Enhancement of Nutrient and Organic Removal from Poultry Litter Using Anaerobic Digestion and Subsequent Algal Growth

by

Elizabeth M Bankston

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 3, 2019

Keywords: anaerobic digestate, poultry litter, algae, nutrient removal, COD removal, activated sludge

Approved by

Brendan Higgins, Chair, Assistant Professor
Jeremiah Davis, Associate Professor & Associate Director National Poultry Technology Center
David Blersch, Associate Professor
Yucheng Feng, Professor

Abstract

Research has indicated anaerobically digesting poultry litter can recover energy and reduce pathogen concentrations, which can be used as a nutrient rich media to support algal growth and simultaneously further treat the effluent of ammonium, phosphate, and COD. However, interactions between wastewater microbes and algae are only partially understood. To further understand algal-microbial interactions, algal growth was tested on digestate treatments in 300mL indoor batch reactors. There were three treatments – algae grown on sterile digestate, algae grown on non-sterile digestate, and digestate grown alone. Digestate bacteria did not significantly affect the growth of two *Chlorella sorokiniana* strains, but it did enhance *Auxenochlorella protothecoides* growth. Starch content in both *C. sorokiniana* strains increased 17 to 38% when grown on digestate, regardless of microbial presence. Adding aerobic bacteria to algae and digestate microbes facilitated COD removal and increased the capacity of aerobic bacteria to remove COD as well as carry out nitrification reactions. Combining aerobic and digestate microbes with algae enhances nutrient and COD removal through nitrification.

Acknowledgments

I would first like to thank my thesis advisor Associate Professor Brendan Higgins of the Biosystems Engineering Department at Auburn University. Dr. Higgins was always available whenever I ended up getting lost in the 'cloud' or had a question about my research or writing. He consistently allowed this paper to be my own work but steered me in the right the direction whenever he thought I needed it.

Many thanks to the Alabama Agricultural Experiment Station (HATCH) for providing the funding received towards my thesis.

I greatly appreciate the support received from my fellow graduate and undergraduate lab-mates. Thank you for your camaraderie, friendship, and support from time spent in and out of the lab.

Finally, I must express my very profound gratitude to my parents, Boyd and Regina, and to my brother, Holland, for providing me with endless support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AD	Anaerobic Digestate
COD	Chemical Oxygen Demand
AS	Activated Sludge
VS	Volatile Solids
TS	Total Solids
OD	Optical Density
DW	Dry Weight
PCR	Polymerase Chain Reaction
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
qPCR	Quantitative Polymerase Chain Reaction
NTC	No Template Control

1.0 Introduction

In 2016, the U.S. Department of Agriculture estimated that the U.S. poultry inventory, which includes broilers, layers and turkeys, produces approximately 550 million tons of manure each year (Coker, 2017). In Alabama, broiler production makes up 60% of Alabama's livestock production and Alabama itself supplies 12% of the nation's broiler products (NSTATE LLC, 2017). Poultry production and processing adds $15 billion to Alabama's economy (ALFA Farmers and Alabama Farmers Federation, 2015); thus determining how to best manage poultry litter manure is relevant from an optimization stand point as well as an environmental one. Currently there are two primary methods the industry uses for managing poultry litter: one is application to land as a fertilizer, the other is to compost it (Kelleher et al., 2002; Moore et al., 1995). Compost can be used as an organic fertilizer, soil amendment or potting media for gardens (Moore et al., 1995). Often agricultural wastes, like poultry litter, are applied based on their nitrogen content, thus more phosphorus is applied than necessary (Power et al., 2000; Preusch et al., 2002). Both methods can cause nutrient loading problems in the surrounding watershed and they also have the potential to cause bacterial or viral pathogen levels to rise in surface waters (Mallin and Cahoon, 2003; Nicholson et al., 2005).

In recent years, anaerobic digestion of agricultural wastes has been studied as an effective way to remove nutrients and simultaneously obtain a useable biogas (Kelleher et al., 2002). Anaerobic digestion converts complex molecules, such as proteins, carbohydrates & lipids, into simple molecules like amino acids, starch & sugars, and fatty acids. As a byproduct of the

anaerobic digestion process, a biogas is produced which ends up being a mixture of carbon dioxide and methane (Chen et al., 2008) (Figure 1). In terms of waste treatment, anaerobic digestion has a low sludge production rate compared to an activated sludge (AS) process and requires less energy to operate the process (Chen et al., 2008). Research has also shown that mesophilic anaerobic digestion can reduce viable human pathogens, such as *Escherichia coli* and *Salmonella* species (Kearney et al., 1993; Resende et al., 2014; Saunders et al., 2012); however, the decline of these pathogenic bacteria varies depending on the pathogenic species, the hydraulic retention time, temperature, pH, and pre-digestion manure particle sizing (Kearney et al., 1993; Saunders et al., 2012; Smith et al., 2005; Ward et al., 2008). Additionally, growing algae on wastewater has been linked with increased fecal coliform and pathogen inactivation (Ansa et al., 2012; Gupta et al., 2016; Muñoz and Guieysse, 2006).

The mineralization of complex biomolecules makes anaerobic digestate (AD) an ideal nutrient-rich media for algal growth. Studies have shown that mixotrophic algal strains are robust enough to withstand the AD's high nutrient concentrations and still yield ~8-14% neutral lipid contents (Singh et al., 2011; Woertz et al., 2009). Oils produced from algae that are high in neutral lipids are desirable for biofuels due to their high potential diesel fuel yields (Pienkos et al., 2011; Zhao et al., 2013). Algae can produce 15-300 times more oil for biodiesel than traditional food crops (soybean and corn) when comparing the growth area required for algae and the area required for traditional crops (Dragone et al., 2010). Some research has shown that algal growth in digestate can lead to higher lipid content than growth on chemical medium, in certain situations (Singh et al., 2011; Woertz et al., 2009). Thus, by coupling anaerobic digestion followed by algal cultivation on the digestate effluent (Figure 1), the process can remove a large fraction of the chemical oxygen demand (COD) remaining in the effluent as well as lowering dissolved nitrogen and phosphorus

concentrations (de-Bashan et al., 2002; García et al., 2000; Higgins et al., 2018; Su et al., 2011). The net effect is a reduction in pollutants entering the environment.

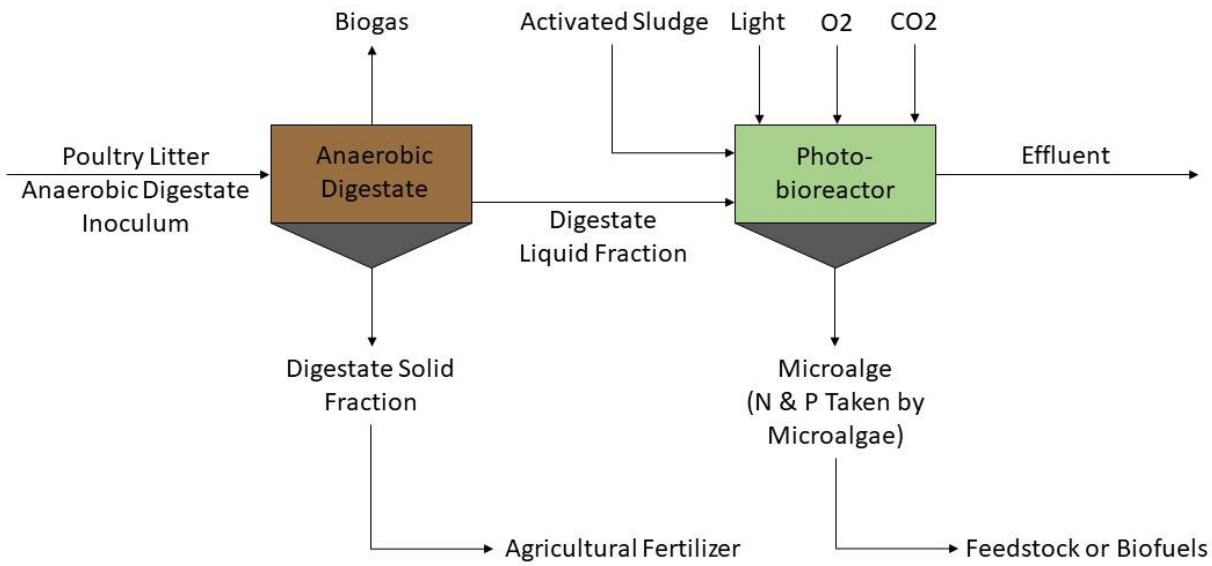


Figure 1: Coupling of anaerobic digestion & algae cultivation to treat poultry litter waste. N represents nitrogen while P equates to phosphorus.

There are currently an abundance of research on using anaerobic digestion to treat agricultural wastes (Borowski and Weatherley, 2013; Higgins et al., 2018; Preisser, 2018; Ward et al., 2008; Y. Zhang et al., 2011); however, there are only 206 operational anaerobic digesters on farms located in the US (American Biogas Council, 2018). Lack of commercial implementation is largely due to the long hydraulic retention times required for process stabilization (Bolzonella et al., 2005; Ziganshina et al., 2015), as well as the variability found in organic conversion efficiencies as a result of different waste compositions (Chaump et al., 2019; Preisser, 2018).

Additionally, inorganic compounds, such as ammonium and phosphate, are poorly consumed during the digestion process which leads to an abundance of these constituents being released into the environment (Higgins et al., 2018). In comparison, algae can take up nitrogen and phosphorus from wastewaters but are ineffective at degrading complex organic molecules (Franchino et al., 2016; Woertz et al., 2009). Other research has demonstrated that algae can grow on swine (Bjornsson et al., 2013; Franchino et al., 2016), cattle (Kobayashi et al., 2013), poultry (Higgins and VanderGheynst, 2014; Kobayashi et al., 2013; Pires et al., 2013; Singh et al., 2011), winery (Higgins et al., 2018), and municipal wastes (Su et al., 2011; Wang et al., 2018; Woertz et al., 2009). Despite previous efforts by Higgins et al., there is still little knowledge about how digester microbes impact algal growth, composition, and nutrient removal from AD. No prior studies have been carried out to investigate how bacteria and algae interact in the treatment of ADs of poultry litter slurry.

The research objective of this thesis was to test the following hypotheses:

- Growing algae in the presence of digestate microbes has minimal impact on algal biomass growth rates and composition given the results of a previous study on winery digestate (Higgins et al., 2018).
- Algae reduce nitrogen and phosphorus nutrient concentrations in the digestate proportional to algal growth.
- Microbes in the digestate help lower COD, including photosynthate secreted by algae, thereby improving water quality.

The experiments conducted resulted in minimal changes in COD. This led to an alternate hypothesis which was subsequently tested:

- Aerobic, rather than anaerobic microbes, are primarily responsible for COD removal from digestate.

