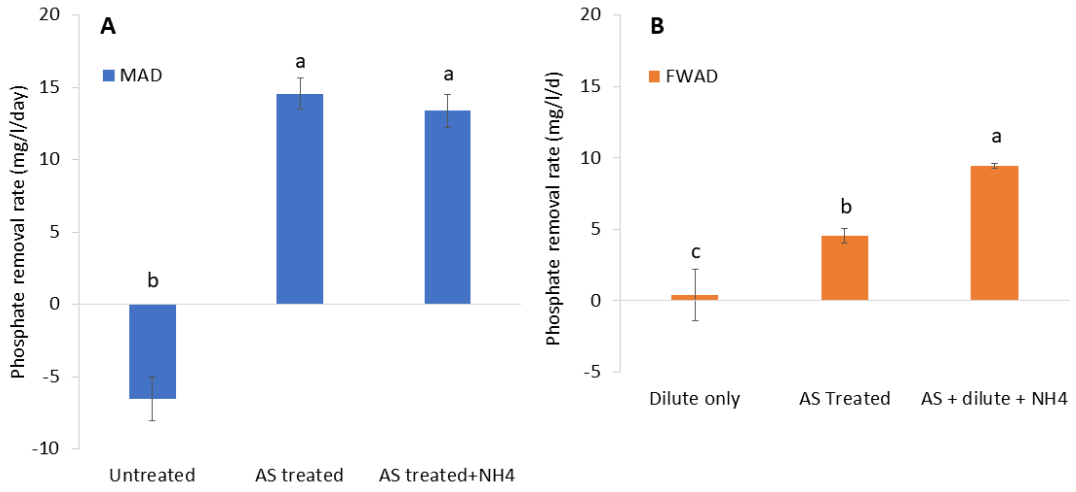


Figure 3.10 Phosphate removal rates by algae grown on municipal anaerobic digestate (A) and food waste digestate (B).
Phosphate removal was determined through the difference of phosphate concentration between t = 0 hr and t = 120 hr in the spent medium as measured by anion chromatography. Negative values indicate net phosphate release. Error bars are SD, n = 3 biological replicates. Bars with the same letter are not significantly different at the 0.05 level.

3.4. Discussion

The results obtained through our experiments suggest that the most commonly-cited factors for algal growth inhibition on digestate, namely ammonia (Cho et al., 2013), turbidity (Wang et al., 2010), and heavy metals (Wong et al., 1994), do not provide a complete picture of algal inhibitors present in digestate. High ammonium concentrations are likely to be inhibitory to a range of algae species, however, ammonium does not appear to be a significant problem for nutrient-tolerant species of the genera *Chlorella* and *Scenedesmus* (Ayre et al., 2017), so long as pH is controlled. The *Chlorella* species in this study grew robustly even at ammonium concentrations of 3,500 mg/L. Heavy metals including aluminum and copper, which are known to inhibit algae (Wong et al., 1994), had lower concentrations in the two digestates than in chemical growth medium. Hence, these metals cannot explain algal inhibition on digestate. Finally, the use of filtration can largely alleviate the problem of digestate turbidity, another inhibitor of algal growth. Filtration is already widely used in wastewater treatment processes for

separation of solids and liquids. For example, the wastewater treatment plant that supplied the municipal anaerobic digestate in this study employs a belt-press filter to separate digestate solids and liquid.

Nevertheless, we observed strong growth inhibition in *C. sorokiniana* in both digestate types. This inhibition could be partially or, in the case of municipal digestate, fully alleviated through pretreatment with an aerobic bacterial consortium. This finding suggests that organic constituents are likely inhibitors of algal growth in the digestates studied. Indeed, Franchino et al. (2016a) have suggested that unknown organic constituents may contribute to inhibition. Tigini et al. (2016) have also cited COD in digestate as an inhibitor of algae.

In past work, we have found that volatile fatty acids (VFAs), particularly propionic and butyric acid that are sometimes present in anaerobic digestate significantly inhibit algae (Wang et al., 2018b). Those studies revealed EC50 concentrations of propionate and butyrate of roughly 450 mg/L (Wang et al., 2018b) which are within the ranges found in many digestates from commercial-scale operations (Franke-Whittle et al., 2014b). However, the digestates used in the present study did not contain detectable VFAs, making this an unlikely explanation for inhibition observed in the present study. Many digestates also contain long chain free fatty acids as a result of lipid hydrolysis (Alves et al., 2009; Sousa et al., 2013) and these are known to be lethal to certain algae including *Chlorella* (Wu et al., 2006a). Lipids are present in food waste, and large volumes of waste cooking oil are processed in the anaerobic digester at the municipal wastewater treatment plant. Thus, free fatty acids, even at low concentrations could contribute to algal inhibition. A range of phenolic compounds are also present in digestates (Hecht & Griehl, 2009; Hernandez & Edyvean, 2008) and many algal species have been shown to be severely inhibited by a wide range of phenolics (Nakai et al., 2001; Pillinger et al., 1994; Wang et al., 2016b).

However, clear links between specific phenolics found in digestates and algal inhibition are the subject of ongoing investigations.

It is possible that organic constituents interact with ammonium to suppress algal growth. However, our results show that removal of inhibitory constituents by aerobic bacteria alleviates inhibition even at very high ammonium concentrations (e.g. 2,000 mg/L). Praveen et al. (2018) also utilized aerobic bacteria to pretreat anaerobic digestate prior to algae growth and found that this approach reduced inhibition. However, they largely attributed this effect to nitrification of ammonium, which they assumed to be the primary inhibitor. During treatment of digestate with activated sludge, there were indeed reductions in ammonium. However, no concomitant increase in nitrite or nitrate was observed suggesting little to no ammonium oxidation during pretreatment. Instead, much of the ammonium loss was likely due to ammonia volatilization. Our results showed that full-strength digestate completely inhibited ammonium oxidizing organisms: ten-fold dilution of the digestate allowed for a resumption of ammonium oxidation and production of nitrate. However, observation of significant nitrite production also indicates continued partial inhibition of nitrite oxidizing bacteria. Indeed, Praveen et al. (2018) used dilution rates of 10-fold for all of their inhibition studies which likely explains their observation of nitrification. It is interesting to note that algae, but not nitrifying bacteria, could process ammonium in full-strength digestate, underscoring the potential niche that hyper-eutrophic algae can play in treatment of high-strength wastewaters.

There are several major problems that make the dilution approach impractical for advancing algae treatment of anaerobic digestate. First, freshwater is a scarce and valuable resource, particularly for agricultural and industrial wastewater generators, who may lack access to large quantities of dilution water. Second, critical nutrients needed for algal growth are diluted

at the same rate as the inhibitors, leading to sub-optimal algal growth rates and nutrient removal, as we observed in digestate dosing studies. It was interesting that dilution led to slightly faster initial algal growth rates than the chemical control medium. However, this effect was likely due to the presence of ammonium in the digestate versus nitrate in the control medium. *C. sorokiniana* preferentially consumes ammonium over nitrate (Ogbonna et al., 2000) and our results indicate that growth on ammonium (Fig. 3.2) was faster than that on nitrate medium (Fig. 3.1). Moreover, dilution led to a lower plateau in growth, indicative of nutrient limitation despite supplementation with a micronutrient solution. Finally, sub-optimal growth rates necessitate a large reactor volume for algal growth. This leads to greater cost and thus lower likelihood of technology adoption. A better approach is to remove or destroy the inhibitors present in the digestate, thus allowing rapid algal growth on full-strength digestate. Fast growing algae, in turn, remove nutrients more quickly, shrinking the footprint (and cost) of the required treatment facility. That said, our results suggest that aerobic bacterial treatment does not always fully remove inhibitors in the digestate, as was the case with FWAD. In such cases, mild dilution can be helpful in maximizing algal growth and nutrient removal rates. The pretreatment approach discussed here would benefit from additional process optimization. Moreover, given the very high nutrient levels in digestate, a multi-stage algal treatment system is likely required in order to reduce nutrient concentrations to levels acceptable for environmental discharge.

3.5. Conclusions

1. Severe algal inhibition was observed on high-strength LD, but the main source of inhibition was not due to the commonly-cited reasons of ammonium toxicity, turbidity, or heavy metal toxicity.
2. Using aerobic bacteria as a pretreatment step effectively alleviated algal inhibition and increased nutrient removal rates. Pretreatment was more effective with municipal sludge digestate than with food waste digestate.
3. Organic compounds in LD are likely to be important algal inhibitors and the pretreatment process led to initial reduction in digestate COD levels.

Chapter 4 Factors impacting the effectiveness of biological pretreatment for the alleviation of algal growth inhibition on anaerobic digestate

Abstract

Algal growth is often inhibited in full-strength anaerobic digestate. The objective of this study was to investigate conditions under which digestate pretreatment using bacteria is effective in promoting algal growth, nutrient removal, and favorable changes in algal biomass composition. Batch culture experiments were carried out using low- and high-strength municipal sludge anaerobic digestate, two algae strains of varying sensitivity to digestate inhibitors, short and long pretreatment periods, and axenic vs. non-axenic algal cultures. Pretreatment of digestate increased algal growth up to 40%, N assimilation (up to 29%), and P removal (340%) by *Chlorella sorokiniana* (resilient algae) when grown on high-strength digestate. Pretreatment did not increase algal growth or nutrient assimilation when *Chlorella sorokiniana* was combined with low-strength digestate. The more sensitive strain, *Auxenochlorella protothecoides*, benefitted from pretreatment on both low- and high-strength digestate, even preventing complete cell death in the latter. Pretreatment increased starch content but not lipid content of algae.

Keywords: Algae; anaerobic digestate; inhibition; nutrient removal; pretreatment

4.1. Introduction

Anaerobic digestion has long been used to treat organic waste materials. The incorporation of modern engineering (temperature control, mixing control, adjustable retention time, and feed ratios) into anaerobic digestion has largely increased the efficiency of organic breakdown and nutrient mineralization (Wickham et al., 2016). However, the aqueous effluent still requires further treatment methods to remove and transform the large flux of re-mineralized nutrients (e.g. nitrogen and phosphorus) that are present (Qin et al., 2015). Typically, the aqueous effluent is separated from the solid sludge by centrifugation and/or filtration. In municipal wastewater treatment plants that employ anaerobic digestion of excess sludge, this aqueous stream is typically redirected to the headworks of the treatment plant, creating an inefficient parasitic load on the system (Halfhide et al., 2015). In agricultural settings, digestate is often applied to land as a soil amendment but aqueous material is confined to local application immediately surrounding the digester. This can lead to over-application of nutrients thereby contributing to nutrient run-off and eutrophication (Lory et al., 2006).

Controlled growth of algae is an alternative use of the aqueous digestate, henceforth referred to simply as digestate. Algae are among the most efficient photosynthetic organisms and thrive in nutrient-rich environments (Chislock et al., 2013a). Uncontrolled algal blooms often occur in eutrophic natural waterways (Graham et al., 2017). While algal blooms can be deleterious in nature, controlled microalgal production has been used in a variety of applications such as biofuel production (Chew et al., 2017b), polymer production (Mathiot et al., 2019), agricultural feedstock (Dineshbabu et al., 2019), and human dietary supplements (Rizwan et al., 2018). Producing useful algal biomass by assimilating nutrients from wastewater (including anaerobic digestate) is a sustainable solution to waste recycling, provided technical challenges can be overcome.

A variety of researchers have found that high strength anaerobic digestate contains not only nutrients but also growth inhibitors such as high concentrations of free ammonia, heavy metals, and organics (Ayre et al., 2017; Franchino et al., 2016a; Praveen et al., 2018). The primary solution to this challenge has been to dilute digestate (10-30 fold) with water (Cho et al., 2013). Our past research has shown that dilution is a sub-optimal strategy for overcoming growth inhibition on digestate (Wang et al., 2019e), largely because key nutrients are also diluted along with inhibitors. Moreover, use of freshwater for dilution is unattractive to stakeholders. In our past work, we developed an aerobic bacterial pretreatment approach for high-strength anaerobic digestates that allows for robust algal growth without the need for dilution (Wang et al., 2019e). Activated sludge from a municipal wastewater treatment plant was used as the bacterial inoculum for pretreatment. However, this approach was only tested with one axenic algae strain, under one pretreatment duration, and only on high-strength digestates. Moreover, the effects of pretreatment on downstream algal biomass composition were not measured. Biomass composition is an important consideration for end-use of the final algal product whether it is as a feed ingredient or for biofuel production (Khan et al., 2018).

The objective of this study was to investigate the conditions under which digestate pretreatment is effective in promoting algal growth, nutrient removal, and favorable changes in algal biomass composition. Anaerobic digestate of municipal sludge was obtained from a large wastewater treatment plant in Columbus, GA. This digestate varies significantly in ionic strength over time so lower-strength (~400 mg/L ammonium) and higher-strength samples of this digestate (~1,500 mg/L ammonium) were tested with two algae strains: *Chlorella sorokiniana* and *Auxenochlorella protothecoides*. The former strain is very robust in high strength digestate (Wang et al., 2019e) whereas the latter was expected to be more sensitive. We also tested two

durations (1-day and 4-day) for the pretreatment process. In our past research, we only grew algae in sterile-filtered digestate after the pretreatment process in order to understand how algae interact with chemical constituents in the digestate. In this study, we investigate the effects of leaving some of the bacteria from the pretreatment process in the digestate during algae culture. The latter is much more realistic in real-world applications.

4.2. Materials and methods

4.2.1. Wastewater collection and preparation

Municipal anaerobic digestates (AD) and activated sludge (AS) were collected at the South Columbus Water Resources Facility (Columbus GA, USA). Both digestates and activated sludge were immediately transported and stored in a cold room (4℃) at Auburn University. The digestate was settled for 7 days in the cold room. In order to limit the cell density of anaerobic microorganisms, the upper portion of the digestate was centrifuged (4696xg, 15min) and filtered through a series of filter paper (Whatman No. 4, No. 1, No. 2 No. 5, Advantec GA-55, GC-50, GF-75) until all liquid digestate passed through 0.7 μm pore size, thus improving digestate clarity.

4.2.2. Activated sludge pretreatment (AS pretreatment)

Activated sludge (solids content of 6.7 g/L) was mixed at a ratio of 2% (v/v) with filtered AD (pH adjusted to 7.5 with 3M HCl, 0.7 μm filtered) in 500 ml bottles. To pretreat the AD, the slurry was aerated at 1 vvm air for 1 or 4 days followed by immediate centrifugation and filtration (Whatman No. 5, Advantec GC-50, and then GF-75) to reduce the total suspended solids in pretreated AD while allowing individual bacteria to pass through the filter (0.7 μm). A subset of pretreated AD underwent additional filtration to create sterile-filtered digestate. This

was achieved by further filtering AD with Advantec 0.65μm, 0.45μm, and 0.2 μm mixed cellulose ester membranes followed by a 0.2 μm sterile filtration apparatus (VWR PES filter).