Culture experiments were carried out at lab scale using poultry litter collected from a local broiler farm in Eastern Alabama. The collected litter was anaerobically digested in mesophilic conditions (35°C) for 30 days. The AD was made in house due to a lack of a regionally operating anaerobic digester facility for poultry litter. To determine the effect of AD microbes on algae, three experimental treatments were used. Algae were cultivated on sterile digestate, on non-sterile digestate, and non-sterile digestate was grown by itself. Algae was cultivated on sterile digestate to demonstrate that any growth effects observed were a result of the microbial presence in the digestate rather than a result of compounds present in the digestate. Three strains of algae were tested [*Auxenochlorella protothecoides, Chlorella sorokiniana* (UTEX 2714), and *C. sorokiniana* (UTEX 2805)] and 2-4 mL samples were taken every 24 hours for 5-day experimental periods. Samples were analyzed using ion chromatography to determine nitrate, nitrite, phosphate, sulfate, and ammonium concentrations. The harvested biomass compositions were analyzed using the following series of processes and chemical assays: lipid extraction, crude lipid and neutral lipid analyses, starch purification, starch assay, total nitrogen assay, soluble protein extraction, and protein assay. COD was analyzed by HACH DR 900. Lastly, bacterial DNA from the following samples, in the secondary experiment, was extracted, quantified and sent for sequencing at CD Genomics (Shirley, NY): AD inoculum, AS inoculum, algae and AD microbe cultures, AD microbes, algae and AD microbes with AS cultures, and AD microbes with AS cultures.

2.0 Literature Review

2.1 Current Practice in Poultry Litter Treatment and Land Application

It is estimated that by the year 2050 that the world's population will grow to more than 9 billion, with an ever-increasing demand for meat. As of 2018, chicken is the number one protein consumed in the United States, more than 93.5 pounds per capita, and Americans consume more poultry products than any other nation in the world (National Chicken Council, 2019). Currently, the United States also has the largest broiler industry in the world with 17% of the United States' broiler products, about 6.77 billion pounds in 2017 (US Poultry and Egg Association, 2019), being exported internationally to Mexico, Canada, Cuba, Angola, and Taiwan (National Chicken Council, 2019; US Poultry and Egg Association, 2019).

In Alabama, over 1.1 billion dollars in broilers and other meat-type chickens were sold during 2017 (Perdue and Hamer, 2019). Broiler production makes up 60% of Alabama's livestock production and Alabama itself supplies 12% of the nation's broiler products (NSTATE LLC, 2017). Poultry production and processing adds $15 billion to Alabama's economy (ALFA Farmers and Alabama Farmers Federation, 2015); thus, determining how to best manage poultry litter manure is relevant from an optimization stand point as well as an environmental one.

Due to projected meat demand, the poultry industry is projected to increase; thus, disposal of organic wastes, like poultry litter, will need to be managed carefully. The U.S. Department of Agriculture estimated in 2016 that the U.S. poultry inventory, which includes broilers, layers and

turkeys, produces approximately 550 million tons of manure each year (Coker, 2017). Organic wastes are one of the major contributors to nutrient pollution in surrounding waterbodies (Logan, 1993). Nutrients like nitrogen and phosphorus are found in poultry litter in high concentrations and are known to cause eutrophication in waterbodies (Conley et al., 2009; Wang and Wang, 2009). Additionally, the breakdown of poultry litter contributes to greenhouse gases, through methane release (Sánchez et al., 2015), and can provide a pathway for pathogenic contamination, through food and water supplies, to humans and livestock (Manyi-Loh et al., 2016; Nicholson et al., 2005; Spiehs and Goyal, 2007).

One way of disposing poultry litter is through composting. Composting uses aerobic degradation to reduce organic concentrations in wastes. To achieve a stabilized biodegraded material through composting, organic wastes must be left to degrade for 4-6 weeks (Kelleher et al., 2002). Composting is a relatively simple process to facilitate and free of any residual pathogens, (Kelleher et al., 2002). However, it has been found that composting poultry litter can lead to losses in nitrogen, via ammonia volatilization, directly into the atmosphere (Elwell et al., 1998; Kithome et al., 1999). After treating composted poultry litter, the decomposed material still needs to be disposed of, which is often then applied to agricultural fields as a fertilizer (Kelleher et al., 2002). However, using compost as a fertilizer increases dissolved organics in soils, decreased available nitrate for plant uptake, and increased phosphorus concentrations in soils (Wright et al., 2008).

The other and most popular disposal method is to land apply poultry litter as a fertilizer to agricultural lands. Poultry litter typically has large concentrations of desirable nutrients, such as nitrogen and phosphorus, and poultry litter has been found to improve physical and chemical properties in soil (Santos Dalólio et al., 2017). However, poultry litter can vary widely in its

physical and chemical constituents, which can affect broiler health, carcass quality, and the litter's ability to efficiently absorb manure. Disposal of poultry litter via land application distributes essential nutrients to plants but it is often over applied to fields when located near livestock operations. This is because agricultural wastes can only be transported in short distances, typically distances less than 50 miles from the livestock operation, thus the same fields receive agricultural wastes repeatedly (Keplinger and Hauck, 2006). Additionally, poultry litter is applied to fields based on nitrogen content which leads to an excessive application of phosphorus in soils (Edwards and Daniel, 1993). Excessive phosphorus is a result of an imbalance between plant phosphorus demand and the phosphorus applied with the poultry litter (Sims et al., 2000). The excessive phosphorus can runoff into waterbodies and result in eutrophication (Conley et al., 2009; Wang and Wang, 2009).

Land applying poultry litter also provides a contact path for pathogens – like *Escherichia coli*, *Salmonella sp.*, and *Campylobacter jejuni* (Bolan et al., 2010) - to contaminate other livestock and humans (Kelleher et al., 2002; Millner, 2009; Santos Dalólio et al., 2017). Awareness of potential disease transmission through environmental sources associated with agricultural land management practices has increased in recent years for the food supply (Smith et al., 2005). Such pathogenic organisms can cause communicable human diseases, some of which have significant mortality rates, like sever acute respiratory syndrome, Ebola virus, and Nipha virus (Woolhouse and Gowtage-Sequeria, 2005). While antibiotics can prevent infection and reduce pathogen risks in close-quarter poultry production units, using non-therapeutic antibiotics in animal feed can cause pathogen mutation resulting in antibiotic resistance which is transferable and may affect treatment in humans if contracted (Marshall and Levy, 2011; Silbergeld et al., 2008; Smillie et al., 2011). However, it is still unclear on whether or not more intense livestock production and thus

increased risk for contamination leads to an increased infection rate in humans (Liverani et al., 2013).Thus, developing alternative treatment and disposal methods for poultry litter that reduce nutrient build-up in soils and waterbodies but also reduce contact risk for pathogens is of great interest. Anaerobic digestion is an attractive alternative method to land application due to its ability to recover energy from organics and reducing pathogen concentrations.

2.2 Anaerobic Digestion of Agricultural Wastes

In recent years, anaerobic digestion of agricultural wastes has been studied as an effective way to remove nutrients and simultaneously obtain a useable biogas (Kelleher et al., 2002). Anaerobic digestion converts complex molecules, such as proteins, carbohydrates & lipids, into simple molecules, like amino acids, starch & sugars, and fatty acids. As a byproduct of the anaerobic digestion process, a biogas is produced which ends up being a mixture of carbon dioxide and methane (Chen et al., 2008). In terms of waste treatment, anaerobic digestion has a low sludge production rate compared to an AS process and requires less energy to operate the process (Chen et al., 2008).

Anaerobic digestion of agricultural wastes has yet to be implemented widely in commercial settings due to several risks and cost associated with the process. One such risk is associated with stability of the microbial community in the anaerobic digester. The acid forming and methane forming bacteria involved in the digestion process will vary widely in physiology, nutritional needs, growth kinetics, and environmental sensitivity; this means that no two batches of anaerobic digestion will contain the same microbial community and thus methane and biomolecule mineralization rates will vary between batches (Chen et al., 2008). In addition, anaerobic digestion of an organic waste can take anywhere from 20 to 40 days for the digestion process to complete (Bolzonella et al., 2005; Ziganshina et al., 2015), which is not always ideal with high organic waste

streams since larger organic loadings decrease methane conversions and thus organic removal (Bujoczek et al., 2000). Moreover, digestion of poultry litter has distinct challenges compared to digestion of cattle or swine manure. Several substances have been reported to be inhibitory to the anaerobic digestion process: such as inorganic materials, like plastic, sand, and dirt; ammonia; pH; temperature; specific ions like sodium, calcium and magnesium; and competition between anaerobes, sulfur reducing bacteria, hydrolytic bacteria and acidogenic bacteria (Barry Hilton and Oleszkiewicz, 1988; Chen et al., 2008; Ishida et al., 1980; Khalid et al., 2011)

In the Southeastern US poultry litter is primarily composed of bedding material, which is typically made from lignocellulosic materials like wood shavings, peanut hulls, or rice hulls (Santos Dalólio et al., 2017). Poultry litter also contains variable amounts of other compounds such as manure, feedstock, and bird feathers (Kelleher et al., 2002). The digestion process has difficulties breaking down cellulose, pectin and hard proteins, meaning these compounds can remain mostly intact when the digestive sludge is discharged out of the system (Benner et al., 1984). It is thought that bedding material is not readily biodegraded in anaerobic conditions due to the presence of lignin binding and protecting cellulose and hemicellulose, which would otherwise be amendable to hydrolysis (Abdel-Hamid et al., 2013; Singh et al., 2010). This makes treating lignocellulosic rich biomass with anaerobic digestion difficult but not impossible. Successful biogas production from digestion can be achieved by co-digesting poultry litter with other high solid wastes, like food waste and municipal waste, but methane yields still remain low (Brown and Li, 2013)(Borowski and Weatherley, 2013).

In order to further reduce nutrient loads and organics found in poultry litter digestate, it has been suggested that algae be grown on the AD effluent. Algae can be used to remove a range of constituents that may be leftover in digestate, such as coliform bacteria, COD, BOD, nitrogen

phosphorus, and some heavy metals (Abdel-Raouf et al., 2012) and are often used as a tertiary treatment step in some wastewater treatment plants.

2.3 Algal Growth on Digestate

Algae have been used in waste treatment processes to remove organics and nutrients from domestic waste streams for decades (Craggs et al., 1997; De La Noie et al., 1992). Algae is used as an economically feasible option for tertiary wastewater treatment, since it has been estimated that chemical tertiary treatment of nutrients can be up to four times as expensive as a primary treatment operation is (Abdel-Raouf et al., 2012). Culturing algae on wastewater can reduce the need for fresh water and reduce nutrient concentrations remaining in the effluent, making algal cultivation on wastewater a technical and economically feasible option compared to tertiary treatment through chemical means (Pires et al., 2013). In recent years, concern over fossil fuel reserves and the increase in the global demand for energy has led to algae emerging as an attractive alternative for various biofuel production options, like corn and soybean (Chisti, 2007). However, algae grown for biofuels alone has not proven economically feasible due to high startup costs including reactor construction, fresh water costs, and nutrient supply costs (Lundquist et al., 2010; Pittman et al., 2011). Thus, it has been determined that the only way to produce algal biomass for biofuels is to couple the wastewater treatment process and biofuel production (Rawat et al., 2011)(Uggetti et al., 2014).

Additionally, algae have been grown on many different digestate types, including cattle (Kobayashi et al., 2013), swine (Bjornsson et al., 2013; Franchino et al., 2016; Prandini et al., 2016), poultry (Higgins and VanderGheynst, 2014; Singh et al., 2011), and winery (Higgins et al., 2018) digestate. Considering digestate can vary in nutrient composition, algal nutrient removal

efficiencies can vary between digestates (Pires et al., 2013). In this context, anaerobic digestates can be seen as viable sources of nutrients for algal growth (Kelleher et al., 2002) while reducing the need for fresh water in both processes and overall reducing operational costs (Lundquist et al., 2010). Additionally, the energy produced from anaerobic digestion would offset part of the energy required to cultivate and extract lipid content from algal biomass (Uggetti et al., 2014). Although the coupling of anaerobic digestion and subsequent algal growth is appealing, many compounds in digestates have been found to be inhibitory to algal growth. The most common inhibitory compounds have been found to be ammonium (Cai et al., 2013; Cho et al., 2013; Park et al., 2010), heavy metals (Clijsters and Van Assche, 1985; Muñoz and Guieysse, 2006; Wong et al., 1978), and light inhibition (Marcilhac et al., 2014).

Heavy metals are inhibitory to algal grown all in very low quantities (< 45 μM), some of which include lead, zinc, copper, and cadmium. Moreover, such heavy metals can cause health problems like cancer, organ failure, as well as reductions in growth and development (Barakat, 2011). The elements lead, mercury, copper and cadmium all inhibit photosynthesis in algae wither through inhibition of phosphorylation or enzyme activity in the chloroplast (Clijsters and Van Assche, 1985). In fact, some heavy metals or the combination of some can decrease algal productivity by up to 70% (Wong et al., 1978). Heavy metal toxicity is mainly a concern only when growing on industrial waste, which contain heavy metals in concentrations of tens of parts per million.

Digestates are typically dark in color which are known to reduce light penetration and thus limit the amount of light available for algal photosynthesis. Often digestates are diluted to help reduce nutrient concentrations but dilution also helps to negate light-limitations (Marcilhac et al., 2014). Algae can experience up to a 60% decrease in productivity when grown in high optical

density (OD) digestates, in other words turbid digestates (Marcilhac et al., 2014). However, there have been some cases where algal growth was not inhibited by digestate turbidity but by self-shading due to overgrowth of algae in a culture (Guieysse et al., 2002; Uggetti et al., 2014). Overall, light inhibition due to either digestate turbidity or algal shading can reduce algal growth rates which can be mitigated through dilution.

Although there are some exception such as one *C. sorokiniana* strain (Wang et al., 2018), it is generally accepted that high concentrations of ammonium (>1000 mg/L) are inhibitory to algae growth (Cai et al., 2013; Cho et al., 2013; Park et al., 2010). Additionally, at high ammonium concentrations, some *Chlorella* strains have shown reductions in their ability to intracellularly take up ammonium possibly due excess production of chlorophyll and light inhibition from self-shading (Cai et al., 2013; Uggetti et al., 2014). Typically, ammonium inhibition in digestates is reduced by diluting it, although the dilution factor needed can vary between digestates (Cho et al., 2013; Franchino et al., 2016; Prandini et al., 2016).

The bulk of research on co-culturing algae with digestates has been done on cattle and swine wastes; thus, there is little work and understanding on how poultry litter digestate can affect algae. Additionally, most studies only report nutrient reductions when algae is co-cultured with digestate and there is even less knowledge on why a specific sets of algal-microbial communities are effective.

2.4 Algal-microbial interaction

There is a significant body of knowledge within the microbial ecology literature surrounding interactions of algae and bacteria in general (de-Bashan et al., 2002; Gonzalez and Bashan, 2000; Higgins et al., 2018, 2015; Higgins and VanderGheynst, 2014; Mouget et al., 1995;

Su et al., 2011; Wang et al., 2018). It also has been established that bacteria impact algal growth which is important for making algal technologies cost effective: specifically faster algal growth means processing facilities can be smaller which leads to lower costs overall (Davis et al., 2011). However, understanding of how algae interact with microbes found in anaerobic digestate is still poorly understood.

A. brasilense is a plant growth promoting bacterium and is normally associated with terrestrial plant growth promotion. Growth promoting bacterium, like *A. brasilense*, have been found to have a positive effect on microalgal growth and significantly improve nitrogen and phosphorus removal (de-Bashan et al., 2002)(Gonzalez and Bashan, 2000). It is though that the production of plant hormones, such as auxins, in growth promoting bacterium interacts with plant and algal hormone mechanisms to induce cell proliferation and growth; however, for plant growth promoting bacteria to have any affect on algal growth, they need to be held in close association with each other which has been done through co-immobilization of the cells (Gonzalez and Bashan, 2000). However, these studies do not cover algal growth and nutrient removal when dealing with the natural wastewater community.

Higgins et al. (2015) determined the effect of bacterial contamination on mixotrophic algal biomass, and lipid production. They observed that co-culturing *E. coli* and a *Chlorella sp.* can enhance lipid production which was likely due to a combination of the co-culturing process increasing substrate uptake and nitrogen limitations as a result of rapid growth (Higgins et al., 2015). Co-culturing *E. coli* and algae also enhanced lipid production by 210-722% compared to algae grown alone (Higgins and VanderGheynst, 2014). While *E. coli* is more commonly found in wastewater and the effects it has on algae are significant, the *E. coli* used in these studies was from a pure culture. Similar to research done with growth promoting bacteria, *E. coli* used was from a

pure culture rather than included in a wastewater consortium, in order to isolate the effects that just *E. coli* has on algal biomass and composition, which ignores how bacteria behave communally and those communal effects on algal growth. Research done by Higgins et al. also suggests that culturing an *Auxenochlorella sp.* with *E.coli* creates a mutualistic environment for the two organisms though the exchange of hormones and cofactors (Higgins et al., 2016). This study confirmed that *A. protothecoides-E. coli* symbiosis occurred through transfer of cofactors such as thiamine degrading products. However, any symbiosis observed was with pure bacterial cultures inoculated into synthetic wastewater. These results do not further improve knowledge on why native bacterial communities can enhance algal growth. Additionally, this study does not include how *E. coli* symbiosis with *A. protothecoides* influences biomass composition.

This area study has been expanded to include groups of wastewater communities and their effects on algae's ability to treat wastewater. Higgins et al. also cultivated a winery digestate microbial community and discovered that digestate microbes alone can reduce COD levels by 30-60% from their initial concentration (Higgins et al., 2018). It was also found that co-culturing this digestate community and algae resulted in higher nutrient and COD removal than algae grown on sterile digestate or digestate microbes alone. Increased net COD removal was attributed to the bacterial community's ability to consume algal photosynthate, which caused increases in COD when algae was grown on sterile digestate, however, the presence of live bacteria appeared to suppress algal growth in some algae (*C. sorokiniana*) but have no effect in another (*A. protothecoides*) (Higgins et al., 2018).

All works in this field show that growing algae with live bacteria can benefit a waste treatment process by either enhancing nutrient and COD removal, or by increasing biomass growth and lipid production for biofuels. Live wastewater can live in symbiosis with algae by consuming

photosynthate, produced by algae, or secrete cofactors which are necessary for algal growth, all of which enhance algal growth. Work in this thesis aims to confirm that enhanced COD removal and nutrient removal is a result of the presence of live microorganisms consuming algal photosynthate and providing cofactors which enhance biomass growth.

2.5 Aerobic bacterial treatment of wastewater

Aerobic processes include AS process, aerated lagoons, and sequencing batch reactors (Ahammad and Sreekrishnan, 2016). Aerobic treatment of municipal wastewaters is typically done through an AS process. AS removes organic compounds, as COD or BOD, and converts ammonium to nitrate (Ranade et al., 2014). Air is mechanically supplied to the microorganisms in order to metabolize organics as a food source. However, AS processes are expensive due to the large quantities of energy required to supply oxygen (Kassaveti, 2008; Oyewo et al., 2018) and it is less effective at removing nutrients than an anaerobic process (Kassaveti, 2008).

AS processes can partially remove organics and nutrient concentrations, but to achieve full nutrient removal the process requires the feedwater to be recycled through the AS process several times or coupled with another treatment process. While aerobic processes are effective at removing organic carbon from wastewaters, strong organic wastes, like agricultural wastes, are hard to treat aerobically. Organic wastes are hard to treat mainly due to the high costs associated with providing oxygen for aerobic treatment (Ramírez-García et al., 2019). Often aerobic and anaerobic treatment processes are coupled to completely remove nutrients (Ahammad and Sreekrishnan, 2016). In these cases, the anaerobic reactor first digests the organics in the agricultural waste to denitrify nitrate or nitrite in to biogas, followed by the aerobic process which is used to remove organic carbon and oxidize ammonium to nitrite (Bernet et al., 2000).

One of the dominant species sequenced from an AS process is the phylum *Protobacteria*; however, *Firmicutes, Bacteroidetes,* and *Actinobacteria* dominate AS communities (Snaidr et al., 1997; T. Zhang et al., 2011). The phylum *Proteobacteria* contains a range of genera in that from pathogenic bacteria, like *Escherichia, Salmonella*, and *Campylobacter,* to bacteria responsible for nitrogen fixation, such as *Azotobacter*, and nitrification, such as *Nitrosomonas* (Reece et al., 2014). On the other hand, *Bacteroidetes* contains bacteria that often can be found in the guts of humans and animals (Kostic et al., 2013; Reece et al., 2014) but they are also just as abundant in the rhizosphere microbiome (Mendes et al., 2013). *Actinobacteria* can be found in soil and fresh and marine biomes and are commonly responsible for decomposing complex polysaccharides, like cellulose and chitin, and phenolics but also contain some species of pathogens (Větrovský et al., 2014). *Actinobacteria* are also known for their use in the pharmacology industry for their ability to produce secondary metabolites like antibiotics (Manivasagan et al., 2014). Only generic knowledge is known about the *Firmicutes* phylum, specifically that they inhabit a variety of habitats such as human and animal gut biomes (Jumpertz et al., 2011; Kostic et al., 2013) and that *Firmicutes* bacteria are pathogenic to a broad range of plants (Hogenhout et al., 2008; Hogenhout and Loria, 2008), including corn, tomatoes and lettuce, and some insects (Hogenhout et al., 2008).