4.2.3. Algal culture experimental design

Four batch culture experiments were conducted to test two strains of algae (*C. sorokiniana*, UTEX 2805 (de-Bashan et al., 2008b) and *A. protothecoides*, UTEX 2341 (Higgins et al., 2015b)) in two anaerobic digestate (low- and high-strength). These two strains of algae were chosen due to their capability of treating anaerobic digestate (Higgins et al., 2017). Our past study showed that AS pretreatment could alleviate growth inhibition for this strain of *C. sorokiniana* in two types of high strength AD (Wang et al., 2019e). *C. sorokiniana* is a starch-accumulating organism (Tanadul et al., 2014a) whereas *A. protothecoides* accumulates neutral lipid under growth stress (Higgins & VanderGheynst, 2014). A previous study also indicated that aerobic bacteria had a positive impact on growth in this strain (Higgins et al., 2016).

In order to better understand the conditions under which AS pretreatment of anaerobic digestate is most effective, four experimental conditions were tested within each batch culture experiment. These conditions varied the duration of aerobic pretreatment and the sterility of the digestate medium. The four conditions were: 1. AD without pretreatment and sterile-filtered to create axenic culture conditions (No treat (A)); 2. AD pretreated for 1 day using AS and sterile-filtered (1DPretreat (A)); 3. AD pretreated for 4 days and sterile-filtered (4DPretreat (A)); and 4. AD pretreated for 4 days and filtered to 0.7 μm pore size, thus retaining a portion of live anaerobic and aerobic bacteria (4DPretreat (N)). Each treatment was conducted in biological triplicate. At the start of each experiment, the digestates were added to bubble column photobioreactors and inoculated with the algae strain specific to the batch culture.

4.2.4. Algal cultivation methods

The algal cultivation methods have been described in detail previously (Wang et al., 2019c). Briefly, algae were plated on ATCC No. 5 sporulating agar plates for 5-7 days. Single colonies were picked and inoculated in 1 L bottles with autoclaved aqueous media (N8 for *C. sorokiniana* (Tanadul et al., 2014a), and modified N8 with ammonium as the nitrogen source for *A. protothecoides* (Higgins & VanderGheynst, 2014). The bottles were placed under fluorescent growth lights (170 mmol photons/m^2/s on a 16 h:8 h light-dark cycle) and aerated (0.5 vvm air with 2% CO_2) for approximately 5 days to create stock cultures of algae. The stock cultures were stopped when the algal optical density (wavelength 550 nm) reached 0.2-0.3 and the cultures were settled on the bench (24-48 hours). After removing 90% of the supernatant, 6 ml of settled algal slurry was inoculated into each bubble column photobioreactor (200 ml working volume) which contained 150 ml pretreated AD as described in section 2.3. The photobioreactors were placed in a water bath at 26 ℃. The same aeration and lighting as the stock cultures was provided to experimental cultures. A 2 ml sample was collected daily from each bioreactor to check optical density and pH. The pH was readjusted as needed to 7.5 using 3M NaOH. The 2 ml samples were also centrifuged and syringe filtered (VWR 25 mm nylon), and the supernatant was stored at -80 ℃. After five days of cultivation, 100 ml of algae culture was harvested, dewatered, freeze-dried and weighed. All cultures were handled in a biosafety cabinet using sterile technique.

4.2.5. Lipid determination

4.2.5.1. Lipid extraction and Nile red assay

Lipid extraction from freeze-dried algal cells was conducted using a modified Folch method which was described step-by-step in a previous publication (Wang et al., 2019c). Briefly,

20 mg dried algal cells were homogenized (6.5 m/s, 6 times 20 s) in Folch solvent (2:1 chloroform: methanol). 0.9% (w/v) sodium chloride in water was added to induce phase separation. The bottom (chloroform) phase was retained for neutral lipid and fatty acid analyses.

The Nile red neutral lipid assay was adapted from Higgins et al. (Higgins et al., 2014b) with the modifications described in Wang et al. (Wang et al., 2019c). In brief, lipid extracts were diluted with 2 parts of methanol, pipetted into 96-well plate and placed on a 55 ℃-hot plate for 20-30 minutes to evaporate solvents. The dried residue was resuspended in 30 µl isopropanol. 200 µl of 1 µg/ml Nile red Dimethyl sulfoxide (DMSO) solution was added into each well. After incubating for 5 minutes, 10 µl of 10% bleach was added. The fluorescence read at 530 nm excitement and 575 nm emission with auto cutoff at 570 nm was measured every 5 minutes by a plate reader (SpectraMax M2, USA) 30 minutes after bleach addition. This chemical assay used canola oil as a standard to estimate the neutral lipid content in the chloroform extracts from algae.

4.2.5.2. Transesterification and GC-MS FAME determination

The extracted lipids (section 2.5.1) underwent transesterification through reaction with methanolic HCl as described previously with slight modifications (Higgins et al., 2014a). Briefly, nonadecanoic acid and the Folch extracts were added to Pyrex vials and the solvent was evaporated under a gentle stream of nitrogen gas. Hexane and methanolic HCl were added, and the mixture was heated for one hour at 100 °C. Highly characterized canola oil (Sigma) was also transesterified and used as a reference standard. Sodium bicarbonate solution was added to induce phase separation. The fatty acid methyl esters (FAME), which were dissolved in hexane, were analyzed by gas chromatography-mass spectrometry (Agilent 6890 with 5970 MSD, USA). A Restek RXI-5Sil MS column (Length: 30m, ID: 0.25mm, d_f: 0.25µm) was used for separation

using the following oven temperature program: Initial Temperature: 100 ℃, 2 minutes; Ramp to 240 ℃ in 10 minutes (rate: 14 ℃/ min); Hold at 240 ℃ for 47 minutes. Helium was used as the carrier gas with a flow of 6.4 ml/min and a split ratio of 8:1 (0.8 ml/min on column).

4.2.6. Starch determination

The undissolved pellet, which contained starch and cell wall after lipid extraction, was washed with acetone and dH_2O. It was digested with α-amylose and amyloglucosidase to hydrolyze starch to glucose as previously described (Higgins & VanderGheynst, 2014). The glucose concentration was determined with the dinitrosalicylic (DNS) method using glucose as an external standard. Released glucose was multiplied by 0.9 to correct for water addition during starch hydrolysis.

4.2.7. Total algal nitrogen and total spent medium phosphorus (HACH)

Dried algae (1.5 mg) were homogenized (6.0 m/s, 20s for 3 times) in 1.5 ml dH_2O with zirconia/silica beads to create a slurry. Total nitrogen content of this slurry was determined by the standard HACH Persulfate digestion total nitrogen method (low range). The phosphorus concentration in the syringe filtered spent medium (daily sample from section 2.4) was determined by the standard HACH Molybdovanadate total phosphorus method (high range). Samples were diluted with dH_2O to fit into the assay range. Algal nitrogen content was used to determine the rate of algal nitrogen uptake by multiplying nitrogen content by average growth rate.

4.2.8. Ion chromatography

Ion chromatography was carried out on the filtered spent culture medium using a previously-described method (Chaump et al., 2018a). Briefly, a Dionex IonPac CS12 column

coupled with Dionex CERS 500 suppressor was used to determine cation concentrations (NH_4^+, Na^+, K^+, Mg^{2+}, Ca^{2+}). A Dionex IonPac AS22 column coupled with Dionex AERS 500 suppressor was used to determine anion concentrations (Cl^-, NO_2^-, NO_3^-, PO_4^{3-}, SO_4^{2-}).

4.2.9. Data analysis and statistics

All experimental data were collected from 3 biological replicates. Statistical analysis (ANOVA and Turkey's HSD test) were carried out in R with the "car" and "agricolae" packages.

4.3. Results and discussion

4.3.1. Activated sludge pretreatment affects algal biomass growth

The effectiveness of digestate pretreatment for the alleviation of algal growth inhibition was dependent on several factors: the strength (high/low) of AD; microalgal strain; duration of AS pretreatment; and axenic/non-axenic conditions for algal cultivation (Figure 4.1). Based on our previous study, AS pretreatment could alleviate the algal growth inhibition from high strength municipal sludge AD and food waste AD (Wang et al., 2019e). In that previous study, the AD had roughly 2,000 mg/L ammonium and the food waste AD had roughly 3,000 mg/L ammonium. In the present study, the low-strength digestate contained ~400 mg/L ammonium and the high-strength digestate contained ~1,400 mg/L ammonium (see Table S1 for digestate composition details). Both algae strains produced more biomass on pretreated AD than untreated AD in the high-strength digestate (Figure 4.1, $p < 0.01$).

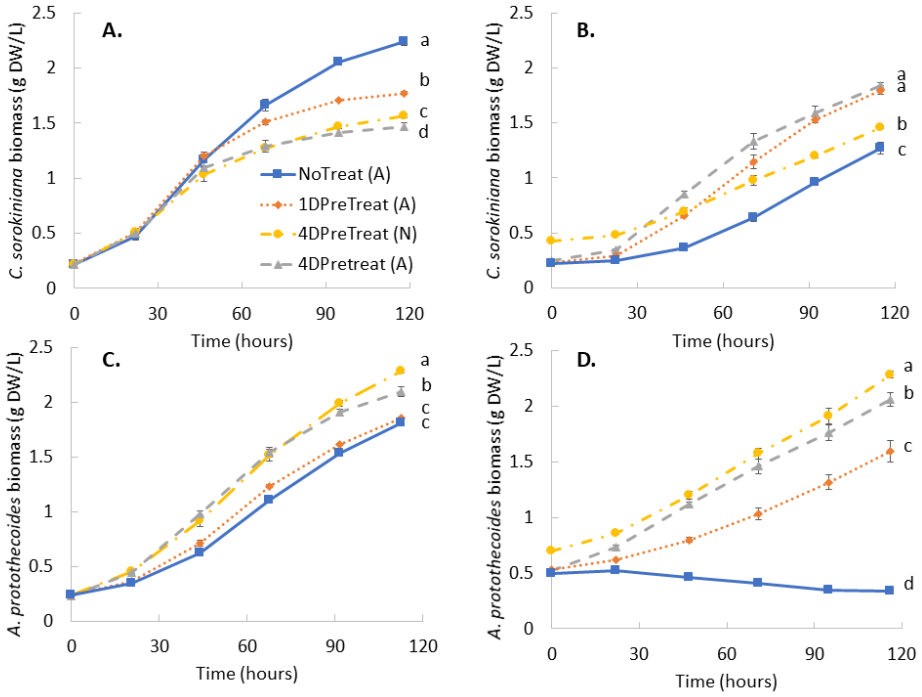


Figure 4.1 Growth curves of microalgae on municipal anaerobic digestate (AD) with varying pretreatments**.** A) C. sorokiniana cultured in low strength AD; B) C. sorokiniana cultured in high strength AD; C) A. protothecoides cultured in low strength AD; D) A. protothecoides cultured in high strength AD. NoTreat(A): algae cultured in sterile filtered (0.2µm) AD without pretreatment (axenic condition); 1DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 24 hours activated sludge (AS) pretreatment (axenic condition); 4DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 96 hours AS pretreatment (axenic condition); 4DPretreat(N): algae cultured in clarified (0.7µm) AD with 96 hours AS pretreatment (non-axenic condition). Biomass concentration is reported on dry mass basis. Error bars represent the standard deviation (n = 3, biological replicates). Different letters indicate statistical significance at < 0.05 based on Tukey's test.

The strain of *C. sorokiniana* used in this study (Figure 4.1A, B) was originally isolated from a wastewater treatment plant in Mexico (de-Bashan et al., 2008b), and past results have shown that this strain is highly tolerant of ammonium (up to 3,500 mg/L at pH 7.5) (Wang et al., 2019e). The high tolerance of this strain to extreme nutrient levels may partially explain why *C. sorokiniana* did not show growth inhibition in the low strength AD (Figure 4.1A) even without AS pretreatment. When grown on low-strength digestate, *C. sorokiniana* reached the log growth stage at ~70 hours, which was earlier than cultures grown in high-strength digestate. This outcome suggests some nutrient limitation may have occurred in the low strength digestate although N, P, Mg were not depleted. Nevertheless, ammonium concentrations declined to as

low as 29 mg/L (Figure 4.2), which may have slowed the growth rate toward the end of batch culture. A previous dose-response study showed that *C. sorokiniana* growth was maximized at 500 mg/L ammonium with a rapid decline in growth when ammonium was <50 mg/L (Wang et al., 2019e). It is also possible that certain micronutrients were limited in low-strength AD towards the end of algal cultivation although micronutrient concentrations (aside from Ca and Mg) were not measured in this study. Our previous compositional analysis of high-strength municipal sludge digestate showed that it had iron and zinc concentrations that were only 16% and 25% of those found in chemical N8 medium and manganese was almost undetectable in the digestate (Wang et al., 2019e). All of these nutrients are essential for algae growth (Walker, 1954). AS pretreatment depleted nutrient levels in digestate (Figure 4.2 and Table 4.1), further exacerbating nutrient limitation in the low-strength digestate. The final biomass concentration of *C. sorokiniana* in low-strength digestate had the following trend: untreated AD > 1-day pretreated AD > 4-day pretreated AD. There was only a slight benefit from the presence of bacteria on biomass growth when *C. sorokiniana* was grown on low-strength AD (Figure 4.1A).

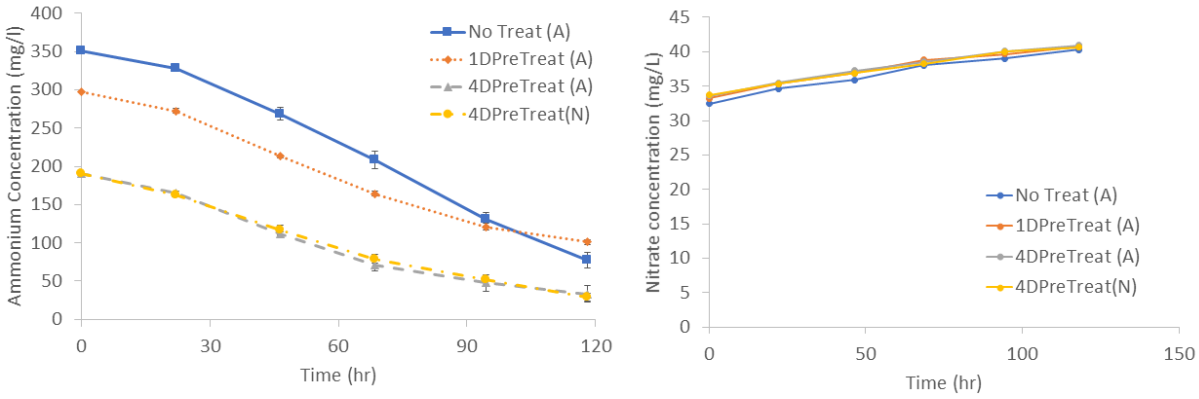


Figure 4.2 Ammonium depletion (A) and nitrate accumulation (B) in *C. sorokiniana* cultures grown on low strength digestate. Error bars are standard deviations based on 3 biological replicates.