The initial experiments conducted in this thesis showed that the presence of live digestate microorganisms resulted in minimal changes in COD, unlike what was observed in Higgins et al. (Higgins et al., 2018). Due to these organism's abundance in AS, it is hypothesized that one or several of these species can improve organics removal from digestate effluent. This led to the alternative hypothesis, that aerobic bacteria, rather than anaerobic microbes, are primarily responsible for COD removal which was subsequently tested.

3.0 Methods

3.1 Waste Collection & Characteristics Determination

Poultry litter was used as the substrate for the anaerobic digestion in the following experiments. The AD was created in house due to the lack of a regionally operating AD facility for poultry litter. The poultry litter was obtained from a local broiler farm in Eastern Alabama and stored at room temperature in zip-lock bags in a fume-hood. The poultry litter used is mainly composed of bedding material, which in this case is 100% pine shavings. The litter collected was taken from the top 4-inches of litter laid in the poultry house. In previous laboratory studies, it has been shown that co-digesting a manure source and municipal solid waste can stabilize digestion pH and increase biodegradation of solid organic waste (Hartmann and Ahring, 2005; Wang et al., 2012).Thus, it was determined that co-digesting municipal solids and poultry litter would be ideal for extracting as much nutrients from the waste as possible. The municipal solids used was collected from a mesophilic anaerobic digester's effluent at the south Columbus (GA) wastewater treatment facility. Chaump et al. (2018) determined that an 80:20 volatile solids (VS) ratio of waste poultry litter to municipal sludge inoculum was ideal to maximize total solids (TS), VS, COD and total organic carbon reduction when anaerobically digesting poultry litter.

To obtain an 80:20 ratio, 1 g of poultry litter and municipal sludge were weighed out onto pre-weighed aluminum pans, in triplicate, and dried at 80°C overnight. After which, the samples were cooled in a desiccator for 30 minutes and again weighed. At this point, the samples were

placed in a furnace which was set to remain idle at 550°C for 30 minutes with a ramp-up rate of 30°C/min. Samples were then placed in the desiccator to cool for 15 minutes and re-weighed again. From the weighed values, the average TS, VS, and moisture content were determined for the sample poultry litter and municipal sludge.

3.2 Preparation of Poultry Litter Digestate

It was decided that three 2 L anaerobic digesters would be grown as media stock for the first batch experiment (AD stock1); however, another batch of three 2 L digesters (AD stock 2) needed to be grown for the second and third batch experiment. Each reactor was created to have a total VS content of 10 g/L. Previous research by others (Chaump et al., 2019; Preisser, 2018; and Webb and Hawkest, 1985) showed that SI ratios below 60% had the highest biomethane potential. However, an SI ratio of 80:20 was found to have the largest quantity of biogas per volume of reactor compared to the lower SI ratios (Preisser, 2018). Efficient volumetric biogas production is important for achieving cost-effective anaerobic digestion and is most likely to be commercially adopted. Consequently, an SI ratio of 80:20 was used to prepare digestates in this study. Thus, for each 2 L reactor to have an 80:20 ratio of poultry litter to municipal sludge, 16 g-VS needed to be from poultry litter and 4 g-VS from the municipal sludge. The numerical amount of poultry litter and municipal sludge to be added was calculated from the TS and VS contents determined. It was determined that 22.41 g of poultry litter and 178 mL of municipal sludge would be added to each reactor. The remaining liquid fraction to reach 2 L was supplemented by deionized water. The reactors were closed with an altered lid, which had a one-way release value fitted to it to allow for off-gassing and to prevent back follow. The altered lids were then screwed on and sealed with plumber's tape to prevent gas leakage. In addition, the reactors were wrapped

in foil to prevent gas reductions due to light intensity (Yao, 2016). All reactors were then incubated at 35°C for 30 days; after which, the reactors were stored in a cold room (4°C) until the digestate was needed for the batch culture experiments.

3.3 Experimental Plan for Algal Treatment of Digestate

Three batch culture experiments were conducted, each with a different strain of algae. The strains used for each experiment were as follows: the first batch experiment with *Auxenochlorella protothecoides,* the second with *Chlorella sorokiniana* (UTEX 2714), and the third with another *Chlorella sorokiniana* strain (UTEX 2805). These strains were chosen because they are known to grow well on high-nutrient wastewaters including digestate (de-Bashan et al., 2008; Higgins et al., 2018). *A. protothecoides* was selected for its ability to accumulate neutral lipids and from previous research revealing that the presence of *E. coli* stimulates increased nutrient uptake in this strain (Higgins et al., 2018, 2015; Higgins and VanderGheynst, 2014). *C. sorokiniana* UTEX 2805 was chosen for its ability to accumulate intracellular starches which can be valuable in biofuel and feedstock production (Higgins et al., 2018; Tanadul et al., 2014).

Each batch experiment consisted of one control and three treatments each grown in triplicate. The treatments consisted of sterile axenic algae, algae & AD bacteria, and AD bacteria. This design allowed for testing of the contributions of algae and heterotrophic microorganisms toward treatment of litter digestate. Moreover, the experimental design should reveal any synergistic or inhibitory interactions between algae and microorganisms native to the digestate.

3.4 Cultivation Methods

In order to inoculate the experimental treatments with their respective algal strains, pre-algal cultures were grown to an early exponential phase, about a week before the batch experiment was to commence. The *A. protothecoides* pre-algal cultures were grown on a N8-NH4 chemical medium (Higgins and VanderGheynst, 2014) while the two *C. sorokiniana* pre-algal cultures were grown on a N8 chemical medium (Tanadul et al., 2014) (see Appendix Table 1 for data). Pre-algal culture bottles and mediums were sterilized via autoclave prior algae inoculation.

All batch cultures were grown in 300 mL cylindrical tubes with 200 mL of their respective mediums. Each of the treated digestate mediums was diluted to a 3:1 ratio of deionized water to digestate; as a previous batch experiment showed that the UTEX 2341 algae were unable to grow on any of the treated, full strength digestates. The pre-culture mediums specified for each algal strain were used for the experimental controls in each batch experiment. All tubes used in the batch experiments and control mediums were sterilized with an autoclave prior to adding the digestate mediums and algae inoculation.

Equal volumes of the liquid AD fraction was removed from each reactor in a batch (AD stock 1 or 2) and then the removed volumes were mixed together before being treated. There were two methods for processing the digestate: a clarification method and step-filtration method. The clarified digestate was used for the algae & AD bacteria, and the AD bacteria treatments; while the step-filtered digestate was used for the sterile axenic algae treatment (Figure 2). The clarification method involved centrifuging the liquid AD fraction for 15 minutes at 5,000 rpm followed by vacuum filtration through a No2 Whatmann filter to remove any large suspended particles remaining after centrifuging. The step-filtration method was also initially processed as the digestate was in the clarification method but then further treated by step-filtering the digestate

through the following filters: 0.8 µm cellulose acetate, 0.45 µm cellulose acetate, twice through a 0.2 µm cellulose acetate filter, and a sterile 0.2 µm filter apparatus. The final treated digestate mediums were stored at room temperature overnight or at 4°C for an extended period of time.

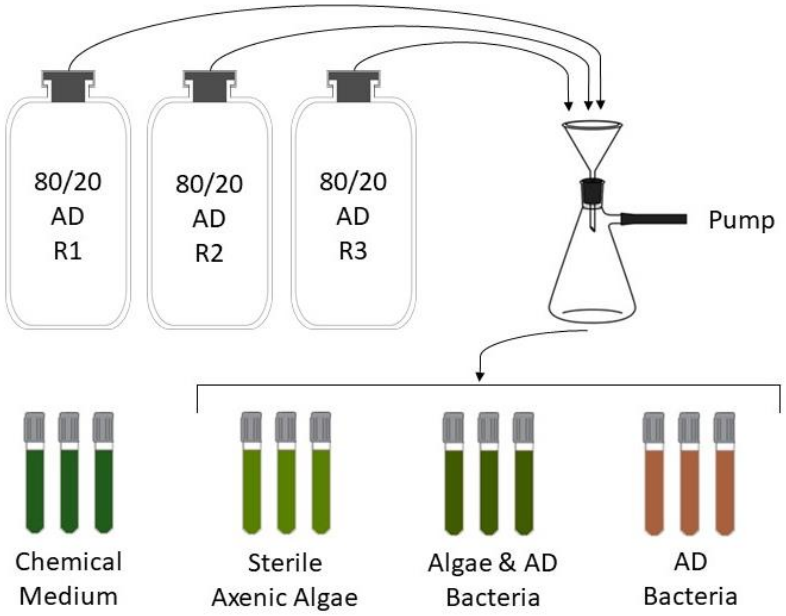


Figure 2: Pre-experimental setup for digestate treatment.
AD represents anaerobic digestate, while 80/20 references the percentage of the reactors' volatile solids as poultry litter to municipal sludge inoculum. R1, R2, and R3 indicates the individual reactors created within a batch.

The experimental cultures were grown in a room temperature water bath, at a 16:8 light-dark cycle and with aeration set to 0.5 vvm and 2% CO2 sparged. Culture pH was adjusted with either 3M NaOH or 3M HCl, as needed throughout the experiments. Every 24 hours a 2 mL sample was removed from each culture, except at hours 0 and 120, and stored at -80°C until the sample could be analyzed. At hours 0 and 120, 4 mL were removed instead. The OD was read for each sample at 550 nm and 680 nm to determine the cell concentration every day. Digestate OD was also determined at 550 nm and 680 nm daily, which was used to account for digestate opacity when determining cell concentration every day. At the end of the 5-day experimental period, the

cultures were harvested for biomass cells and freeze dried to determine the final biomass concentrations on a dry weight (DW) basis

3.5 Harvesting Algal and Bacterial Biomass

Biomass cells were collected after a full 120 hours of culture growth. Before harvesting, twelve 15 mL tubes were pre-weighed in triplicate and their weights were recorded. Each culture was thoroughly mixed before 160 mL of cell suspension, from each culture, was transferred into 400 mL centrifuge bottles. The 400 mL bottle cell suspensions were centrifuged, and the supernatant was aspirated off. During cell harvesting, all centrifuge steps were conducted at 5,000 rpm for 5 minutes. Using less than 20 mL of deionized water, the cell pellets were transferred to 50 mL tubes; after which, the 400 mL bottles were rinsed with deionized water, centrifuged and the remaining cells transferred to the 50 mL tubes. The cell suspensions in the 50 mL tubes followed the same centrifuge, transfer and rinse steps to the 15 mL tubes. After all cell suspensions are transferred to the 15 mL tubes, they are once more centrifuged, and the supernatant is aspirated off. The lids to the 15 mL tubes are loosely placed on the tubes and frozen at -80°C for 1 hour. The tubes were then freeze dried overnight or until the pellets were dry. After which, the 15 mL tubes were re-weighed to obtain the total DW of biomass.

3.6 Determination of Nutrient Concentrations

The nutrient concentrations of interest were nitrate, nitrite, phosphate, sulfate, and ammonium. In order to quantify the removal of each nutrient, soluble cation and anion concentrations were measured using ion chromatography on the media samples collected each day of the batch experiments. Each media sample was first filtered through a 0.2 µm filter before

analysis. A Shimadzu Prominence High Pressure Liquid Chromatography instrument with a conductivity detector was used either with a Dionex CS12 column coupled with a CS500 suppressor at 59mA (for cations) or a Dionex AS22 column coupled with an AS500 suppressor at 26mA (for anions). For both cation and anion measurements, each sample had 20 µL injected into the column and a column flow rate of 1 mL/min. A mobile phase of 20 mM methansulfonic acid in nanopure water was used to analyze cations; while, a mobile phase of 4.5 mM sodium carbonate and 1.5 mM sodium bicarbonate in nanopure water was used to analyze anions. The resulting peaks were integrated using the Shimadzu LC Solutions software.

3.7.0 Assessment of Biomass Composition

The biomass fractions of interest were the crude lipid, neutral lipid, starch, cell wall and other biomass contents. To complete the composition analyses, 20 mg of dry cell mass was weighed out into pre-weighed 2 mL tubes. Biomass composition analyses could not be completed for the AD bacteria treatments since the total DW of biomass for those cultures were less than 1 mg.

3.7.1 Lipid Extraction

Prior to beginning lipid extraction, a 2:1 ratio of chloroform to methanol solution (Folch solvent) and 0.9% NaCl solution were prepared. A volume of 1.5 mL of Folch solvent and 0.5 mm zirconia/silica beads were added to the 20 mg-DW tubes which was then placed in a bead-beater for cell disruption. The bead-beater was operated a total of six times at 6.5 m/s for 20 seconds with 30 second intervals on ice water. The zirconia/silica beads were then filtered out from the cells using a modified 12 mL syringe containing a stainless-steel wire mesh disk (#40 mesh) into 15 mL tubes. The 2 mL tubes and modified 12 mL syringes were intensively washed three times with

Folch solvent into the 15 mL tubes. After washing, 0.9% (w/v) NaCl was added to induce phase separation and the samples vortexed. Samples were then centrifuged at 6,000 xg for 5 minutes, after which the bottom chloroform phase volume was recorded. Two mL of the bottom chloroform phase was collected and stored at -80°C for crude lipid and neutral lipid analyses. The remaining bottom chloroform phase left in the 15 mL tubes were extracted. The 15 mL tubes containing the pellet and top chloroform phase were stored at 4°C until the samples could be analyzed for starch content.

3.7.2 Crude Lipid & Neutral Lipid Analyses

Crude lipid content and neutral lipid content were both determined from the extract collected from the lipid extraction procedure. To determine crude lipid content, aluminum pans were first pre-weigh, one for each sample, and 1 mL of the chloroform extracts were pipetted into their respective pans. The samples were left in a fumehood to dry off any chloroform overnight. The pans were then re-weighed to obtain the combined dried sample and pan weights. Subtracting the pan weight from the combined sample and pan weight was how the crude lipid weight was obtained.

Neutral lipid content was determined using a Nile Red assay and a fraction of the chloroform extracts. Before starting the assay, a microplate block was pre-heated to 55°C. Additionally, the sample extracts were diluted to a 2:1 ratio of methanol to extract volume, to ensure that samples remained within the assay range. A volume of 80 µL of each diluted sample was added to the polypropylene microplate wells in quadruplicate. A 1 mg/mL vegetable oil standard in chloroform was created for the standards and stored at -80°C. The vegetable oil standard, like the extract samples, was diluted to a 2:1 ratio of methanol to standard. Volumes of

10, 30, 60, 90 and 120 µL were added in quadruplicate to the microplate wells as the assay standards. Lastly, a solvent blank was created from a 2:1 ratio of methanol to chloroform and 80 µL added in quadruplicate to the microplate. The microplate was heated on the pre-heated heating block for 30 minutes or until all the solvent is evaporated. During this time a 1.0 µg/mL of Nile Red solution was prepared with deionized water. After the solvent has evaporated, 25 µL of isopropyl alcohol was added to all wells and mixed to re-dissolve the remaining lipids. A volume of 200 µL of Nile Red solution was then added to each well, mixed, and incubated at room temperature for 5 minutes. Once the Nile Red has been added, 20 µL of a 50% bleach solution was added and mixed to all wells. Using a spectrometer, the fluorescence was read at 530/575 nm with an auto cutoff set to 570 nm every 5-10 minutes for about 45 minutes or until the signal from the samples stabilized. From the stabilized fluorescence readings, the standards and solvent blank were used to create a standard curve and calculate the concentration of neutral lipids in the samples.

3.7.3 Starch Purification, Hydrolysis, & Cell Wall Determination

Before starch hydrolysis could be completed, the starch samples needed to be purified. Starch purification requires 2 mL tubes to be pre-weighed as a point of reference of the cell wall content to be determined later on. The 15 mL tubes containing the top chloroform phase and pellet from the lipid extraction procedure were vortexed and centrifuged at 6,000 xg for 5 minutes. The supernatant was then discarded. A volume of 1.5 mL acetone was added to each tube, vortexed, and the contents were transferred to the pre-weighed 2 mL tubes. The 2 mL tubes were then centrifuged, and the supernatant discarded. During the purification procedure, all centrifuge steps were conducted at 12,000 xg for 5 minutes. The 15 mL tubes were rinsed with more acetone and the remaining contents transferred to the 2 mL tubes, vortexed, centrifuged, and the supernatant

discarded. The 2 mL tubes were then rinsed with acetone, in the same manner that the 15 mL tubes were. After the third rinse with acetone, the 2 mL tubes were rinsed three times with 1.5 mL of deionized water. The lids to the 2 mL tubes were loosely placed on the tubes and frozen at -80°C for 1 hour. The tubes were then freeze dried overnight or until the pellets were dry.

A heating block was pre-heated to 80°C before starting the starch hydrolysis procedure. The dried starch pellets from the purification procedure were ground with micro-pestles until the pellets were broken apart. A volume of 1 mL deionized water was used to simultaneously wash any sample on the pestles into the 2 mL tubes. The tubes were heated in the pre-heated heating block for 30 minutes and then left to cool at room temperature. During this time, a starch hydrolysis enzyme mixture was created using 6 U/mL α-amyloglucosidase and 15 U/mL α-amylase in a 100 mM sodium acetate buffer (pH 5). After the tubes had cooled, 900 µL of the starch hydrolysis enzyme mixture was added to each tube, vortexed, and left to incubate at 37°C, with shaking at 150 rpm, overnight. The next day, the samples were centrifuged at 12,000 xg for 10 minutes and the supernatant was collected and stored at -80°C for starch content analyses later. The remaining pellet was washed twice with deionized water and centrifuged at 12,000 xg for 5 minutes. The tubes were frozen at -80°C for 1 hour and then freeze dried overnight or until the pellets were dry. The final tube and pellet weights were recorded as the combined cell wall and tube weight, which was then used to determine the cell wall content. Any cell mass remaining after hydrolyzing the starch was considered to be cell wall mass. Thus, subtracting the tube weight from the final biomass content after starch hydrolysis was considered to be the cell wall weight.

3.7.4 Starch Analysis

Starch content was determined using a Reducing Sugar assay and a fraction of the starch extracts obtained from the starch hydrolysis procedure. Before starting the assay, a DNS regent and a 1mg/mL glucose standard was prepared. The DNS reagent was prepared in a volume of 100 mL of deionized water with the following reagent quantities: 1.4 g DNS, 1.4 g NaOH, 28g Rochelle salts, 293 µL of 90% liquid phenol, and 0.07 g Na_2SO_4. Concentrations of 1.0, 0.8, 0.6, 0.4, and 0.2 mg/mL glucose standard were prepared and 40 µL of each was added to a 96-well PCR plate in quadruplicate. In addition, 40 µL of the starch hydrolysis enzyme mixture was added in quadruplicate as a solvent blank. Each starch extract was diluted as need based on the absorbance curve generated by the standards and 40 µL of each added to the PCR plate in quadruplicate. Then 40 µL of deionized water was used to suspend all samples, standards, and blanks in the 96-well plate. At this point, 80 µL DNS reagent was added to each well and mixed thoroughly. The plate could then be covered with microplate adhesive foil and sealed. The PCR block was used to heat the samples for 10 minutes at 90°C and cool to at least 70°C before removing the plate from the PCR block. The plate was further cooled to room temperature before 100 µL of the samples were transferred to a polystyrene microplate. A spectrometer was used to read the absorbance of the samples at 540 nm. The standards and solvent blank were then used to create a standard curve and calculate the concentration of starch in the samples. If any of the samples were not within the assay range, the assay was run again with a different sample dilution.

3.7.5 Total Nitrogen Assay

A Hach Total Nitrogen (TNT) assay kit was used to analyze the TN in the culture biomass at hour 120. Since the TNT assay kit measures soluble TN, the sample biomass cells first need to

be disrupted. Biomass samples were diluted with nanopure water to a 1 mg/mL concentration and 0.5 zirconia/silica beads were added before cell disruption. Nanopure water is used throughout to ensure no soluble TN is introduced into the assay from the water. The bead-beater was operated a total of four times at 6.0 m/s for 20 seconds with 30 second intervals on ice water. Disrupted cell samples were also diluted with a 4:1 ratio of nanopure water to sample to ensure that samples were not above the upper limit of the assay range. The remaining disrupted cell samples was stored a 4°C until soluble protein could be extracted. A HACH DR 900 spectrometer was used to analyze samples and samples were prepared by following the manufacturing instructions.

3.7.6 Soluble Protein Extraction

Beforehand, a 150mM disodium phosphate buffer, adjusted to pH 7, was prepared. The prepared Na_2HPO_4 buffer was added in a ratio of 1:2 buffer to cell suspension to the tubes containing the cell suspension. The samples were disrupted again using the bead-beater at 6 m/s for 30 seconds and then centrifuged at 13,500 xg for 30 seconds. After centrifuging, 1 mL of the supernatant was transfer to a new 2 mL tube for storage at 4°C until protein content could be analyzed.