Table 4.1 Chemical composition of low and high-strength AD

Component	Low strength AD	High strength AD	Low strength AD After pretreatment	High strength AD After pretreatment
COD (mg/L)	137.3±1.5[a]	1359.4±19.2	103.3±13.3	1122.8±26.7
Ammonium (mg/L)	433.4±1.7	1372.7±9.9	205.2±8.6	994.8±15.3
Nitrite (mg/L)	N.D.[b]	N.D.	10.0±0.6	N.D.
Nitrate (mg/L)	20.8±0.1	N.D.	2.6±0.1	N.D.
Phosphate (mg/L)	133.1±0.5	503.1±1.5	118.8±1.9	464.7±9.0
Sulfate (mg/L)	47.2±0.1	34.0±0.1	41.5±0.4	15.3±0.3
Chloride (mg/L)	139.8±0.6	474.3±5.3	154.1±1.0	472.1±7.8
Potassium (mg/L)	16.9±0.1	72.3±1.1	15.7±0.7	80.0±0.1
Sodium (mg/L)	76.2±4.3	86.8±1.7	68.2±0.4	78.4±2.9
Magnesium (mg/L)	N.D.	7.8±0.1	4.3±0.4	N.D.
Calcium (mg/L)	N.D.	42.4±0.1	9.4±0.1	5.7±0.3

a value after ± is standard deviation (n = 3, biological replicates).
b measured but not detected

Unlike low-strength AD, *C. sorokiniana* was inhibited by high strength AD, especially during early exponential growth (Figure 4.1B). AD pretreatment shortened the lag phase in *C. sorokiniana* growth. Meanwhile, *C. sorokiniana* in all AS pretreated conditions showed increased biomass production (maximum 40% higher) compared to no pretreatment after 5-days of cultivation. This result indicated that, in contrast to low-strength digestate, the high strength digestate was inhibitory to *C. sorokiniana* but that pretreatment could alleviate inhibition. Nutrient limitation was also not an issue given the high nutrient load in high-strength digestate.

Interestingly, *C. sorokiniana* co-cultured with AS bacteria grew to 20% less final biomass compared to the corresponding axenic cultures. The comparatively lower biomass in co-cultures could be the result of competition (e.g. light, nutrients) between algae and bacteria (Liu et al., 2017).

The inhibitory effects of anaerobic digestate on algal growth were more obvious in the comparatively sensitive microalgae, *A. protothecoides* (Figure 4.1C, D). *A. protothecoides* growth on the AD increased with the duration of aerobic pretreatment, and co-culturing the algae with AS bacteria further increased growth. These growth trends were consistent in both the low and high strength AD and suggest growth inhibition by AD could be partially-alleviated by AS pretreatment. However, the benefit of pretreatment was more dramatic in the high-strength digestate. Use of untreated high-strength digestate killed this algae. In addition to being more sensitive to high-strength digestate, *A. protothecoides* is known to benefit from co-cultivation with bacterial consortia (Higgins et al., 2017). This algae is a thiamine auxotroph and depends on thiamine metabolites secreted by bacteria (Higgins et al., 2016). In contrast, *C. sorokiniana* could thrive under strict autotrophic conditions (Cecchin et al., 2018). This major difference was likely the key reason for the different performance of these two strains under axenic versus non-axenic conditions.

Growth of both algae strains on pretreated digestate compares favorably to results reported in the literature. *C. sorokiniana* grown on pretreated high-strength digestate had average 5-day productivites exceeding 300 mg/L/d. *A. protothecoides* grown on pretreated low and high strength digestate also had productivity exceeding 300 mg/L/d, and the non-axenic cultures even reached 400 mg/L/d. Prandini et al. reviewed ten studies that cultivated algae on non-pretreated, diluted anaerobic digestates and, in all but two of the studies, algal growth rates were well below

100 mg/L/d (Prandini et al., 2016). In two of the studies, the growth of *Scenedesmus* species reached approximately 150 mg/L/d, still far below the growth rates observed in our system. This further underscores that pretreatment can be more effective at promoting algal growth than digestate dilution.

Inhibition of algal growth on anaerobic digestate is still poorly understood. Many researchers have hypothesized that ammonia (Cho et al., 2013) and light limitation (Wang et al., 2010) are the main algal inhibitors. Our past research showed that neither of these factors explained growth inhibition in *C. sorokiniana* (Wang et al., 2019e). At the time, we hypothesized that phenolic and free fatty acid compounds found in digestate could be inhibitory to algae given that such compounds are common in digestates (Hecht & Griehl, 2009; Hernandez & Edyvean, 2008) and known to inhibit various algae types (Nakai et al., 2001; Pillinger et al., 1994; Wang et al., 2016b). That said, high ammonium concentrations could be inhibitory to *A. protothecoides* so a dose-response study was carried out in defined medium to determine its tolerance. Inhibition became apparent around 500 mg/L ammonium with an approximate EC50 of ~1000 mg/L ammonium (Figure 4.3). This indicates that ammonium was very likely inhibitory to this strain in the high strength digestate. However, even at 1,500 mg/L ammonium, growth of *A. protothecides* was still robust (reaching 1.4 g/L within 5 days) whereas in the high-strength digestate (1,400 mg/L ammonium), this algae completely died. Similar to past findings with *C. sorokiniana*, this result indicates that additional inhibitors besides ammonia are present in the digestate.

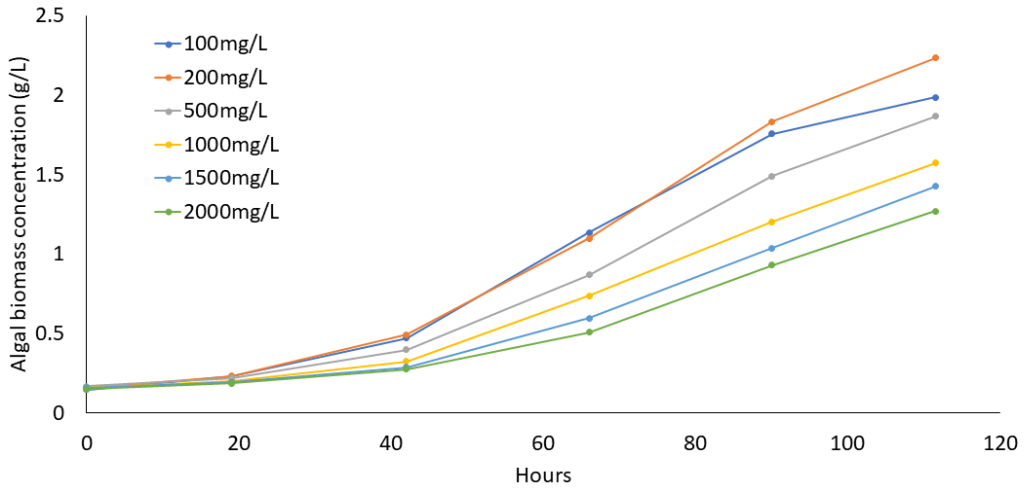


Figure 4.3 Growth of *A. protothecoides* on varying concentrations of ammonium.

4.3.2 Biomass composition

4.3.2.1 Starch content

Starch is valuable for its biofuel potential (Rehman & Anal, 2019) and also is a nutrient in animal diets (Murillo & Granados-Chinchilla, 2018). *C. sorokiniana* is a known starch-accumulating organism (Tanadul et al., 2014a). In the present study, the highest starch accumulation by *C. sorokiniana* was achieved in experimental treatments that reached the stationary growth stage most quickly (Figure 4.4). *C. sorokiniana* was observed to accumulate starch when cultivated in both low and high strength AD (Figure 4.4 A, B). In low-strength AD, *C. sorokiniana* had 6-12% starch content, with the highest concentration achieved in the non-axenic cultures. In the high-strength digestate, starch content was not higher than 7%, with the highest levels observed in the fastest-growing cultures, and almost no starch was observed in cultures grown on untreated digestate. Starch can accumulate in algae under stress conditions (Markou & Nerantzis, 2013a) but is also associated with growing cultures (Tanadul et al., 2014a) since it serves as an easily-accessible energy reserve.

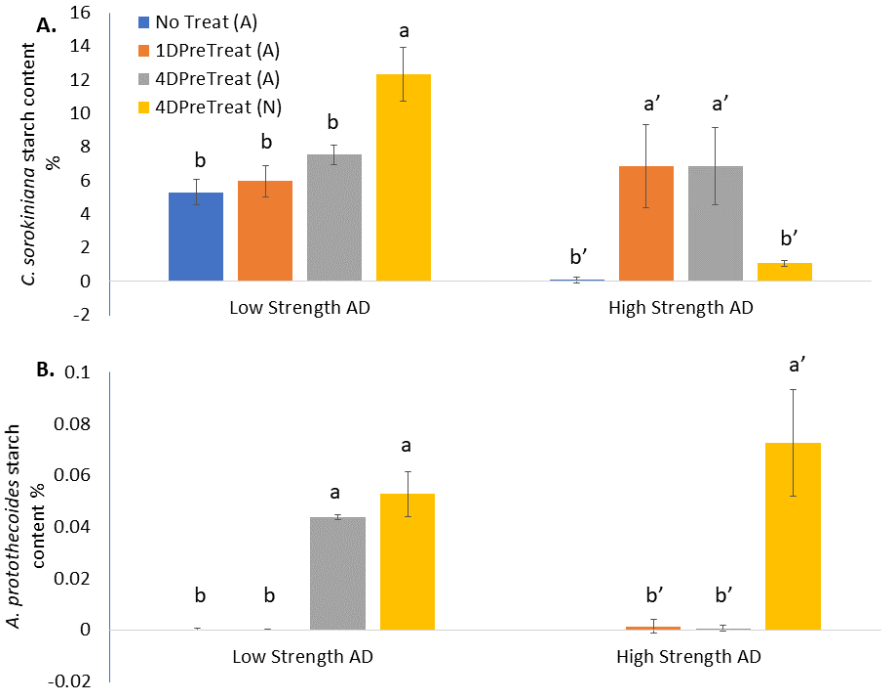


Figure 4.4 Percentile starch content in dry mass of *C. sorokiniana* (A) and *A. protothecoides* (B) cultured in anaerobic digestate.
NoTreat(A): algae cultured in sterile filtered (0.2µm) AD without pretreatment (axenic condition); 1DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 24 hours activated sludge (AS) pretreatment (axenic condition); 4DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 96 hours AS pretreatment (axenic condition); 4DPretreat(N): algae cultured in clarified (0.7µm) AD with 96 hours AS pretreatment (non-axenic condition). Note: There was no A. protothecoides harvested in high strength AD without pretreatment due to cell death, the starch content was not measurable in that case. Error bars represent the standard deviation (n = 3, biological replicates). Different letters indicate statistical significance at < 0.05 based on Tukey's test.

Interestingly, the observed starch contents in this study were much lower than the >40% starch content observed when this algae was grown on poultry litter slurry digestate (Bankston & Higgins, 2020) and the 20% starch content observed on chemical N8 medium (Tanadul et al., 2014a). There has been a general trend that *C. sorokiniana* grown on manure anaerobic digestates has yielded significantly higher starch content than cultures grown on chemical medium (Kobayashi et al., 2013). However, in the municipal anaerobic digestates used in this study, the opposite outcome was observed. Many algae are known to accumulate starch under conditions that cause oxidative stress, such as nutrient limitation (Dragone et al., 2011) or high light intensity (Cheng et al., 2017). In this regard, this digestate may not have led to oxidative

stress conditions, with the possible exception of the pretreated low-strength digestate which likely became nutrient-limited. Cultures grown on pretreated low-strength digestate exhibited 12-130% increases in starch content compared to untreated digestate. *A. protothecoides* is not a strong starch-accumulating algae but does accumulate starch under mixotrophic conditions, particularly when bacteria are present (Higgins & VanderGheynst, 2014). There was very little starch accumulation (<0.1%) observed in *A. protothecoides* in both high- and low-strength AD. The non-axenic cultures nominally had the highest starch accumulation but this is unlikely to be of practical significance.

4.3.2.2 Neutral lipid and FAME

Both strains of algae used in this study are known to accumulate neutral lipid under nutrient stress conditions (Higgins et al., 2014a). Relatives of these organisms also accumulate neutral lipids under a wide range of oxidative stress conditions (Burch & Franz, 2016; Chen et al., 2017; Chokshi et al., 2017). One of the classic symptoms of oxidative stress is growth suppression (Yilancioglu et al., 2014). Because growth of both *C. sorokiniana* and *A. protothecoides* was inhibited to varying degrees on anaerobic digestate, it was initially hypothesized that algal growth inhibition could be linked to oxidative stress. Thus more-inhibited cultures were expected to yield a higher neutral lipid content. However, there was no significant difference in neutral lipid content among any of the experimental treatments for both *C. sorokiniana* and *A. protothecoides* (Table 4.2). Moreover, the strength of AD did nothing to alter the neutral lipid content. This finding strongly suggests that algal growth inhibitors in these digestates did not induce an oxidative stress response in these algae.

Table 4.2 Neutral lipid content in dried algal biomass

Neutral Lipid Content %		No treat (A)[a]	1DPreTreat (A)[b]	4DPreTreat (A)[c]	4DPreTreat (N)[d]
C. sorokiniana UTEX 2805	Low strength AD	2.11±0.79[e]	2.13±0.7	2.75±0.43	3.73±0.74
	High strength AD	2.03±0.13	2.33±1.02	3.54±0.86	3.11±0.28
A. protothecoides UTEX 2341	Low strength AD	3.67±1.27	3.19±0.40	2.21±0.88	2.57±0.44
	High strength AD	N.A.[f]	2.22±0.88	2.28±0.78	2.79±0.41

[a] NoTreat(A): algae cultured in sterile filtered (0.2µm) AD without pretreatment (axenic condition).
[b] 1DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 24 hours activated sludge (AS) pretreatment (axenic condition).
[c] 4DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 96 hours AS pretreatment (axenic condition).
[d] 4DPretreat(N): algae cultured in clarified (0.7µm) AD with 96 hours AS pretreatment (non-axenic condition).
[e] value after ± is standard deviation (n = 3, biological replicates).
[f] There was no *A. protothecoides* harvested in high strength AD without pretreatment due to high algal inhibition, neutral lipid content was not able to be measured in that case.

Similar to the neutral lipid, the total FAME in algae did not show statistical significance with AS pretreatment (Table 4.3). However, it is of economic relevance that both strains of algae maintained high levels of the ω-3 fatty acid, α-linolenic acid, when they were cultured in anaerobic digestate (25% and 90% of total fatty acids for *C. sorokiniana* and *A. protothecoides*, respectively). ω-3 fatty acids are very important component of animal feed, and supplementation of these fatty acids in animal diets results in a more nutritious fatty acid profile in the animal product (Kouba & Mourot, 2011). A long-term goal is to use the algae from this process as a feed ingredient, provided feed safety and other nutritional needs are adequately addressed.

Table 4.3 Fatty acid methyl ester content in dried algal biomass

μg FAME /mg Dry algae			Palmitic Acid (C16:0)	Hexadecaienoic Acid (C16:2)	Palmitoleic Acid (C16:1w7)	Stearic Acid (C18:0)	Linoleic Acid (C18:2w6)	α-Linolenic Acid (C18:3w3)	Total FAME
C. sorokiniana UTEX 2805	Low strength AD	No Treat (A)	51.96±5.52[a]a[c]	18.78±1.41a	8.63±0.47a	8.76±1.32a	51.29±4.87a	52.43±5.97a	191.85±18.73a
		1DPreTreat (A)	52.47±1.26a	14.85±1.25b	6.13±0.56b	10.24±0.13a	50.79±3.39a	49.92±1.29a	184.41±7.66a
		4DPreTreat (A)	45.68±1.89a	12.24±0.95b	5.30±0.20b	9.79±0.43a	40.98±2.10b	45.44±1.98a	159.44±7.10a
		4DPreTreat (N)	53.48±4.20a	13.00±0.75b	3.11±0.19c	10.42±0.76a	43.72±3.20ab	48.13±3.95a	171.87±12.12a
	High strength AD	No Treat (A)	34.67±5.47a	15.28±2.55b	2.36±0.70b	6.33±1.00a	37.24±6.25a	45.64±8.96a	141.52±24.75a
		1DPreTreat (A)	43.43±12.52a	25.78±6.63a	3.78±0.71ab	8.15±3.85a	53.76±14.92a	51.93±10.96a	186.84±47.20a
		4DPreTreat (A)	33.43±2.06a	21.11±0.57ab	3.96±0.47ab	7.19±0.78a	40.52±1.26a	45.53±1.21a	151.74±3.25a
		4DPreTreat (N)	31.46±4.70a	20.58±1.64ab	4.73±0.62a	7.356±0.64a	37.55±3.99a	42.95±6.19a	144.62±16.62a
A. protothecoides UTEX 2341	Low strength AD	No Treat (A)	3.88±3.50ab	N.D.[d]	N.D.	N.D.	4.78±4.14a	75.82±4.56a	84.48±11.35a
		1DPreTreat (A)	0b	N.D.	N.D.	N.D.	7.47±0.13a	68.63±1.08a	76.10±6.95a
		4DPreTreat (A)	5.08±0.46a	N.D.	N.D.	N.D.	9.76±0.80a	78.74±8.30a	93.58±1.79a
		4DPreTreat (N)	5.22±0.33a	N.D.	N.D.	N.D.	8.68±0.90a	82.58±8.90a	96.47±0.98a
	High strength AD	No Treat (A)	N.A.[b]	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
		1DPreTreat (A)	2.01±3.48a	N.D.	N.D.	N.D.	8.82±0.27b	75.49±3.43a	86.32±5.03a
		4DPreTreat (A)	0a	N.D.	N.D.	N.D.	11.80±0.99a	74.51±5.81a	86.31±2.58a
		4DPreTreat (N)	3.37±2.92a	N.D.	N.D.	N.D.	10.73±1.23ab	77.12±9.61a	91.22±7.15a

[a] Value after ± is standard deviation (n = 3, biological replicates).
[b] Not available. There was no *A. protothecoides* harvested in high strength AD without pretreatment due to high algal inhibition.
[c] Within a row, same letter means no statistical significance at < 0.05 based on Tukey's test.
[d] No FAME detected.

4.3.3 Nutrient removal

4.3.3.1 Nitrogen uptake and removal

Both *C. sorokiniana* and *A. protothecoides* showed excellent ability to assimilate ammonium nitrogen (Figure 4.5). The maximum ammonium uptake rate of both algal strains was around 30 mg/L/day, and higher ammonium assimilation occurred in bioreactors with higher algal biomass production. As expected, the nitrogen removal rate (nitrogen concentration reduction rate in AD) was higher than the uptake rate in nearly all cases. This observation indicated that there were other pathways besides algal assimilation (such as volatilization) which

lowered the ammonium concentration in AD. Nitrification was negligible in both digestate types (Figure 4.2). Nitrifying bacteria are generally inhibited above 350 mg/L ammonium (Kim et al., 2008).

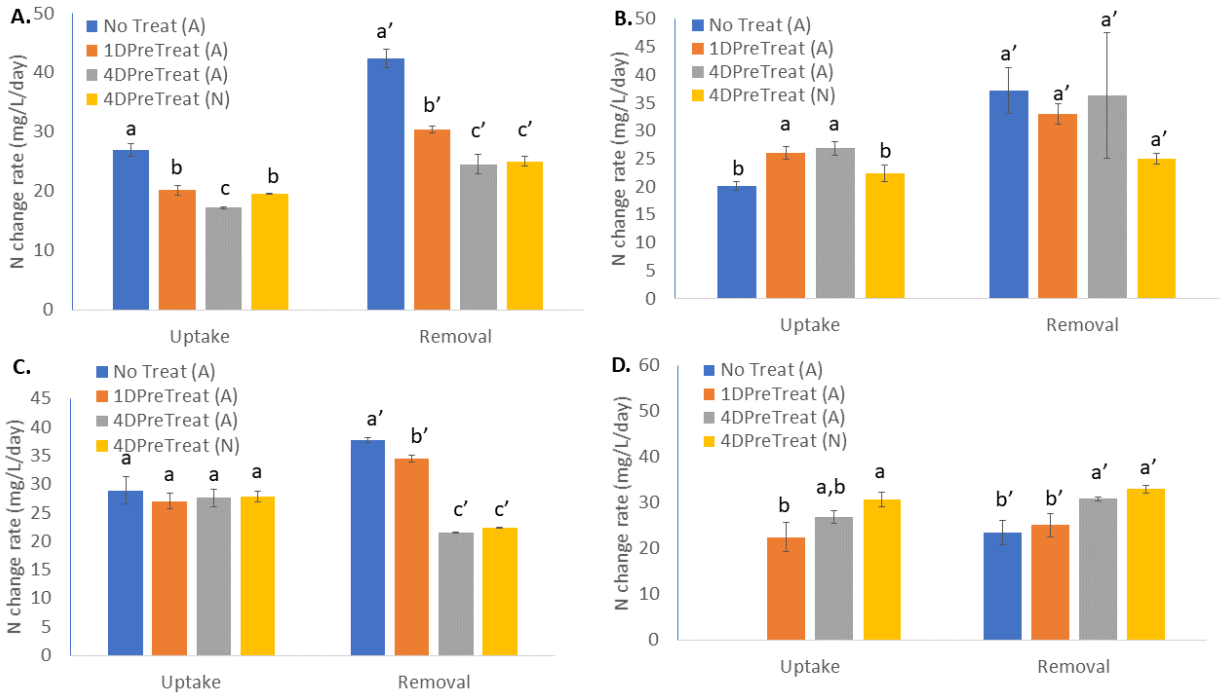


Figure 4.5 The changing rate of ammonium nitrogen in A) *C. sorokiniana* cultured in low strength AD; B) *C. sorokiniana* cultured in high strength AD; C) *A. protothecoides* cultured in low strength AD; D) *A. protothecoides* cultured in high strength AD.
NoTreat(A): algae cultured in sterile filtered (0.2µm) AD without pretreatment (axenic condition); 1DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 24 hours activated sludge (AS) pretreatment (axenic condition); 4DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 96 hours AS pretreatment (axenic condition); 4DPretreat(N): algae cultured in clarified (0.7µm) AD with 96 hours AS pretreatment (non-axenic condition). Uptake represents the ammonium nitrogen that was assimilated into the algae cells. Removal represents the reduction of ammonium nitrogen in the spent media. Note: There was no A. protothecoides harvested in high strength AD without pretreatment due to high algal inhibition, ammonium nitrogen uptake was not able to be measured in that case. Error bars represent the standard deviation (n = 3, biological replicates). Different letters indicate statistical significance at < 0.05 based on Tukey's test.

Similar to the growth results, AS pretreatment decreased the algal nitrogen uptake rate by *C. sorokiniana* on low-strength AD (Figure 4.5A). No difference was observed in the 5-day average rate of ammonium nitrogen uptake when *A. protothecoides* was cultured in low strength AD (Figure 4.5C). This result was confounded by the depletion of ammonium nitrogen from the digestate in 4-day pretreated cultures after 90 hours (Figure 4.6). This also can explain the lower

total ammonium removal in the 4-day pretreated cultures (ammonium had already been partially removed during pretreatment, as shown in Figure 4.6), thus there is less potential for removal in the algal stage.

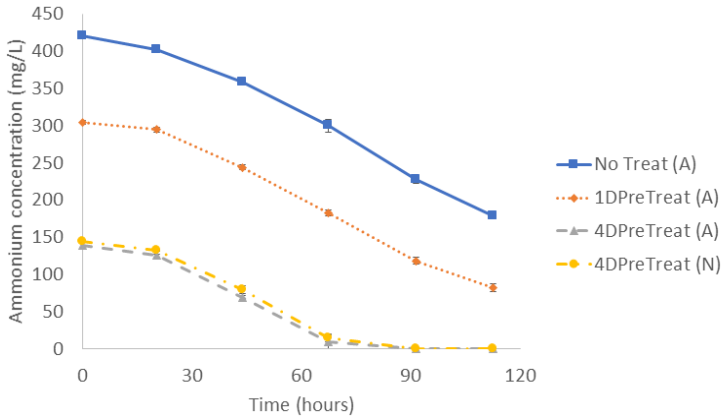


Figure 4.6 Ammonium depletion in *A. protothecoides* cultures grown on low strength digestate. Error bars are standard deviations based on 3 biological replicates.

The high-strength AD had higher initial ammonium concentrations, but experienced similar removal and uptake rates as in the low-strength digestate (Figure 4.5B, D). This is because algal nitrogen uptake is correlated with growth and growth rates were similar between high and low-strength digestate. This also explains why pretreatment improved nitrogen uptake in most cases. The nitrogen content of the biomass, however, exhibited little difference among treatments (Table 4.4). The extent of ammonia volatilization during pretreatment and during algal treatment clearly complicated the story around total ammonium removal. There was no pattern for *C. sorokiniana* cultures: high removal was observed even in untreated digestate, despite slower algal growth in these cultures. The ammonium removal trend followed the nitrogen uptake trend in *A. protothecoides* cultures on high-strength digestate: pretreatment and the presence of bacteria were helpful. Ayre et al. (Ayre et al., 2017) also cultured algae on full-strength anaerobic digestate and found that algal ammonium-N uptake rates were on the order of 1-1.5 mg/L/d which were an order of magnitude lower than those observed in this study (17-30

mg/L/d). Ayre et al. did not use any pretreatment method but maintained ammonium levels that were similar to the digestate in this study. Many other researchers have used digestate dilution to increase growth and thereby ammonium uptake. Franchino et al. (Franchino et al., 2016a) observed ammonium-N removal rates of 13-22 mg/L/d from piggery digestate diluted 10-20 fold with water. Likewise, Prandini et al. (Prandini et al., 2016) observed 11-21 mg/L/d ammonium-N removal from 16.7-fold diluted swine anaerobic digestate using *Scenedesmus* cultures. Algal assimilation rates are expected to be lower than total removal rates. Our pretreatment approach led to 17-30 mg/L/d ammonium-N assimilation rates without the use of dilution water, underscoring the potential effectiveness of this approach.

Table 4.4 Total Nitrogen content in dried algal biomass

Nitrogen Content %		No treat (A)[a]	1DPreTreat(A)[b]	4DPreTreat(A)[c]	4DPreTreat(N)[d]
C. sorokiniana UTEX 2805	Low strength AD	6.01±0.23[e]ab	5.68±0.28b[g]	5.87±0.20ab	6.28±0.09a
	High strength AD	7.95±0.65a	7.24±0.37a	7.32±0.24a	7.66±0.49a
A. protothecoides UTEX 2341	Low strength AD	7.98±0.67a	7.29±0.33ab	6.58±0.29bc	6.10±0.17c
	High strength AD	N.A.[f]	7.03±0.57a	6.55±0.45a	6.71±0.39a

[a] NoTreat(A): algae cultured in sterile filtered (0.2µm) AD without pretreatment (axenic condition);
[b] 1DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 24 hours activated sludge (AS) pretreatment (axenic condition);
[c] 4DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 96 hours AS pretreatment (axenic condition);
[d] 4DPretreat(N): algae cultured in clarified (0.7µm) AD with 96 hours AS pretreatment (non-axenic condition).
[e] value after ± is standard deviation (n = 3, biological replicates).
[f] There was no *A. protothecoides* harvested in high strength AD without pretreatment due to high algal inhibition, neutral lipid content was not able to be measured in that case.
[g] within a row, same letter means no statistical significance at 0.05 level based on Tukey's test.

4.3.3.2 Phosphorus removal

The removal of phosphorus from AD is mostly the result of assimilation into biomass or precipitation. Either way, phosphorus can be recovered in the biomass solids. Faster phosphorus removal coincided with faster algal growth rates. In low-strength AD, *C. sorokiniana* removed phosphorus most rapidly (15.6 mg/L/d) in untreated AD (Fig. 4.7A). This was also the condition with highest growth. When *C. sorokiniana* was grown on high strength AD (Figure 4B), the order of P removal rate reversed where the AS-pretreated AD led to 4-fold faster P removal (12 mg/L/d) compared to untreated digestate (2.8 mg/L/d). These results compared favorably to those of Franchino et al. (Franchino et al., 2016a) who grew *C. vulgaris* on diluted anaerobic digestates and observed 1.4-2.4 mg/L/d P removal. In our study, no difference was observed among pretreatment durations and P removal was not impacted by the presence of bacteria. This last point was notable because this algae strain grew more slowly when bacteria were present, indicating that bacteria may also have contributed to phosphorus removal. Indeed, a recent study has shown that *C. sorokiniana* can support phosphate accumulating bacteria in anaerobic digestate (Bankston et al., 2020b).