3.7.7 Protein Analyses

Protein content was analyzed using a bicinchoninic acid (BCA) assay on the stored soluble protein extracts. A microplate block was pre-heated to 37°C on a heating block before starting the assay. Concentrations of 250, 125, 62.5, 31.25, 15.625, and 7.8125 µg/mL of bovine serum albumin (BSA) standard were prepared from a 1 mg/mL BSA stock provide by the assay kit. Each sample, standard, and deionized water blank were added in triplicate to a polypropylene microplate

in volumes of 25 µL. The buffer reagent and BCA reagent were combined in a 50:1 ratio of buffer to BCA and 200 µL of the working reagent was added to each well. The microplate was then covered and heated for 30 minutes. A spectrometer was used to read the absorbance of the samples at 562 nm. The standards and water blank were then used to create a standard curve and calculate the percentage of TN contained in the sample biomasses.

3.8 COD Determination

A COD assay kit was used to analyze the COD remaining in the culture mediums at hour 0 and hour 120. Medium samples were filtered through a 0.2 µm filter before analysis with the assay to measure soluble COD. Sample mediums were diluted as necessary to ensure that samples were not above the upper limit of the assay range (1500 mg/L). A HACH DR 900 spectrometer was used to analyze samples and samples were prepared by following the manufacturing instructions (Chaump et al., 2019).

3.9 Extraction of Bacterial DNA in Culture Biomass

By quantifying the number of bacteria cells in each culture, it's possible to determine if any visible growth increases are due to growth promotion by these bacterial cells or if growth increases are a result of increases in bacterial cell counts. In order to quantify bacteria cell counts, a quantitative polymerase chain reaction (qPCR) technique is used. Before qPCR can be used to determine the bacteria cell count, bacterial DNA first needs to be extracted from the harvested culture biomass. A FastDNA Spin kit (MP Biomedicals, Solon OH) was used to extract bacteria DNA genes from the freeze-dried biomass (Higgins and VanderGheynst, 2014). As per the manufacturing instructions, a small fraction of harvested biomass was taken from each culture, in

one of two ways, to be used for DNA extraction depending on the final DW of the culture. For samples that had a final DW larger than 20 mg, 1 mg of biomass was weighed out and suspended in 1 mL of deionized water. For samples with a final DW less than 20 mg, the entire biomass harvested was suspended in 1 mL of deionized water. The remainder of the DNA extraction process was carried out as per the manufacturing instructions.

3.10 Quantification of Bacterial DNA

Since poultry digester microbial communities can vary widely (Smith et al., 2014; Y. Zhang et al., 2011; Ziganshina et al., 2015) the conserved regions of total bacterial 16s rRNA genes were amplified to determine total bacterial cell counts. However, generic 16s rRNA primers are known to amplify algal chloroplast genes along with the desired bacterial genes (Higgins et al., 2018). Thus, it was decided that the 16Sv5F799mod3 and 16Sv5R926 primers were to be used, in order to capture the wide range in possible species present in the digester community without having algal chloroplast amplification interference (Hanshew et al., 2013). All qPCR reactions were prepared in a biosafety cabinet to ensure that the DNA extracts and any other qPCR materials were not contaminated during the preparation process. The 100 µM primer stocks were pre-diluted to 0.5 µM, with nuclease free water, before being added to the working master mix. Extracted DNA samples were also pre-diluted to a 10x dilution before being added to each reaction master mix, to ensure that the DNA concentration is no greater than 10^9 bacterial cells (as per manufacturing instructions). The working master mix was based off of 20 µL reaction volumes and composed of SYBR Green fluorescence dye and the previously specified forward and reverse 0.5 µM primers. PCR tube strips were used to house each sample, no template control (NTC), and standard reactions during the qPCR run. Each qPCR run contained a 10x dilution of each DNA

extracted sample in triplicate, 2 NTCs as reaction blanks, and DNA standards made from each bacteria culture in singles. The DNA standards consisted of a 100x and a 1000x dilution of each digester bacteria culture; while, the NTCs used were made from the working master mix. After all reactions are pipetted into the PCR tubes, the tubes were closed taking care not to mark the lids. The PCR tubes were centrifuged with a swing bucket centrifuge at 1100 xg for 1 minute.

An analytic qTower3G and its software was used to run PCR on the prepared reactions. Before starting qPCR, each reaction tube was labeled in the software as an unknown or an NTC. In the software, the qTower3G thermocycler profile had to be set at specific temperatures for TAQ activation and elongation to occur for these general primers to work. For TAQ activation, the program ran at 50°C for 2 minutes and then 95°C for 2 minutes for the duration of 1 cycle for each temperature change. Elongation of the sequence was run on a loop for 45 cycles with the following settings: 15 seconds at 95°C, 15 seconds at 47°C, and 30 seconds at 72°C. After all 45 cycles had been completed, a melt curve was developed by reading the fluorescence as the program ramps up to 95°C from 72°C within a 6 second duration. After the qPCR sequence had run, the reaction samples were removed from the qPCR block and stored at 4°C.

The fraction of biomass as algae and bacteria was calculated by correlating the cycle thresholds and the final harvested biomass concentrations. From the qTower3G software, cycle thresholds were obtained for all samples run. The AD bacteria sample DWs used in DNA extraction were used to create a standard curve for digester bacteria. The average DW of DNA extracted in the undiluted samples was divided by each of the dilution factors (10x, 100x, and 1000x) to obtain the DW of DNA extracted from each dilution. The natural logarithm of each AD bacteria DNA DW dilution was taken and plotted with the average cycle threshold against each dilution to create a standard curve of digester bacteria. From this standard curve, the slope and

intercept were obtained. The standard curve was then used to correlate the mixed culture thresholds to the mass of bacteria present in the mixed cultures, which was corrected for the 10x DNA dilution used and then divided by the mixed culture sample DWs to obtain the percentage of the mixed cultures as bacteria. A binary logarithm was also taken of the AD bacteria DNA DWs to determine the amplification efficacy. The amplification efficiency was obtained by taking two raised to the power of the negative inverse of the binary logarithm subtracted by one. It was determined that the 16S primers used had a 70.9% amplification efficiency. This low efficiency is likely the result of the low melting temperature of this primer set (~50 °C).

3.11 Bacterial DNA Sequencing

The remainder of the bacterial DNA extracts were sent to CD Genomics (Shirley, NY) for flow-cell sequencing using an Illumina MiSEQ instrument (Higgins et al., 2018). All submitted extracts had the 300 bp paired-ends read, which were obtained from the V3-V4 region of the 16s rRNA gene. CD Genomics quality-filtered and demultiplexed the submitted reads using a proprietary software pipeline. The Quantitative Insights Into Microbial Ecology (QIIME) software was used to read and remove chimeric sequences from the resulting FASTA files returned from CD Genomics. Once the FASTA files were cleaned the Greengenes database used to pick OTUs at 97% identity.

3.12 Experimental Plan for Follow up Study on AS Treatment of Digestate

From the experiments conducted on *A. protothecoides* and the two *C. sorokiniana* species, it was revealed that there were only minor changes in COD over time. These results indicated that the microbes in the digestate were unable to help lower COD and photosynthate secreted in the

medium. From these results it was hypothesized that aerobic, rather than anaerobic microbes are primarily responsible for COD removal from digestate. In order to test whether or not aerobic microbes are responsible for COD removal, another experiment was conducted using a combination of digestate and AS. The AS was collected from an aeration pond at the wastewater treatment plant in Columbus, GA (Wang et al., 2018) and then stored at 4°C until it was needed.

To test how the addition of aerobic microbes affect organic uptake by algae, one batch culture experiment was conducted using *C. sorokiniana* UTEX 2805. The batch experiment consisted of two controls and two treatments, each grown in triplicate. In this experiment the first control was algae & AD bacteria, while the second control was AD bacteria. The treatments were then algae & AD bacteria with AS, and AD bacteria with AS. The AS added to each culture was 1 mL of the solid and liquid AS fractions homogenized. This design allowed for testing of the contributions of aerobic bacteria towards the treatment of litter digestate in terms of organic removal.

The AS follow up study used the clarification method (see Cultivation Methods), harvesting (see Harvesting Biomass), and biomass compositional analyses (see Assessment of Biomass Composition) methods previously described.

3.13 Data Analyses

Basic statistics such as mean and standard deviation were performed in Microsoft Excel. ANOVA and Tukey method of the multiple comparison tests were performed in SAS University Edition (Westfall et al., 2011).

4.0 Results & Discussion

4.1 Poultry Waste Characteristics

Poultry litter was anaerobically digested for roughly one month in order to create digestates suitable for testing algae cultures. Reactors were prepared to achieve a VS loading rate of 10 g/L VS. To achieve this loading rate, the waste characteristics of the sampled poultry litter and municipal sludge first needed to be determined. The poultry litter was found to be 85% solids and 14% moisture; while the municipal sludge was found to be 4% solids and 96% moisture (Table 1). Additionally, it was determined that 84% of the poultry litter solids were volatile, while 56% of the municipal sludge solids were volatile.

Table 1:VS content for poultry litter & municipal anaerobic digestate solids inoculum.

	Poultry Litter	Municipal Solids Inoculum
Sample Mass (g)	1.005 (0.002) †	1.008 (0.005)
Total Solids (%) *	85 (<1)	4 (<1)
Volatile Solids (%) *	84 (2)	56 (<1)
Moisture Content (%) *	14 (<1)	96 (<1)

* All percentages are on a mass basis.

† Reported values are averages of the triplicates for each treatment; Standard deviations are in parenthesis.

From additional analyses, initial TS, VS, soluble ions, and COD concentrations were determined for each batch of four-fold diluted AD used in each batch experiment (Table 2). These 4-fold diluted digestates were used for algae cultivation. The AD VS loadings decreased to 2.5 g/L when diluted to four-fold. Soluble ion concentrations and COD measurements of the four-fold

diluted digestates show that there is variability among all four digestate batches before algal cultivation (Table 2). Additionally, no nitrite was introduced into the treatment culture with the four-fold diluted digestate for all experiments, but some nitrate was introduced for the *A. protothecoides* and *C. sorokiniana* (UTEX 2714) experiments.

Table 2: Initial four-fold diluted AD concentrations for all batch experiments conducted.