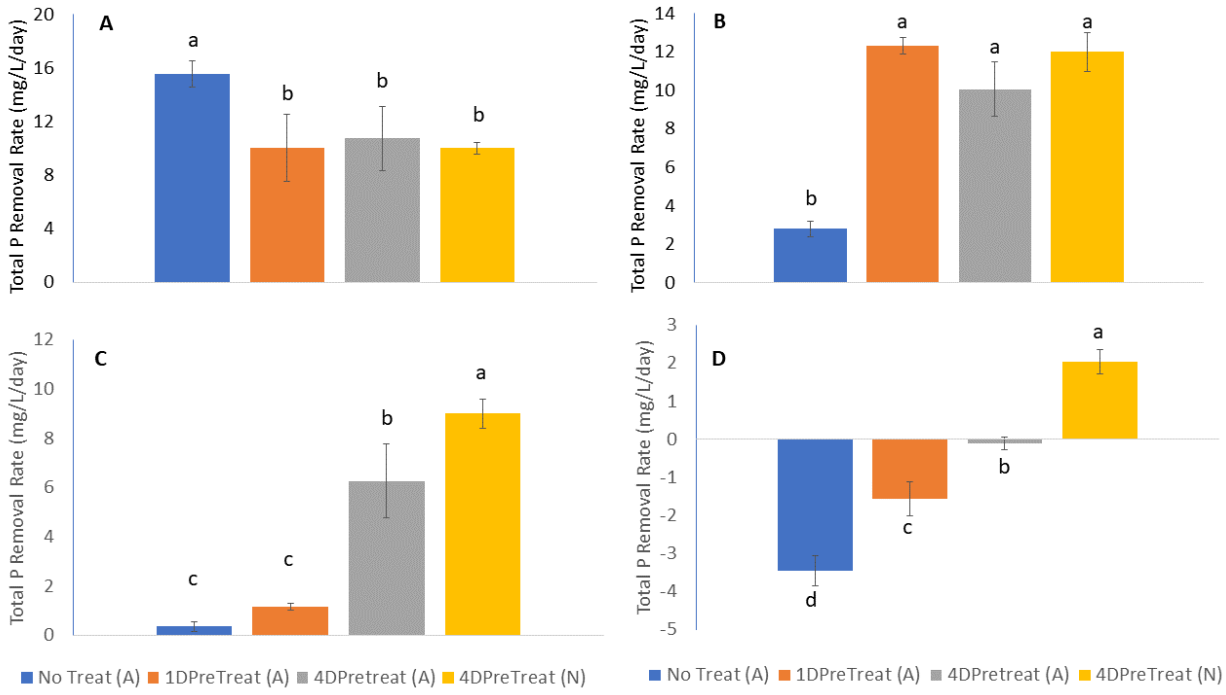


Figure 4.7 Total phosphorus removal rate in the spent media of A) *C. sorokiniana* cultured in low strength AD; B) *C. sorokiniana* cultured in high strength AD; C) *A. protothecoides* cultured in low strength AD; D) *A. protothecoides* cultured in high strength AD.
NoTreat(A): algae cultured in sterile filtered (0.2µm) AD without pretreatment (axenic condition); 1DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 24 hours activated sludge (AS) pretreatment (axenic condition); 4DPretreat(A): algae cultured in sterile filtered (0.2µm) AD with 96 hours AS pretreatment (axenic condition); 4DPretreat(N): algae cultured in clarified (0.7µm) AD with 96 hours AS pretreatment (non-axenic condition). Error bars represent the standard deviation (n = 3, biological replicates). Different letters indicate statistical significance at < 0.05 based on Tukey's test.

The highest phosphate removal rate by *A. protothecoides* (~9 mg/L/day) was observed in cultures grown on 4-day AS pretreated low-strength AD (Figure 4.7C). As discussed previously, *A. protothecoides* was more sensitive to the algal inhibitors in AD. The longer AS pretreatment improved biomass growth by roughly 26% on low-strength digestate, yet the phosphorus removal rate increased by nearly 20 to 25-fold versus untreated digestate. This suggests that pretreatment not only increased algal growth potential but also its ability to assimilate phosphorus. It is known that many algae are able to accumulate polyphosphate and thereby assimilate phosphorus at rates far exceeding their growth rate (Sells et al., 2018). It is not currently known if *A. protothecoides* has the ability to synthesize polyphosphate, however, this

capability has been known for a long time in other green algae including *Scenedesmus* (Rhee, 1973) and *C. vulgaris* (Eixler et al., 2006). In addition, the presence of bacteria increased phosphorus removal from low-strength digestate by 43% in *A. protothecoides* cultures compared to their axenic counterparts. This increase was greater than the 9% increase in growth stimulated by bacteria, underscoring that bacteria may also have played a role in P uptake, similar to the case of *C. sorokiniana*. These results are particularly noteworthy because P removal is one of the main goals of using algae in wastewater treatment (Wang et al., 2016a). Many wastewater treatment plants are currently searching for efficient P removal and recovery systems in order to meet discharge regulations (personal communication, William Kent, Columbus Water Works).

An unusual result was observed for P removal by *A. protothecoides* cultured in high strength AD (Figure 4.7D). A negative P removal (-1.1 mg/L/d) was found in cultures grown on untreated high-strength AD. These cultures died and likely released their phosphorus content into the digestate. Such an outcome was expected to be outweighed by the more robust growth in pretreated cultures but the results were mixed. In the one-day pretreated digestate, a small but negative (-0.5 mg/L/d) phosphate uptake was recorded and is difficult to reconcile with the fact that this culture clearly grew (213 mg/L/d). One possible explanation is the presence of moderately high concentrations of ions in the high-strength digestate that potentially interfered with the total P assay (e.g. Na^+, K^+, SO_4^{2-}, and CO_3^{2-}). However, such interference also would have applied to experiments with *C. sorokiniana* using the same digestate, yet the P uptake rates for that strain matched well to its growth rate. This further indicates that *A. protothecoides* phosphorus metabolism is decoupled from growth, in contrast to *C. sorokiniana*. This latter point merits further investigation of this strain's ability to accumulate polyphosphate as a means of engaging in luxury P uptake.

4.4. Conclusion

Digestate pretreatment was most beneficial when culturing algae on high-strength digestate and/or when using more sensitive algae strains. The robust algae, *C. sorokiniana,* grew well in low-strength digestate without pretreatment whereas pretreatment was helpful in high-strength digestate. *A. prototheco**ides*** was more sensitive to AD growth inhibitors, and a longer pretreatment was helpful on both high- and low-strength digestate. *A. prototheco**ides*** also benefited from the aerobic bacterial community whereas *C. sorokiniana* did not. Pretreatment led to increases in starch production in both strains but had no effect on neutral lipid content or the fatty acid profile.

Chapter 5: Acclimation of an algal consortium to sequester nutrients from anaerobic digestate

Abstract

The objective of this research was to investigate the growth, community composition, and digestate treatment performance of a local algae consortium that was adapted to bacteria-pretreated digestate. The approach was to subculture a local consortium on pretreated dairy manure digestate and then municipal wastewater sludge digestate, allowing the community to adapt before assessing its performance. The adapted consortium was then tested for growth and nutrient removal performance on the digestates and compared to the model organism, *Chlorella sorokiniana*. Dramatic restructuring of the consortium took place when subcultured on the digestates with Scenedesmaceae and Chlorellaceae almost completely replacing Euglena. The consortium was consistently less productive than *C. sorokiniana* (184 vs. 248 mg/L/d in dairy digestate and 32 vs. 48 mg/L/d in municipal digestate, $P<0.01$). Pretreatment increased growth by 81% and 500% for *C. sorokiniana* and the consortium, respectively, in dairy digestate ($P<0.01$), and allowed for algal growth in municipal digestate.

Key words: Adaptation; Aerobic bacteria; Green algae; Nutrient removal, Pretreatment

5.1. Introduction

Growth of microalgae on anaerobic digestate for biomass production and nutrient removal has attracted attention as a means of waste upcycling in the food and agriculture sector (Chuka-ogwude et al., 2020d). Using anaerobic digestate as a growth medium for microalgae cultivation holds the potential to improve agricultural sustainability: the process simultaneously reduces nutrient pollution and results in a stream of protein-rich algal biomass suitable for feed applications (Bauer et al., 2021; Hyman et al., 2021a). A variety of algal species and consortia have been tested in different types of anaerobic digestate (Åkerström et al., 2014; Cheng et al., 2015a; Wang et al., 2010; Yang et al., 2015) proving that anaerobic digestate contains the right blend of macro- and micro-nutrients to support robust biomass production of algae (Chuka-ogwude et al., 2020d; Veronesiv et al., 2017).

However, full strength anaerobic digestates usually cause significant algal growth inhibition for a variety of possible reasons: high (>1,000 mg/L) ammonium concentration (Shen et al., 2020), high (>1 g/L) suspended solids content (Deng et al., 2019), and toxic residual organic compounds (Zhu et al., 2019). Dilution (Franchino et al., 2013), ammonia stripping (Bauer et al., 2021), and biological pretreatment (Sekine et al., 2020; Wang et al., 2019e) are potential methods for alleviating algal growth inhibition from anaerobic digestate. Dilution, ranging from 10-30 fold, is the simplest and most common approach used in research (Franchino et al., 2016a; Krzeminska et al., 2019; Prandini et al., 2016), but it is not efficient economically (increased cost for larger facilities) or environmentally (consumption of large amounts of freshwater). Ammonia stripping requires input of base to raise the digestate pH as well as scrubbing technology to recover the ammonia, increasing capital and operational cost. Moreover, previous research has shown that, in the case of hyper-eutrophic algae, very high levels of ammonia nitrogen (e.g. up to 3000 mg/L) in digestates are not problematic so long as pH is

neutral (Wang et al., 2021b; Wang et al., 2019e), hence stripping alone may not improve algal growth. Moreover, high suspended solids in digestate can be managed through digestate filtration, mitigating light blocking effects on algal photosynthesis (Wang et al., 2019e). Regardless, full strength digestate has proven inhibitory to even these hyper-eutrophic algae (Jiang et al., 2018b), likely due to the presence of toxic residual organic compounds (Wang et al., 2019e; Zhu et al., 2019). In previous research, it was shown that biological pretreatment of digestate with heterotrophic aerobic bacteria can significantly improve the growth (up to 500 mg/L/d) and nutrient removal performance of axenic green algae strains obtained from culture collections (Wang et al., 2021b; Wang et al., 2019e).

Although this biological pretreatment worked successfully on pure cultures of hypereutrophic algae, use of such organisms in real-world treatment systems is unlikely for two reasons: 1) they are non-native organisms and 2) they may be outcompeted by locally adapted algal species. It is therefore important to understand how locally obtained consortia of algae perform when grown on pretreated anaerobic digestate. However, native algal consortia are unlikely to be adapted to the extreme environment of digestate (even when pretreated), necessitating a gradual adaptation and community restructuring prior to testing the consortium's effectiveness in terms of growth and nutrient removal from digestate. Although other researchers have investigated the use of algal consortia for the treatment of anaerobic digestate (Ayre et al., 2017; Pizzera et al., 2019), none have investigated growth of such consortia on biologically-pretreated digestate nor have they investigated how such consortia adapt to the extreme digestate environment. Better understanding of how algal consortia adapt to extreme environments can aid in the treatment of a variety of concentrated waste streams (e.g. undiluted digestate, source separated urine).

The objective of this research was to investigate the growth, community composition, and digestate treatment performance of a local algal consortium that was adapted to pretreated digestate (either dairy manure or municipal sludge digestate). The approach was to subculture this local consortium on two types of pretreated digestate, allowing the community to adapt before assessing its performance. Also of interest were the bacteria taxa that dominated during the aerobic pretreatment process given a previous hypothesis that such organisms eliminate molecules that are inhibitory to algal growth. In this study, it was hypothesized that nutrient-loving extremophiles (e.g., *Chlorella* sp., *Scenedesmus* sp.) would self-select in the pretreated digestates based on the findings of Ayre et al. (2017) and lead to growth and nutrient removal comparable to the successful *C. sorokiniana* monocultures demonstrated in past research. It is also possible that (potentially toxic) cyanobacteria would come to dominate some of these consortia, and it was therefore worth knowing which types were highly abundant. The types of algae are important because the long-term goal is to feed the digestate-grown algae to zooplankton, generating a high-protein fish feed (Hyman et al., 2021a).

5.2. Material and Methods

5.2.1. Collection of native algal consortia

A native algal consortium was collected from a sludge settling container which is connected to the biofloc fish production tank at Auburn University's aquaponics system. A robust algal consortium was growing in the supernatant of this concentrated waste stream and was therefore promising for the treatment of high strength digestate waste. A 500 ml sample of the aquaponics consortium was collected. The collected algae sample was placed on a stir plate under household fluorescent lights (164 μmol/s*m^2 on 16 h:8 h light/dark cycle) at room temperature to increase biomass density. A 2ml sample of this initial consortium was centrifuged

and the pellet was stored at -80℃ for DNA extraction and microbial community analysis. In addition to the successful consortia from aquaponics system, a few other consortia were collected from Auburn University fishponds.

5.2.2. Pretreatment of anaerobic digestate

Dairy anaerobic digestate was collected from a dairy farm in north-central Florida. Municipal sludge anaerobic digestate (MAD) was collected from a mesophilic digester at the South Columbus Water Resource Facility in Columbus, GA. Both digestate types were first centrifuged (4696 x g, 15min) to remove the majority of solids prior to pretreatment. The soluble nutrient composition of the two digestates is shown in Table 1. The pretreatment of digestate was conducted in 500 ml bottles filled with 400 ml centrifuged anaerobic digestate. To these bottles, 4 ml (1% v/v) of activated sludge with a solids content ranging from 0.7% to 1.0% (also collected from the South Columbus Water Resource Facility) was added. More details on the pretreatment process and its rationale can be found in a previous publication (Wang et al., 2021b). Pretreatment reactors were aerated with 200 ml/min air (0.5 vvm) for 96 hours. pH was maintained at 7.5 by adding 3M HCl daily to overcome the continuous increase in pH during pretreatment. After aerobic pretreatment, dairy manure digestate was vacuum filtered through a series of 47 mm Advantec glass fiber filters down to 0.7 μm pore size. The municipal sludge digestate had a greater abundance of small particles, making filtration more challenging than the dairy digestate. The municipal sludge digestate was filtered through 150 mm VWR qualitative-410 filter paper with a pressure filtration system (Advantec, Japan) down to (1 μm). Samples (2 ml) were collected from the pretreatment reactors at the beginning and end of the batch process for DNA extraction and microbial community analysis.

5.2.3. Sub-culturing of consortium on pretreated digestate

The algal consortia, which are a mixture of algae and bacteria, were subcultured first on pretreated dairy anaerobic digestate. The concentration of pretreated dairy digestate was gradually increased with each subculture to allow for a smooth adaptation of the local consortia. The consortia were first cultured in 10% pretreated dairy digestate in 200 ml bottles under fluorescent lights for 5 days. The fish pond consortia died out in the early stages of this acclimation process and were not explored further. The aquaponics consortium showed robust growth and continued in the subculture sequence. 150 ml of the resulting culture slurry was harvested by centrifugation (4,696 x g, 5min). The remaining 50 ml residue slurry was refilled with 150 ml of 20% pretreated dairy digestate. The same 5-day procedure was conducted with 33%, 50%, 67%, 80% and eventually 100% pretreated digestate. Growth on 100% digestate was repeated to ensure a stable culture. A daily 2 ml sample from each reactor was taken for optical density (OD) measurements at 550 nm and 680 nm. A 2 ml sample was collected from the initial and final adapted consortium for DNA extraction and microbial community analysis. This adapted consortium was also used for the subsequent growth and nutrient removal performance testing on dairy digestate.

This dairy digestate adapted consortium was then cultured in full strength pretreated MAD until the algal growth stabilized. This digestate has a higher ammonium concentration (~1,800 mg/L) than the dairy digestate (~900 mg/L). The adapted dairy consortium was used in place of the original aquaponics consortium for subculture on pretreated MAD since it was expected to be more resilient to MAD than the original aquaponics consortium which failed to grow in diluted MAD. The stabilized consortium was semi-continuously cultured in 1L activated sludge pretreated MAD in 2L PET bottles with 0.5 vvm aeration (2% CO_2 supplemented) outdoors for community stabilization prior to testing its treatment performance.

5.2.4. Experimental design for consortium performance test

Adapted local consortia were tested by cultivating them side-by-side with a model green algae strain (*C. sorokiniana*) used in previous research (Wang et al., 2021b; Wang et al., 2019e). The dairy-adapted algal consortium was tested first using dairy digestate as the growth medium. *C. sorokiniana* and the dairy-adapted consortium were separately inoculated into 200 ml hybrid tube photobioreactors (Wang et al., 2019b). These bioreactors were filled with either pretreated or non-pretreated dairy digestate. In all cases, the digestate was vacuum filtered through 0.7 μm pore size filters (45mm, Advantec GF-75). Reactors were maintained with 0.5 vvm aeration (2% CO_2 supplementation) under fluorescent plant growth lights (170 mmol photons/s*m^2 on a 16 h:8 h light-dark cycle) for 5 days similar to past work (Wang et al., 2019e). pH was maintained at 7.2 by adding either 3M NaOH or 3M HCl. The coding used for the treatments were: 1. *C. sorokiniana* in untreated Dairy digestate (C. soro untreated); 2. *C. sorokiniana* in pretreated dairy digestate (C. soro pretreated); 3. Dairy-adapted consortium in untreated dairy digestate (Consortium untreated); and 4. Dairy-adapted consortium in pretreated dairy digestate (Consortium pretreated). A daily 2 ml sample was collected from each photobioreactor to measure OD at 550 nm and 680 nm. The remaining sample was centrifuged (13,201 x g, 5 minutes), and syringe filtered (VWR 0.2 μm Nylon). The supernatant was stored at -80℃ for nutrient analysis. After 5-days of cultivation, algal slurry from each photobioreactor was harvested, washed with deionized water to remove soluble compounds, freeze dried, and weighed. The final biomass concentration was correlated to OD to estimate the growth curve.

A similar experiment was conducted for the performance test on MAD (1. *C. sorokiniana* untreated; 2. *C. sorokiniana* pretreated; 3. Consortium untreated; 4. Consortium pretreated). In this case, however, the consortium that was tested had been adapted to pretreated MAD. All MAD (with or without pretreatment) was filtered through 150 mm VWR qualitative-410 filter

paper (1 μm) with a pressure filtration system (Advantec, Japan). All treatments were conducted with 3 biological replicates.

5.2.5. Water quality analyses

Soluble inorganic macronutrients were analyzed by suppressed ion chromatography on a Prominence Liquid Chromatography (LC) system (Shimadzu). A Dionex IonPac CS16 column (4x 250mm, Thermo Scientific) and Dionex CERS 500e 4mm regenerative suppressor were used to measure cations (Na^+, K^+, NH_4^+, Ca^{2+}, and Mg^{2+}). A Dionex IonPac AS22 column (4x 250mm, Thermo Scientific) plus regenerative suppressor (Dionex AERS 500 carbonate 4 mm) was used for the analysis of anions (Cl^-, NO_2^-, NO_3^-, PO_4^{3-}, and SO_4^{2-}). Nitrogen assimilation into biomass was calculated from the growth rate and nitrogen content of the algal biomass. Total nitrogen in biomass was analyzed with the HACH persulfate total nitrogen assay based on a previously published method (Wang et al., 2019e). Briefly, 1.5 mg dried biomass was homogenized (6.0 m/s, 20s for 3 times) in 1.5 ml deionized water with zirconia/silica beads. This homogenized slurry was oxidized by persulfate and nitrogen content was determined by the HACH TN (low range) assay using a HACH DR900 colorimeter. Total phosphorus concentration in syringe-filtered samples (section 2.4) were analyzed by the HACH Molybdovanadate total phosphorus method (high range) per the manufacturer's instructions. Soluble COD was also measured using the HACH high range assay kit. All nutrient removal rates were calculated from the following equation: $R_{removal} = (C_i - C_f)/days$ where $R_{removal}$ was the nutrient removal rate; C_i was the initial nutrient concentration; C_f was the final nutrient concentration; and days was the algal cultivation time.

5.2.6. DNA extraction and sequencing

Biomass solids collected from the initial algal consortium, the digestate-acclimated consortia, and the pretreatment reactor (pre- and post) were used for DNA extraction with the FastSpin Kit (MP Biomedicals) according to the manufacturer's instructions. The extracted DNA concentrations were quantified using the QuanitFluor dsDNA System (Promega). Targeted amplicon sequencing of the 16S rRNA and 18S rRNA genes were performed by Molecular Research LP (Shallowater, TX). Specifically, the V4 hypervariable region of the 16S rRNA gene was targeted with the 515F/806R primers and a region of the 18S rRNA gene was targeted using the euk1391F/EukB-Rev primers. PCR was performed using the HotStarTaq Plus Master Mix Kit (Qiagen, USA) under the following conditions: 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 53°C for 40 seconds and 72°C for 1 minute, after which a final elongation step at 72°C for 10 minutes was performed. PCR products were checked on a 2% agarose gel. Samples were multiplexed using unique dual indices and pooled. Pooled samples were purified using calibrated Ampure XP beads and the resulting product was used to prepare an Illumina DNA library. Sequencing was performed on an Illumina MiSeq instrument per the manufacturer's guidelines. Sequence data were processed using the sequencing company's proprietary analysis pipeline but a summary of this pipeline follows. Reads were demultiplexed and trimmed using a the company's bTEFAP pipeline. Sequences were joined and those that were <150bp were removed. Sequences with ambiguous base calls were removed. Sequences were quality filtered using a maximum expected error threshold of 1.0 and dereplicated. The dereplicated sequences were denoised: unique sequences, PCR point errors, and chimeric sequences were removed using a UCHIME algorithm. The result was a denoised sequence or zOTU (zero distance operational taxonomic unit). Final zOTUs were classified by using BLASTn against a curated database derived from the NCBI database. The original FASTQ files

from this work have been uploaded to NCBI's SRA under the accessions SRR15570328-SRR15570349 for 16S rRNA sequences and under SRR15584257-SRR15584266 for 18S rRNA sequences.

5.2.7. Data analysis

The 'vegan' package in R (Oksanen et al., 2015) was used to analyze the microbial community structure including indices of diversity (Shannon), community richness, and beta dissimilarity (Bray-Curtis) among experimental treatments. Given that large dissimilarity was observed among experimental treatments, similarity percentage breakdown analysis (SIMPER) was performed to understand which particular zOTUs contributed most to community dissimilarity. In this way, community restructuring could be efficiently assessed and responsible taxa investigated in more detail. Comparisons in algal growth and nutrient removal among different experimental treatments were assessed using Tukey's multiple comparison test using the 'agricolae' and 'car' packages in R. A significance threshold of 0.05 was used.

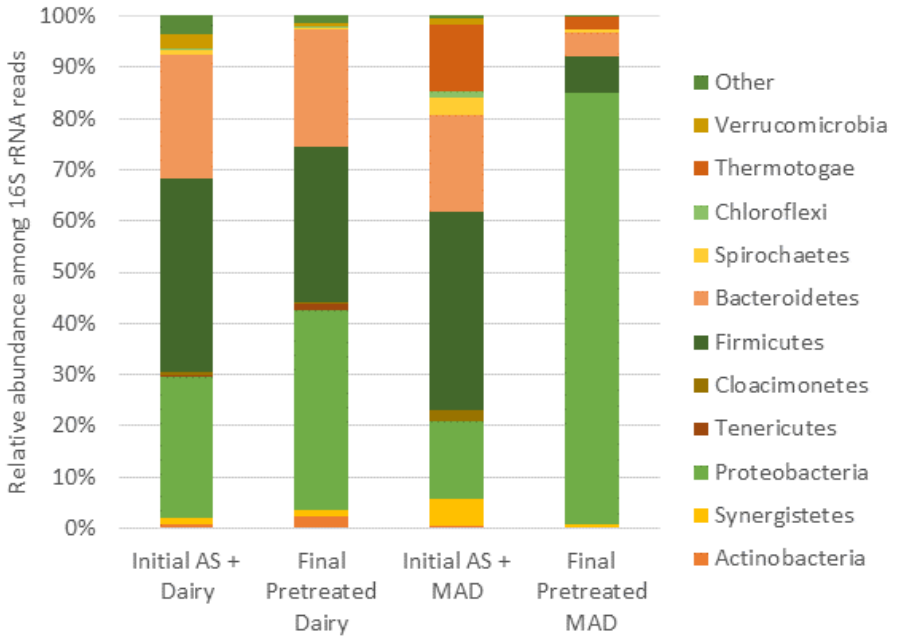
5.3. Results and Discussion

5.3.1. Bacterial community adaptation during pretreatment of anaerobic digestates

The initial microbial community was a combination of bacteria from activated sludge and organisms that were native to the digestate (either dairy or MAD). The bacterial community underwent moderate restructuring at the phylum level during pretreatment of the dairy digestate (Figure 5.1A): there was a 50% increase in proteobacteria and a 15% decline in firmicutes after pretreatment. The community underwent dramatic restructuring in the higher-strength MAD. Proteobacteria abundance increased ~6 fold whereas firmicutes abundance declined 80%. This increase of proteobacteria abundance in MAD was driven largely by an increase in the genera *Acinetobacter* which came to represent >50% of the total prokaryotic organisms in pretreated

MAD (Figure 5.1B). Figure 1B shows the top taxa that contributed most to community dissimilarity among treatments based on SIMPER analysis. Multiple species of *Acinetobacter* thrived during pretreatment of MAD but not in the dairy digestate. This indicates the presence of carbon sources and environmental conditions in MAD (but not in dairy digestate) that this genera prefers. *Acinetobacter* are known to consume a wide range of organic compounds as sole carbon sources (Kämpfer, 2014), including crude oil and other pollutants (Percival & Williams, 2014). This genus may play an important role in removing organic molecules that are inhibitory to algal growth (Wang et al., 2019e). Dairy digestate (which had lower ionic strength than MAD) did not have as strong of a selective effect on prokaryotes with species of *Pseudomonas*, *Clostridium*, and *Rikenella* persisting during the pretreatment process. *Brevundimonas, Pedobacter*, and *Sphingomonas* increased >48 fold during pretreatment of dairy digestate. These non-fermenting, aerobic organisms are generally well-adapted to extreme environments (Göker et al., 2017; Ryan & Pembroke, 2018; Viana et al., 2018), apparently including anaerobic digestate.

A



B

ID	Organism (genus)	Initial AS + Dairy	Final Pretreated Dairy	Initial AS + MAD	Final Pretreated MAD
zOTU4	*Acinetobacter towneri*	0	0.001	0	0.323
zOTU5	*Acinetobacter soli*	0	0	0	0.266
zOTU8	*Pseudomonas*	0.152	0.073	0	0
zOTU10	*Fervidobacterium*	0	0	0.121	0.024
zOTU11	*Clostridium*	0.084	0.079	0	0
zOTU14	*Proteiniphilum*	0	0	0.087	0.017
zOTU15	*Clostridium*	0.059	0.066	0	0
zOTU16	*Bacillus*	0	0	0.086	0.010
zOTU17	*Rikenella*	0.032	0.048	0	0
zOTU18	*Acinetobacter soli*	0	0	0	0.054
zOTU21	*Acinetobacter soli*	0	0	0	0.049
zOTU22	*Brevundimonas subvibrioides*	0.001	0.048	0	0
zOTU23	*Pseudomonas*	0.022	0.007	0	0.023
zOTU32	*Pedobacter*	0	0.035	0	0
zOTU35	*Sphingomonas*	0	0.033	0	0
Total	Fraction of reads	0.350	0.389	0.294	0.765

Scale	<0.002	0.002-0.05	0.05-0.1	0.1-0.2	>0.2

Figure 5.1 Adaptation of the prokaryotic microbial community during pretreatment of anaerobic digestate at the phylum level (A) and either genus or species level (B).
Initial communities were composed of activated sludge (AS) and organisms native to dairy or municipal anaerobic digestate (MAD). Final pretreated digestate represents the evolved community after pretreatment for 96 hours. The heat map shows the fraction of total reads associated with the top zero-radius operational taxonomic units (zOTUs) that contributed most to beta dissimilarity across all conditions based on SIMPER analysis.

5.3.2. Eukaryotic algal community adaptation to pretreated digestates

The eukaryotic microbial community in the native algal consortium underwent complete restructuring after it was adapted to anaerobic digestate through subculturing (Figure 5.2). As expected, eukaryotic community richness declined after cultivation on the digestate as less-fit organisms died off, however, evenness increased as a new set of organisms came to dominate the community (Table 5.1). In fact, only one zOTU, the protozoan *Rhabdostyla*, had >1% relative abundance in all three microbial communities (initial and both acclimated consortia). Euglena occupied >93% of all eukaryotes in the initial algal consortium but completely died out in both digestates. In its place, *Coelastrum* emerged as the dominant algae in both digestate-adapted consortia. Likewise, species mapping to the genera *Scenedesmus*, *Parachlorella*, and *Chlorella* all increased in relative abundance (>2.8 fold) in both adapted consortia. *Chlorella* in particular increased from undetectable (initial) to 2% of the dairy-adapted community and then to 14% of the MAD-adapted community. *Coelastrum* is part of the family Scenedesmaceae and these results support the initial hypothesis that genera related to *Chlorella* and *Scenedesmus* would become dominant in the digestate. This is consistent with the findings of Ayre et al. (2017) who studied algal growth on raw swine digestate. The biggest surprise of the eukaryotic sequencing result was the high abundance of the protozoan *Rhabdostyla*. Such an organism is expected to feed on algae and its relative abundance increased from 2% in the initial consortium to nearly 26% after acclimation on the dairy digestate. This was unexpected because of the very high ionic strength of the digestate. Further acclimation of the community to MAD led to a substantial decline in *Rhabdostyla* abundance to 4% of the eukaryotic community which better aligned with expectations given the high strength of this digestate.