	4x Dilution of AD *A. protothecoides*	4x Dilution of AD *C. sorokiniana* (UTEX 2714) ‡	4x Dilution of AD *C. sorokiniana* (UTEX 2805) ‡	4x Dilution of AD AS Study ‡
TS Loading (g/L)	4.8	4.8	4.8	4.8
VS Loading (g/L)	2.5	2.5	2.5	2.5
Soluble COD (mg/L)	291 (9) †	123 (15) †	249 (6) †	274 (13) †
Chloride (mg/L)	40.0 (2.5)	47.1 (1.3)	44.4 (2.0)	39.2 (0.1)
Sodium (mg/L)	13.1 (3.5)	25.9 (1.0)	31.5 (5.4)	35.5 (2.3)
Potassium (mg/L)	84.6 (3.2)	100.0 (6.2)	97.7 (9.2)	80.5 (0.2)
Magnesium (mg/L)	2.8 (<0.1)	5.8 (0.1)	4.7 (0.7)	4.0 (<0.1)
Calcium (mg/L)	18.5 (0.3)	15.4 (0.7)	17.7 (2.3)	13.6 (0.1)
Nitrite (mg/L)	- *	- *	- *	- *
Nitrate (mg/L)	19.8 (0.2)	225.0 (0.2)	- *	- *
Ammonium (mg/L)	196.8 (7.9)	209.0 (14.9)	246.7 (21.3)	208.3 (0.7)
Phosphate (mg/L)	32.4 (1.7)	67.6 (2.1)	120.2 (2.9)	103.3 (0.3)
Sulfate (mg/L)	27.5 (0.2)	43.0 (0.1)	42.8 (0.2)	37.5 (0.2)

***** Measured but not detectable.

† Reported values are averages of the triplicates for each experiment; Standard deviations are in parenthesis.

‡ AD used for an experiment is from the same stock as other experiments indicated.

4.2 Algal Growth on Different Digestate Concentrations

Three different algal strains, *A. protothecoides*, *C. sorokiniana* (UTEX 2714), and *C. sorokiniana* (UTEX 2805) were cultured on AD stock 1 (for *A. protothecoides*) and AD stock 2 (for the *C. sorokiniana* species) poultry litter digestates. Past research has shown that full-strength ADs are generally inhibitory to algal growth typically due to light-inhibition via turbidity and ammonium-inhibition (Marcilhac et al., 2014; Uggetti et al., 2014), which can be overcome with dilution. The initial ammonium concentration for the full strength digestate with bacteria (n=3) was 878.3 ± 63.7 mg/L. It was hypothesized that the high ammonium concentrations found

in poultry litter digestate would be the main compound resulting in the inhibitory growth effect on algae. We carried out an initial study in which we cultured *A. protothecoides* on different dilutions of litter digestate. The results (Figure 3A and 3B) showed that a four-fold dilution yielded maximum growth rates and this dilution rate was used in subsequent experiments.

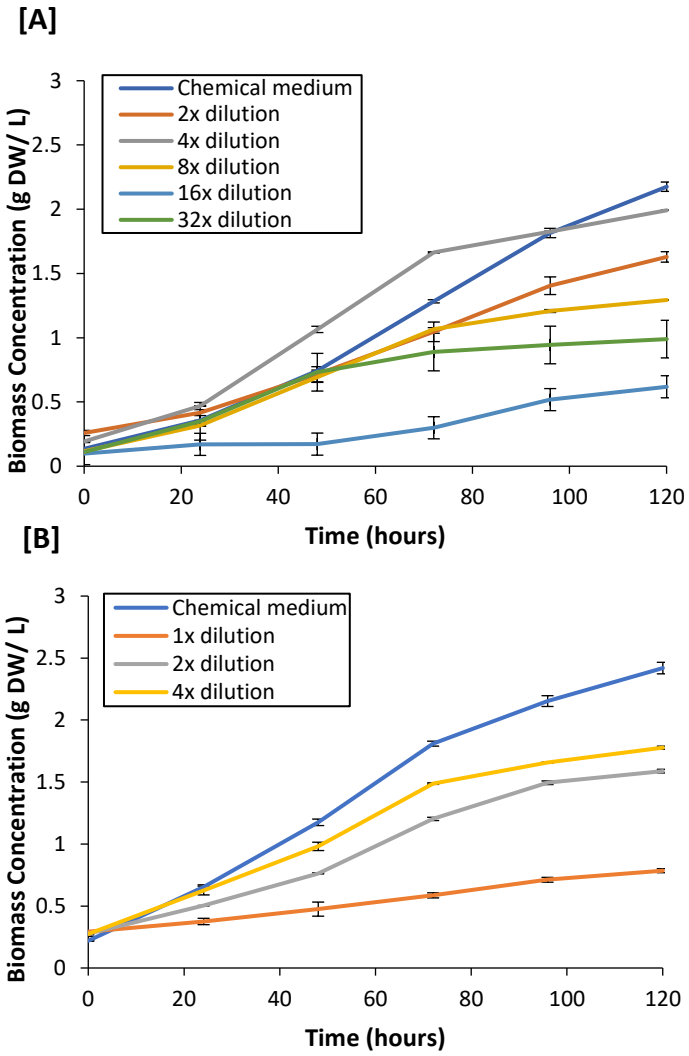


Figure 3: Initial (A) and final (B) dose responses of *A. protothecoides* to various dilutions of AD. Error bars for graph A is with n=2 and B is SD with n=3. Both Graph A and B are using *A. protothecoides*. Graph A compares different dilution rates in duplicate to determine which dilution yields the highest final biomass growth. Graph B confirms a four-fold dilution is best with biological triplicates. The reported biomass concentrations are the average daily algae dry weight concentrations.

4.3 Impact of Digestate Microbes on Algal Growth

We initially hypothesized that growing algae in the presence of digestate microbes would have minimal impact on biomass growth rates given the results of a previous study on winery digestate (Higgins et al., 2018). The results shown in figure 4B and 4C confirmed that the presence of digestate microbes had minimal impact on biomass growth rates in *C. sorokiniana* cultures. However, the presence of digestate microbes led to roughly double the biomass growth in *A. protothecoides* cultures compared to the axenic algal cultures grown on digestate (Figure 4A). The mixed-culture biomass growth is the combination of growth from algae plus growth of heterotrophic and autotrophic microorganisms. In the absence of algae, the microbes in the digestate showed no growth in all three experiments, which is consistent with the results from the winery digestate study (Higgins et al., 2018). These results suggest that, for certain algae species, either microorganisms promote algae growth, algae promote microorganism growth, or some combination of these two possibilities. The positive control (chemical medium) showed rapid algal growth in all cases except for *C. sorokiniana* (UTEX 2714). Compared to the *C. sorokiniana* (UTEX 2714) cultures grown on chemical medium, the axenic and mixed cultures grown on digestate exhibited rapid algal growth. This could be explained by the presence of compounds in the digestate that were conducive to growth of *A. protothecoides*.

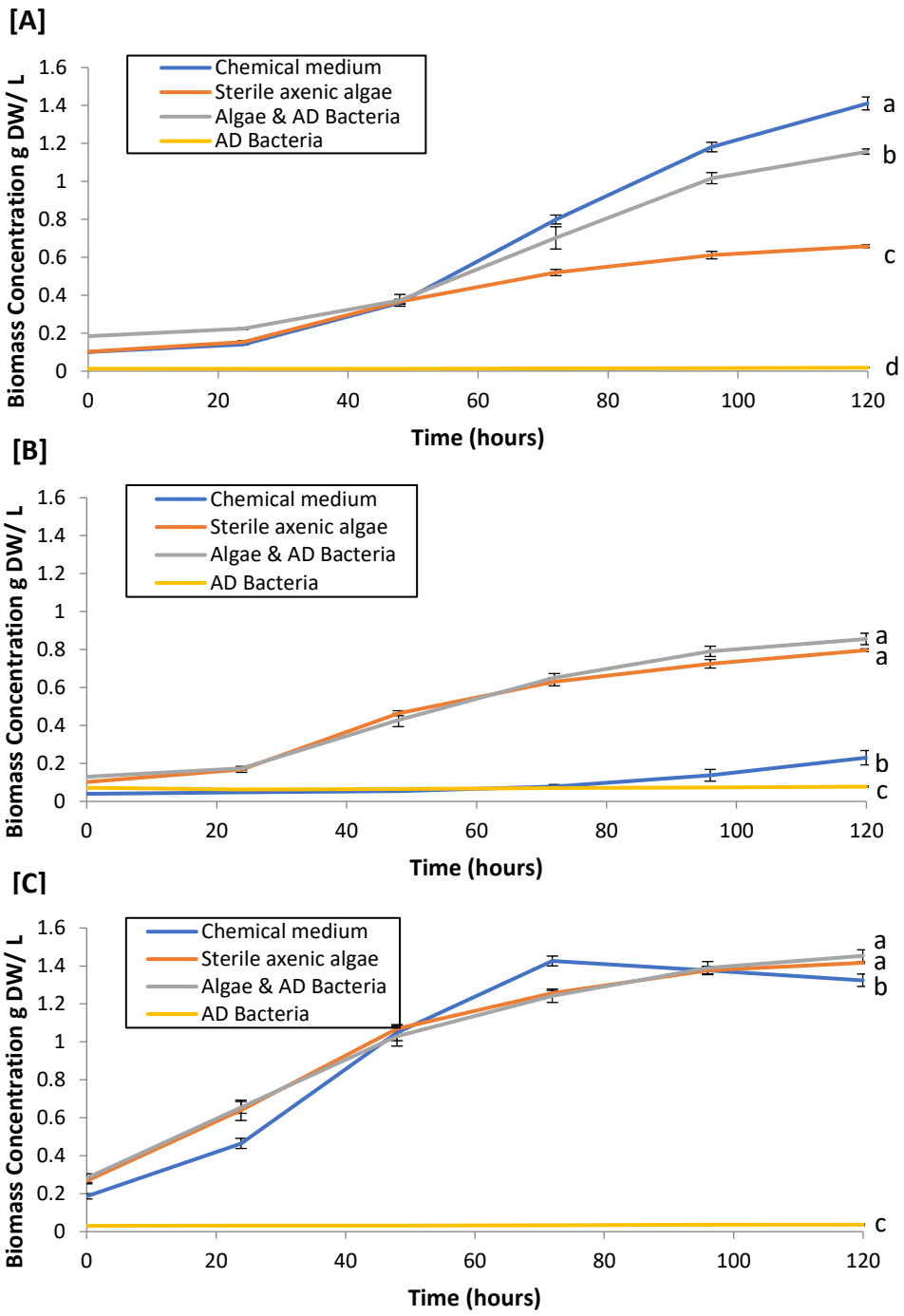


Figure 4: Daily average DW for *A. protothecoides*, *C. sorokiniana* (UTEX 2714), and *C. sorokiniana* (UTEX 2805).

Error bars for graphs A, B and C are SD, n=3. Graph A is using *A. protothecoides*, B is using *C. sorokiniana* (UTEX 2714), and C is using *C. sorokiniana* (UTEX 2805). Graph A shows *A. protothecoides'* biomass growth between treatments. Graph B shows *C. sorokiniana's* (UTEX 2714) biomass growth between treatments. Graph C shows *C. sorokiniana's* (UTEX 2805) biomass growth between treatments. The reported biomass concentrations are the average daily algae dry weight (DW) concentrations after the media ODs, at 550 nm, have been accounted for. Within an algal strain, biomass concentrations at hour-120 with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

Given the apparent growth-promoting effects of the mixed culture in *A. protothecoides*, we next examined the fraction of mixed-culture biomass that was bacteria vs. algae. This analysis showed that only 0.64% (Table 3) of biomass in the mixed culture was bacteria and suggests that algal growth was promoted by the presence of live microorganisms in the mixed culture. In the case of *C. sorokiniana*, the bacteria fraction of biomass was also low (0.99% and 2.24%), indicating that the majority of the culture was algae (Table 3). Thus, our initial hypothesis held true for the species of *C. sorokiniana* – that digestate microbes would have minimal impact on algal growth. In contrast, *A. protothecoides* actually experienced growth promotion in the presence of native digestate microorganisms.