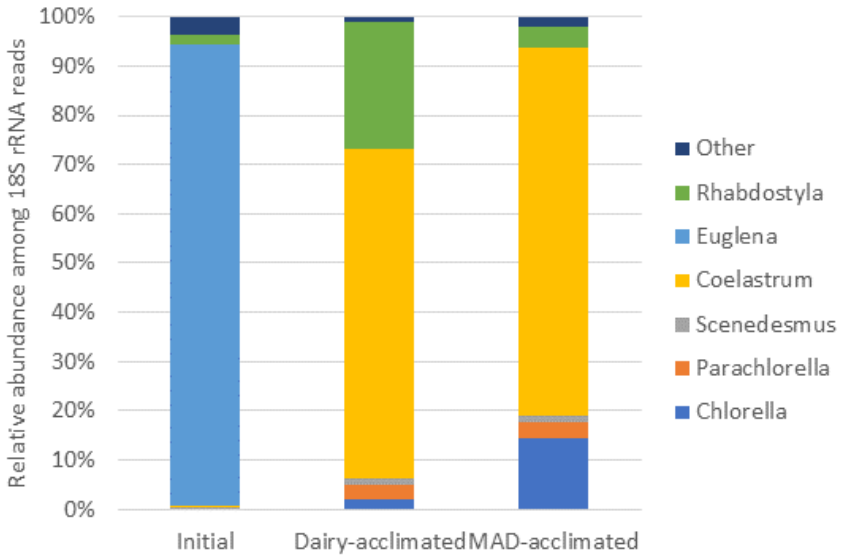


Figure 5.2 Composition of the eukaryotic community in the initial algal consortium and after acclimation to pretreated dairy and municipal anaerobic digestate (MAD). Acclimation was achieved through multiple sub-culture passages on pretreated digestate.

Table 5.1 Composition of anaerobic digestates used in this study

Component	Dairy AD	MAD
COD (mg/L)	5102 (55) *	1937 (91)
Ammonium (mg/L)	860 (8)	1372 (8)
Nitrite (mg/L)	N.D. †	N.D.
Nitrate (mg/L)	N.D.	24 (0.1)
Phosphate (mg/L)	134 (4)	418 (4)
Sulfate (mg/L)	N.D.	3 (1)
Chloride (mg/L)	378 (6)	39 (0.5)
Potassium (mg/L)	571 (2)	98 (0.4)
Sodium (mg/L)	442 (3)	57 (6)
Magnesium (mg/L)	N.D.	17 (0.1)
Calcium (mg/L)	358 (6)	16 (0.3)

* Values in parentheses are standard deviations based on n = 3 biological replicates.
† N.D. means not detected.

5.3.3. The algal consortium's associated bacterial community adaptation to pretreated digestates

Investigation of the prokaryotic microbial community that co-existed with the consortia's eukaryotic community can allow for understanding of changes in cyanobacteria abundance as well as organisms that potentially interact with the eukaryotes. Cyanobacteria are important because they are often perceived as nuisance organisms, potentially capable of producing toxins (Cheung et al., 2013). This could potentially lead to issues if the algal biomass is upcycled back into feed production. Although cyanobacteria are not normally considered to be good food for zooplankton, research has shown that certain *Daphnia* that are capable of subsisting on toxic cyanobacteria (Chislock et al., 2013b).

The results showed a 233-fold increase in taxa mapping to cyanobacteria after acclimation to dairy digestate and a 273 fold increase after acclimation to MAD (Figure 4.3A). Further investigation revealed that this increase was entirely driven by one zOTU corresponding to the genus *Limnothrix*, representing 10% and 14.6% of the total 16S rRNA read counts in the acclimated dairy and MAD consortia, respectively. From 16S amplicon sequencing it is impossible to tell if such an organism produces toxins but a study has now shown that certain strains of *Limnothrix* are capable of making the novel limnothrixin toxin (Lima et al., 2018). This requires further investigation to determine if toxin-producing *Limnothrix* were present and what negative repercussions this could have on downstream use of the algal biomass. The results also showed a roughly 37- to 42-fold increase in bacteroidetes and a corresponding decline in proteobacteria after consortium acclimation to digestate. At the genus level, these changes were driven by a complete die-off of the genus *Aquicella* which represented 48% of bacteria in the initial consortium but could not survive in the digestate (Figure 4.3B). The dominance of *Aquicella* may also explain why prokaryote richness appeared to be lowest in the initial consortium (Table 5.2): it overshadowed low-abundance organisms that later emerged after

cultivation on the digestates. Members of *Aquicella* are known to infect protozoa (Santos et al., 2003) and their die-off may explain why *Rhabdostyla* proliferated in the dairy digestate.

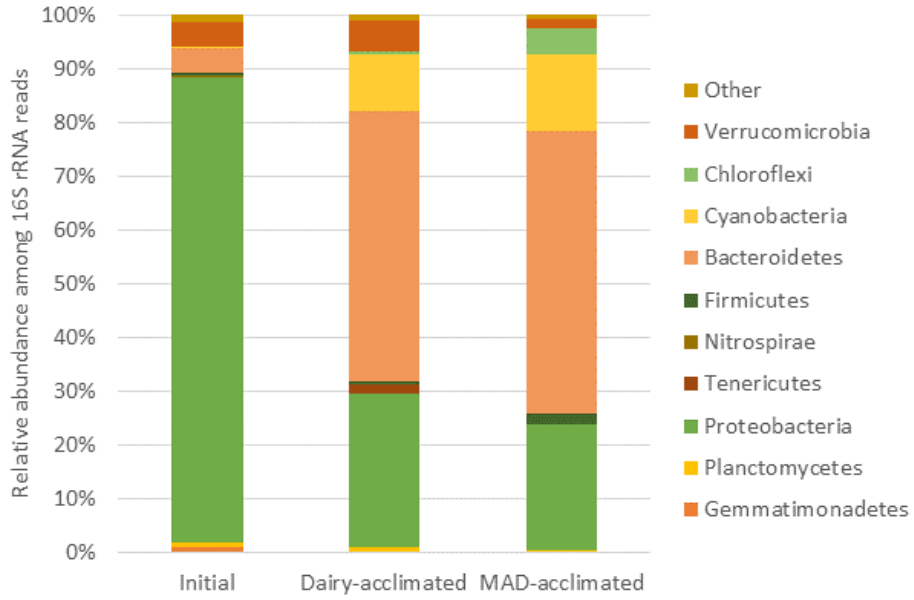
In *Aquicella*'s place, the genus *Emticicia* emerged after acclimation to both the pretreated dairy digestate and MAD (representing 34% of bacteria in the latter). This organism was almost undetectable in all inoculum sources indicating strong selective advantage within the digestate-adapted algal community. *Emticicia* is found in wastewater sludge, soil, and aquatic habitats and is aerobic (Ngo et al., 2017; Saha & Chakrabarti, 2006). There was also complete die-out of the nitrifying phylum, Nitrospirae after digestate acclimation. There were also large losses of the ammonia-oxidizing genera *Nitrosococcus* and *Nitrosomonas* (Ramanathan et al., 2017; Samocha & Prangnell, 2019)

Table 5.2 Community composition indices in the initial and acclimated algal consortia

	Prokaryotic community			Eukaryotic community		
	Shannon	Richness	Evenness	Shannon	Richness	Evenness
Initial Consortium	1.78 (0.09*) b	233 (14) b†	0.33 (0.01) b	1.14 (0.01) a	178 (4) a	0.22 (<0.01) a
Acclimated dairy	3.99 (0.32) a	452 (35) a	0.65 (0.05) a	1.11 (0.17) a	103 (2) b	0.24 (0.04) a
Acclimated MAD	3.20 (0.51) a	389 (34) a	0.54 (0.08) a	1.29 (0.40) a	115 (15) b	0.27 (0.08) a

* Values in parentheses are standard deviations based on n = 3 biological replicates.
† Letters indicate significance at the 0.05 level based on Tukey's HSD test; two values in a column with the same letter are not significantly different.

A



B

ID	Organism (genus)	Initial Consortium *	Dairy acclimated	MAD acclimated	Pretreated Dairy*	Pretreated MAD*
zOTU3	*Emticicia*	0	0.044	0.339	0	0
zOTU7	*Limnothrix redekei*	0.002	0.100	0.146	0	0
zOTU12	*Aquicella*	0.480	0	0	0	0
zOTU13	*Mucilaginibacter gracilis*	0	0.120	0	0	0
zOTU25	*Chloroflexus*	0	0	0.041	0	0
zOTU26	*Dyadobacter fermentans*	0	0.005	0.036	0	0
zOTU28	*Niastella*	0	0.044	0	0	0
zOTU31	*Sphingobacterium*	0	0.005	0.035	0	0
zOTU33	*Anaeromyxobacter*	0	0.008	0.024	0	0
zOTU34	*Luteolibacter*	0	0.029	0.002	0	0
zOTU37	*Pedobacter daejeonensis*	0	0.036	0	0	0
zOTU39	*Dyadobacter*	0	0.023	0.004	0.006	0
zOTU40	*Verrucomicrobium spinosum*	0	0.018	0.009	0	0
zOTU46	*Comamonas testosteroni*	0	0.027	0.001	0	0
zOTU47	*Sediminibacterium*	0.001	0.024	0	0	0
zOTU49	*Crocinitomix*	0	0.025	0	0	0
zOTU52	*Devosia*	0	0.022	0.001	0	0
zOTU53	*Simplicispira*	0	0	0.022	0.003	0
zOTU59	*Lysobacter concretionis*	0	0	0.019	0	0
zOTU60	*Acholeplasma vituli*	0	0.019	0	0	0
Total	Fraction of reads	0.483	0.549	0.679	0.009	0

*Sources of inoculum for the acclimated community

Scale	<0.002	0.002-0.05	0.05-0.1	0.1-0.2	>0.2

Figure 5.3 Composition of the prokaryotic community in the initial algal consortium and after acclimation to pretreated dairy and municipal anaerobic digestate (MAD).
Community at the phylum level (A) and either genus or species level (B) is shown. The heat map shows the fraction of total reads associated with the top zero-radius operational taxonomic units (zOTUs) that contributed most to beta dissimilarity across all conditions, including all sources of inoculum, based on SIMPER analysis.

5.3.4. Growth and treatment performance of adapted algal consortium on dairy digestate

The adapted local consortium was tested in dairy manure digestate with and without pretreatment (Figure 5.4A) and compared to *C. sorokiniana* which showed robust growth in pretreated digestates in previous studies (Wang et al., 2021b; Wang et al., 2019e). The average 5-day biomass productivity of *C. sorokiniana* and the dairy adapted consortium was 248 mg/L/d and 184 mg/L/d, respectively, in the pretreated dairy digestate. Without aerobic pretreatment, the adapted local consortium showed the most severe growth inhibition with only 30 mg/L/d 5-day average productivity whereas *C. sorokiniana* grew more quickly on untreated digestate (137 mg/L/d) but at only 55% the rate of the pretreated cultures. For both *C. sorokiniana* and the local consortium, activated sludge pretreatment led to a significant ($P < 0.01$) increase of biomass production comparing to their corresponding no-treat groups which was also consistent with previous studies (Wang et al., 2019e). The growth rate of the consortium on pretreated full-strength digestate (184 mg/L/d) compares favorably to algal growth rates observed by others on diluted digestate where growth rarely exceeded 100 mg/L/d across eleven studies conducted on a wide range of digestate types (Pizzera et al., 2019; Prandini et al., 2016). Pretreatment was more beneficial to the consortium than it was to *C. sorokiniana* and this was consistent with past findings that less resilient algae benefit more from pretreatment than highly resilient algae (Wang et al., 2021b).

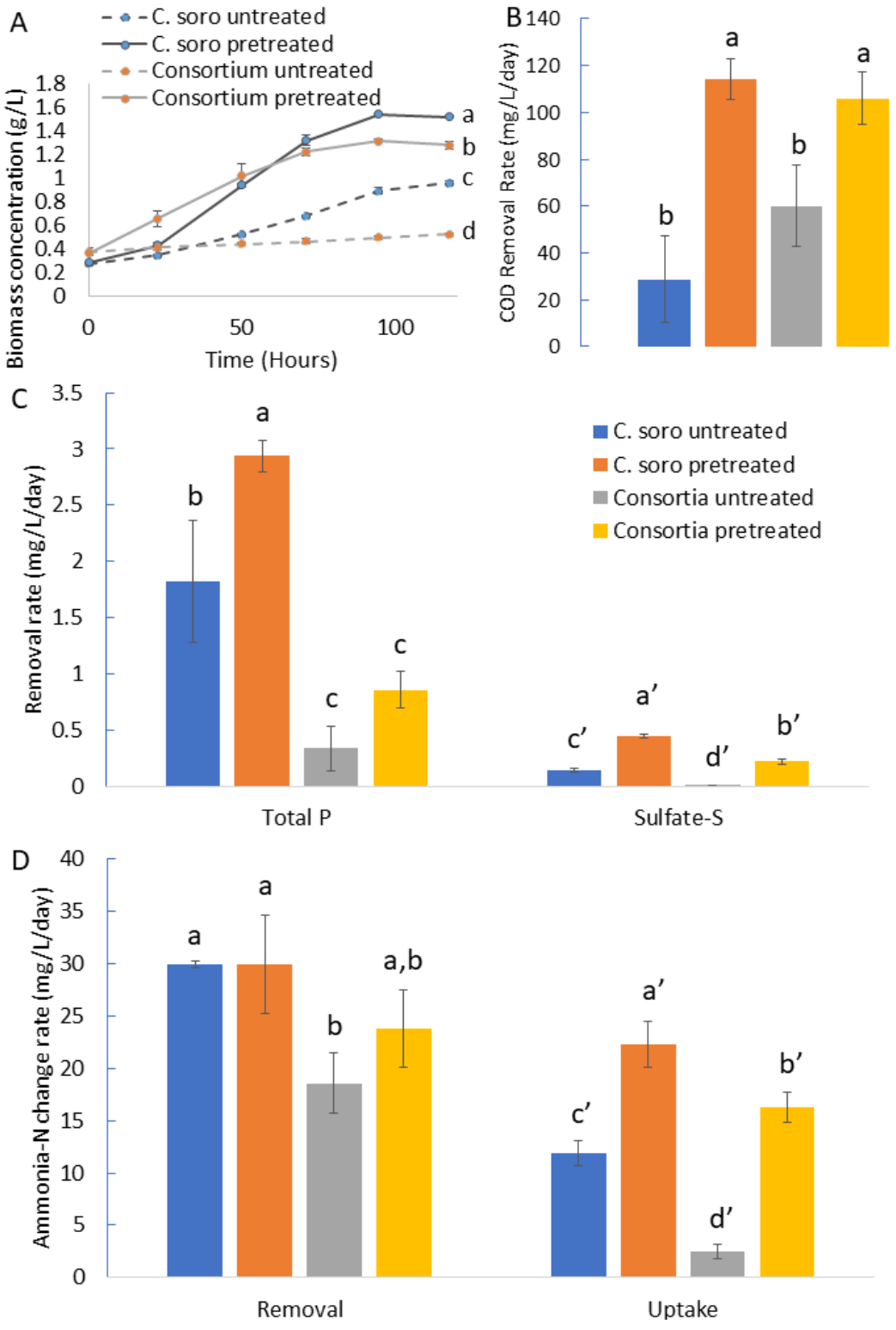


Figure 5.4 Growth curve (A), phosphorus and sulfate removal (B), and nitrogen removal or assimilation (C) by the dairy-adapted algal consortium versus *C. sorokiniana* when grown on dairy digestate.
Both pretreated and untreated digestate were tested with each algae type. Error bars are standard deviation (n = 3 for each condition). Letters indicate statistical significance at the 0.05 level using Tukey's HSD test.

Pretreatment led to 62% higher and 155% higher phosphorus removal rates by *C. sorokiniana* and the adapted consortium, respectively, but only the former was statistically significant ($P = 0.009$). Likewise, pretreatment more than doubled the COD removal rate for both the consortium and *C. sorokiniana* cultures ($P < 0.05$). In both cases, bacteria were present in the algae cultures, so it is unclear to what extent algal mixotrophy accounts for removal of organic molecules in the digestate. Similar to the growth results, *C. sorokiniana* had 3.4 fold greater P removal than the consortium ($P < 0.001$) and 37% greater N assimilation than the consortium ($P < 0.05$). Differences in ammonia-N removal were not significantly different among treatments except for the consortium grown on untreated dairy digestate, which had ~40% lower removal than the *C. sorokiniana* cultures. This removal is a function of cell assimilation, ammonia volatilization, and ammonia-oxidation. Pretreatment of digestate led to roughly double the nitrogen assimilation by *C. sorokiniana* and 6.7 fold higher assimilation by the adapted local consortium because of the higher biomass production rate. This suggests that ammonia volatilization or oxidation occurred at higher rates in non-treated digestate. No evidence of nitrite or nitrate production was observed, however, suggesting volatilization was the main mechanism of ammonium removal similar to past findings (Wang et al., 2019e). There was less ammonia volatilization during the cultivation period on pretreated digestate (less differences between N removal and N uptake). This could be due in part to the more rapid algal assimilation as well as acidification that occurs during ammonium uptake by cells (Bolan et al., 1991). This is undoubtedly a benefit of pretreatment because ammonia-volatilization would be a major source of air pollution if conducted at scale.

5.3.5. Growth and treatment performance of adapted algal consortium on municipal digestate

Municipal digestate, which contains higher ammonium concentration (~1,800 mg/L) and higher suspended solids (higher initial optical density), showed higher algal growth inhibition in all conditions compared to dairy manure digestate for both *C. sorokiniana* and the adapted consortium (Figure 5.5A). However, pretreatment still enabled significantly ($P < 0.001$) higher biomass productivity for both *C. sorokiniana* (48 mg/L/d) and the adapted consortium (34 mg/L/d) compared to untreated groups. *C. sorokiniana* grown on untreated digestate had negative productivity (-8 mg/L/d due to cell death whereas the consortium had no detectable growth. These growth rates for *C. sorokiniana* were substantially lower than those observed in previous studies using ultrafiltered (0.2 μm pore size) pretreated MAD. In those studies, productivity up to 316 mg/L/day was observed (Wang et al., 2019e), and this is likely due to improved light penetration in the ultrafiltered digestate. Poor light penetration in digestate is often cited as a reason for poor algal growth on digestate (Wang et al., 2010) and likely occurred in this case. It is apparent from Figure 5A that pretreatment had some clarifying effect on the digestate as indicated by the lower initial optical density in pretreated cultures. In addition to removal of chemical inhibitors, this could also partially explain the improved algal growth on pretreated digestate.

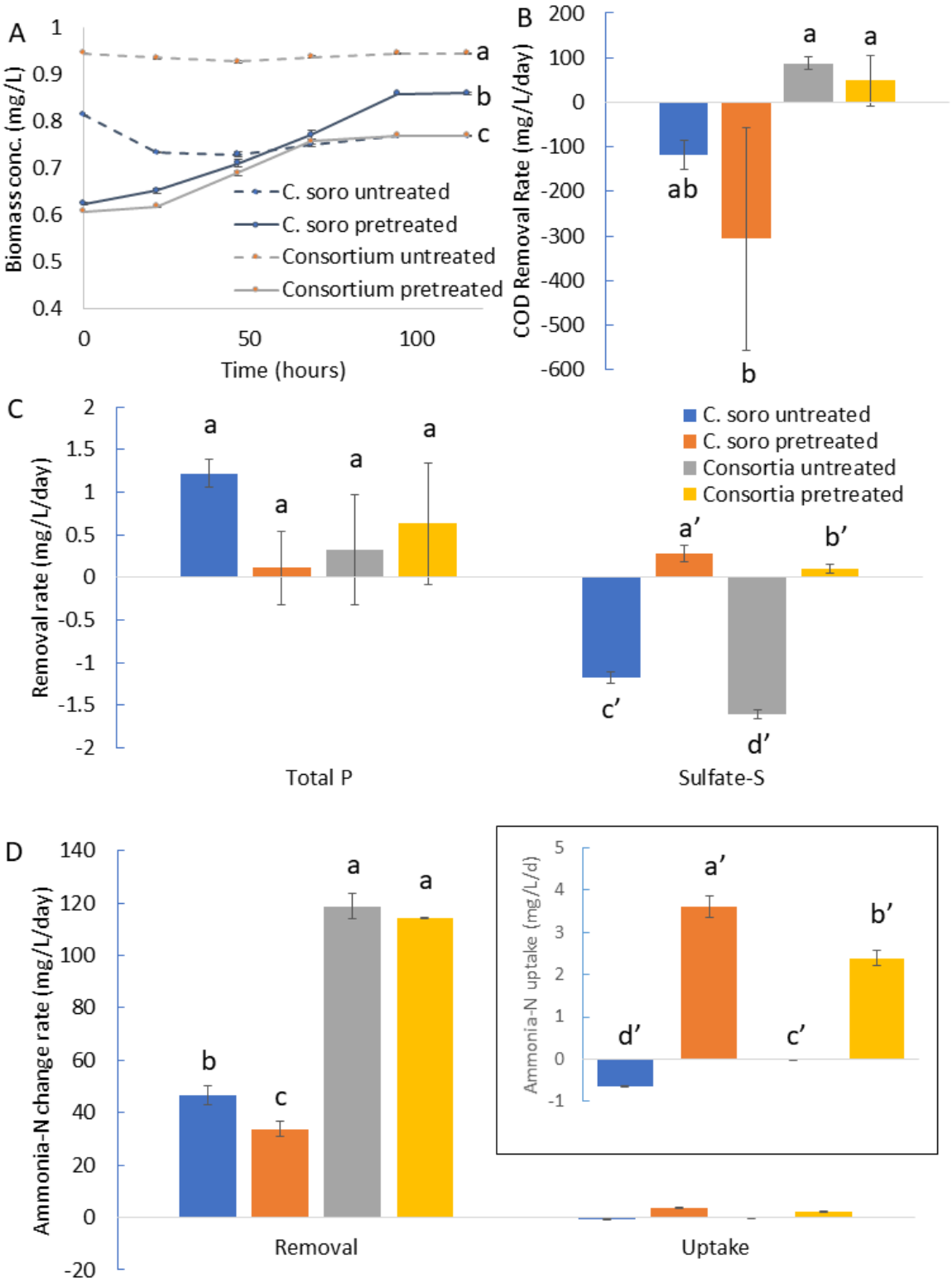


Figure 5.5 Growth curve (A), phosphorus and sulfate removal (B), and nitrogen removal or assimilation (C) by the MAD-adapted algal consortium versus *C. sorokiniana* when grown on municipal anaerobic digestate (MAD). Both pretreated and untreated digestate were tested with each algae type. Error bars are standard deviation (n = 3 for each condition). Letters indicate statistical significance at the 0.05 level using Tukey's HSD test.

Due to the low biomass productivity, the phosphorus removal from MAD was low compared to that observed in the dairy digestate (Figure 5.5B), and no significant difference in removal was observed on pretreated versus untreated digestate. Net COD was released into the wastewater in the *C. sorokiniana* cultures whereas a low rate of removal was achieved by the consortium. Pretreatment, however, had no significant effect in either case. This could be due to the overall low level of culture growth or the presence of recalcitrant COD in the digestate. The untreated digestate treatments had negative sulfate-S removal from MAD for both *C. sorokininana* (-1.17 mg/L/d) and the adapted consortium (-1.6 mg/L/d) which indicates that S mineralization and oxidation exceeded uptake by growing algae. In contrast both pretreated groups showed net sulfate-S removal suggesting that assimilation rates exceeded mineralization and oxidation rates.

The nitrogen assimilation in MAD (Figure 5.5C) was not as obvious as in dairy digestate, also due to the low biomass productivity. The nitrogen assimilation rate in pretreated MAD for *C. sorokiniana* and the adapted consortium were 3.60 mg/L/d and 2.39 mg/L/d, respectively, whereas negative nitrogen assimilation was observed in untreated MAD for both *C. sorokiniana* and the adapted consortium. Nitrogen assimilation in pretreated full-strength digestate (both dairy and MAD) compare favorably to results by others using non-pretreated full-strength digestate sources. Ayre et al. (2017) used full strength digestate with a native consortium and observed only 1-1.5 mg/L/d of N assimilation. This is comparable to the consortium N assimilation on untreated dairy digestate in this study but lower than the 2.4-16 mg N/L/d observed on pretreated digestate.

The ammonia-N removal rate was higher than the nitrogen assimilation rate, consistent with the findings of past studies (Bankston & Higgins, 2020). *C. sorokiniana* had approximately

46 mg/L/d and 33 mg/L/d ammonium nitrogen removal in untreated MAD and pretreated MAD respectively. Since there were no signs of nitrification found in *C. sorokiniana* cultured MAD (both untreated and pretreated), most ammonium nitrogen removal was likely due to ammonia volatilization. Interestingly, the adapted local consortium exhibited nitrification capacity which partially explains the higher ammonia-N removal by consortium cultures compared to *C. sorokiniana* ($p < 0.05$). However, the source of this nitrification capacity was not clear from the sequencing results given the low apparent abundance of nitrifying bacterial taxa in both the adapted consortium and the pretreated digestate. Further investigation is needed to confirm if additional community adaption occurred in the course of experimentation and is the subject of ongoing research.

5.3.6 Practical implications

The practical application of these findings is that pretreatment could enable a locally-derived algal consortium to grow and remove nutrients from two different types of full-strength anaerobic digestate. This has implications for algal biotechnology applications including nutrient recovery, biofuel production, feed production, and carbon sequestration (Fabris et al., 2020). The fact that no dilution water was required is a major advantage, particularly for water-scarce regions. Even if the consortium's performance fell short of the hypereutrophic alga, *C. sorokiniana*, in this short-term test, it is likely that locally-derived consortia will be used in real-world applications of digestate treatment. This is because it is very challenging to maintain pure cultures in outdoor environments across multiple seasons (Godwin et al., 2018), and because use of non-native strains could result in inadvertent microbial pollution. The large-scale outdoor trial of Godwin et al. showed that while consortia could rarely match the performance of the best

algal monocultures, they outperformed monocultures on average and were more resistant to invasion events.

5.4. Conclusion

Subculturing the native algal consortium on pretreated dairy and municipal anaerobic digestate resulted in dramatic algal community restructuring, with Scenedesmaceae and Chlorellaceae replacing *Euglena*. However, the consortium was consistently less productive and generally removed nutrients more slowly than the model organism, *C. sorokiniana*. Pretreatment of digestate using aerobic bacteria improved growth and nutrient removal by *C. sorokiniana* but the improvement was even more dramatic with the consortium. Pretreated digestate also enabled the growth of *Limnothrix* in the consortia (10-14.6% of total 16S rRNA read counts), and further research is needed to prevent toxin-producing organisms from thriving in this environment.

Chapter 6: Conclusion and Future Scopes

6.1 Summary

Algal growth inhibition in anaerobic digestate was widely observed in a variety of research including the studies discussed in this dissertation. Lab scale studies could easily apply dilution as a primary pretreatment for minimizing the impact from algal inhibitors, but dilution approach would never gain market interest in large industrial scales for either algal biomass production or digestate nutrient removal. Therefore, non-dilution pretreatment approaches need to be developed for algal-digestate treatment systems.

This dissertation showcases the development of an effective biological pretreatment process for alleviating algal growth inhibition from undiluted anaerobic digestate. An ammonium tolerant algal strain (*C. sorokiniana*) showed significant increases in algal biomass production and nutrient removal after aerobic bacteria pretreatment when cultivated (under axenic and non-axenic conditions) in undiluted anaerobic digestates. The result not only proved the effectiveness of aerobic bacteria pretreatment but also narrowed the possible algal growth inhibitors in anaerobic digestate by excluding the impacts from ammonium, turbidity, and heavy metals. There are a variety of factors which contribute to the effectiveness of aerobic bacteria pretreatment on anaerobic digestate for algal growth. Generally, longer (4-day) aerobic bacteria pretreatment was mostly observed to have better outcomes than shorter (1-day) pretreatment. Because there was significant shifting of bacteria community during aerobic bacteria pretreatment, it required more time to stabilize the functioning bacteria community. Also, there was a clear difference between different strains of microalgae. *C. sorokiniana*, a high ammonium-tolerant algae, performed better in high strength digestate (MAD) while *A.*

protothecoides performed better in relatively low strength digestate (BMAD). This finding suggested that different types of algae are likely to be selected and can thrive in different types of digestates. Moreover, the presence of bacteria in the algae culture did not seem to have strong impact in most cases and in fact led to benefits in the *A. protothecoides* culture. This is likely due to the thiamine-auxotrophic status of this organism.

The adaptation and growth of local algal consortium in aerobic bacteria pretreated anaerobic digestate sheds more light on the possibility of having a large scale algal-digestate treatment system. Adaptation of a local consortium to anaerobic digestate led to a complete restructuring of the algal community from the initial Euglena dominated consortium to the final Coelastrum and Chlorella dominated consortium. Moreover, the aerobic bacteria pretreatment also significantly ($p < 0.05$) alleviated the growth inhibition for the adapted consortium.

6.2 Limitations and Future scopes

Although aerobic bacteria pretreatment has been proven effective, several critical mechanisms behind this process remains unclear.

1. Lack of knowledge about inhibitors.

This dissertation only narrowed the target list of inhibitory compounds. However, there is not enough information showing evidence that the algal inhibition in the experiments were caused by a specific set of organic compounds.

2. Lack of knowledge on the bacteria pretreatment mechanism

The dissertation showed a massive shifting of bacteria community before and after pretreatment, but what was causing this community change and how this was related to the alleviation of later algal inhibition was not clear.

3. Lack of knowledge on the interactions between microalgae and bacteria

This dissertation mentioned about suppressed nitrification in chapter 3 due to high ammonium concentrations. However, this study did not focus on the interactions after the pretreatment processes. It would be interesting to test how the presence of algae in anaerobic digestate impact other wastewater microbes' activity such as nitrifiers, denitrifiers, and polyphosphorus accumulating organisms.

4. Lack of large-scale and long-term testing

All experiments included in this dissertation were conducted in lab scale photobioreactors over short time horizons. Work is underway on a long-term outdoor study based on the results of this dissertation.

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