Table 3: Fraction of final biomass productivity as bacterial and other biomass growth for *A. protothecoides* and *C. sorokiniana* (UTEX 2714 & 2805).

Species Name	Strain Code	Treatment	Total Average Biomass Productivity (mg/L/d)	Productivity as Bacteria Growth (mg/L/d)	Fraction of Productivity as Other Biomass Growth (mg/L/d) §
A. protothecoides	UTEX 2341	Chemical medium (N8-NH4)	252.89 (20.72) a †	- a *	252.89 (20.72) a
		Sterile axenic algae	99.44 (10.72) c ¶	- a *	99.44 (10.72) c
		Algae & AD Bacteria	169.03 (9.52) b	1.08 (0.34) a	167.95 (9.64) b
		AD Bacteria	-1.39 (0.90) d ‡	-1.38 (0.91) b ‡	- d *
C. sorokiniana	UTEX 2714	Chemical medium (N8)	32.80 (12.32) b †	- a *	32.80 (12.32) b
		Sterile axenic algae	121.47 (3.79) a ¶	- a *	121.47 (3.79) a
		Algae & AD Bacteria	122.80 (7.16) a	1.16 (1.21) a	121.64 (8.34) a
		AD Bacteria	-12.47 (1.63) c ‡	-12.47 (1.63) b ‡	- c *
C. sorokiniana	UTEX 2805	Chemical medium (N8)	217.98 (16.73) a †	- b *	217.98 (16.73) a
		Sterile axenic algae	207.24 (8.11) a ¶	- b *	207.24 (8.11) a
		Algae & AD Bacteria	207.01(13.88) a	4.62 (1.23) a	202.39 (14.19) a
		AD Bacteria	-5.02 (0.95) b ‡	-5.02 (0.95) c ‡	- b *

* Measured but not detectable.

† Reported values are averages of the triplicates for each treatment; Standard deviations are in parenthesis.

‡ Negative values indicated a decrease in productivity rather than an increase.

§ Other biomass growth includes algal growth and any *Archaea* growth present.

¶ Within an algal strain and column, values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

One possible explanation for growth promotion in mixed *A protothecoides*-microbe cultures is differences in concentrations of limiting metal nutrients compared to algae-only cultures. Many limiting metals (e.g. Mg, Ca) for insoluble complexes and may have been removed during the extra filtration step used to remove indigenous microorganisms from the digestate. These sterile-filtered digestates were used to prepare the axenic algal cultures grown on digestate. Magnesium, in particular, has been identified as a limiting metal in many digestates (Huang et al., 2015) and it is essential for chlorophyll production and nitrogen assimilation into microalgae (Bjornsson et al., 2013). Consequently, both the filtered and unfiltered digestates were analyzed for potentially limiting metal nutrients. However, analysis of soluble cations (see Nutrient Removal from Digestate Effluent) showed that there was some removal of magnesium and calcium between the treatments and that it varied depending on the strain. Only *C. sorokiniana* (UTEX 2805) had no significant difference in initial magnesium ($p > 0.5400$) or calcium ($p > 0.2622$) concentrations among the different digestate cultures. For *A. protothecoides*, there was no significant difference in initial magnesium ($p > 0.2445$) concentrations, but the axenic cultures did have significantly lower calcium ($p > 0.0290$) concentrations compared to the mixed cultures and the AD bacteria cultures. *C. sorokiniana* (UTEX 2714) actually had a significant difference in magnesium ($p < 0.0001$) concentration between the axenic cultures and the unfiltered treatments, but all treatments, regardless of filtration, had a significant difference in calcium ($p < 0.0001$) concentrations. This would explain why *C. sorokiniana* (UTEX 2714) experienced an inhibition of growth and the other strains did not. Moreover, only magnesium for *C. sorokiniana* (UTEX 2805) was completely depleted during the culture period, suggesting that inadvertent metal removal during filtration is unlikely to explain high algal growth in algal-microbe cultures for *A. protothecoides*.

One of the most likely explanations for growth promotion in *A. protothecoides* is that native wastewater microorganisms produce vitamin cofactors. *A. protothecoides* is a known auxotroph for thiamine, and past research has shown that it can obtain thiamine and thiamine derivatives from bacteria (Higgins et al., 2016), including those in wastewater (Higgins et al., 2018). In a previous study, Higgins et al. (2018) found that live bacteria were not necessary for *A. protothecoides* to experience a benefit from exogenous thiamine metabolites. Rather, the native bacteria produced and released cofactors into the wastewater where they were available to algae even after removal of the bacteria. In contrast, the results of the present study indicated there was a benefit from having live bacteria. Thiamine metabolites are particularly unstable in the environment (Jenkins et al., 2007), and live bacteria may have been necessary in this environment to continuously supply cofactors to *A. protothecoides.*

The other possible explanation for growth promotion is that the algae and wastewater microbes engaged in a symbiotic exchange of dissolved oxygen and dissolved carbon dioxide. Mouget et al. (1995) concluded that increased algal growth was a result of the aerobic bacterium enhancing photosynthetic activity when oxygen was actively removed rather than a result of the bacterium releasing growth-promoting substances. This mechanism is only possible with live heterotrophs interacting with algae. However, this mechanism also should have enhanced the growth of both *C. sorokiniana* strains which was not the case. Additional research (Li et al., 2014) has shown that even if increased photosynthetic activity was observed in just the *A. protothecoides* strain, there should have been an increase in lipid content observed (~35%) as a result, which was not the case (results presented in Section 4.4 “Biomass Composition”). Moreover, this mechanism assumes an abundance of biodegradable organic material that bacteria can metabolize into carbon dioxide. Results presented in Section 4.7 “COD Removal” suggest that biodegradable organics

were limited in these mixed cultures. There was little to no COD removed from any of the treatments, indicating that either the organics concentrations were too low for the bacteria to utilize or this mechanism was not used to increase biomass growth. Considering that the maximum initial COD observed was 339 mg/L (axenic cultures) and the minimum was 282 mg/L (mixed cultures), it is unlikely that the digestate lacked organics for the mechanism to proceed but that there was a lack of live heterotrophs to interact with the algae. The observed growth productivity for the AD bacteria shows that there was actually a decrease in bacterial biomass which indicated that the community continually died rather than grow throughout the experiment (Figure 5A). The lack of bacterial growth productivity observed indicates that the bacteria present in the digestate community were not facultative aerobes. This points towards photosynthetic symbiosis being an unlikely explanation for increased growth in *A. protothecoides*.

Another potential source of algal growth promotion can be a result of the production of hormones by the native microbial community. There were two main classes of hormones that are likely to either enhance or inhibit algal growth: endogenous hormones and phytohormones. Endogenous hormones have demonstrated a variety of developmental and physiological effects on various organisms (reviewed in Lange et al., 2002); thus, they have the potential to inhibit growth in axenic algal cultures. Additionally, it has been found that chickens excrete endogenous hormones in much lower concentrations compared to other manure wastes (swine and dairy cattle) (Lorenzen et al., 2004) making the likelihood of endogenous hormone inhibition even lower. An extensive literature search did not turn up any significant research on the effects of endogenous hormones on microalgal species. On the other hand, phytohormones have shown to have positive growth effects on algae when grown in a consortium with bacteria (Pires et al., 2013). In some cases, aerobic bacterium, like *Azospirillum brasilense*, can produce phytohormones which have

been shown to increase culture biomass in *C. vulgaris* (Gonzalez and Bashan, 2000). However, the possibility of plant-growth promoting bacteria being naturally present in poultry digestate is unlikely, despite that it has been demonstrated that poultry litter contains around 460 µg/g tryptophan per mass of waste (Arkhipchenko et al., 2006). Tryptophan, while it is the main precursor of indole-3-acetic acid which is the most common phytohormone, is also an amino acid and thus present in all living things. Thus, the small mass ratio of tryptophan to waste indicates that any tryptophan present in the digestate is unlikely to be a precursor to phytohormones.

Overall, the likelihood that endogenous and phytohormones are present in the native microbial community is low and most likely not the cause of growth promotion observed in the *A. protothecoides* mixed cultures. Moreover, symbiotic exchange of dissolved oxygen and carbon dioxide resulting in the observed growth promotion is unlikely due to the lack of bacterial growth in any of the treatments. Additionally, if this mechanism was utilized, growth promotion would have been observed in both *C. sorokiniana* strains as well. Thus, the most plausible cause of growth promotion in *A. protothecoides* is to be a result of secreted vitamin cofactors from the bacterial community which was then taken up by the algae.

4.4 Biomass Composition

It was initially hypothesized that the neutral lipid content in the mixed algal and bacteria cultures would be larger than the axenic algal cultures, due to increased competition for nitrogen resources between algae and digestate microorganisms. Such competition could lead to nitrogen limitation which is known to lead to neutral lipid accumulation in many oleaginous algae including *C. sorokiniana* and *A. protothecoides* (Higgins et al., 2015). Moreover, microbial promotion of A. protothecoides growth has previously been observed to lead to nitrogen depletion followed by

neutral lipid accumulation (Higgins et al., 2015). However, neutral lipid contents were less than 4% (Table 4) suggesting that either none of these strains accumulated neutral lipids under nitrogen stress or that the digestate was not nitrogen limiting to all algal strains. This point was confirmed during subsequent analysis of nutrient levels in the media.

In addition to neutral lipid content, total crude lipid content was also measured. Crude lipid includes neutral lipid, polar lipids, sterols, and pigments. Overall, there was no significant difference in crude lipid contents between any of the treatments ($p > 0.0668$) (Table 4). Only for the *C. sorokiniana* (UTEX 2805) strain was there a significant increase in crude lipid content however this was observed in the chemical medium compared to the axenic and mixed cultures. Moreover, the trends observed in the crude lipid contents followed the trends observed in the neutral lipid contents for each algal strain.

These results show that there is a low neutral lipid accumulation for all algal strains tested, regardless of the treatment, and that co-culturing poultry litter digester microbes and algae decreases the neutral lipid content rather than increasing it. This could result from partial displacement of algal biomass by bacteria, however, the effect is expected to be small given the low (<1%) concentrations of bacteria in the mixed algae cultures.

Table 4: Biomass composition for *A. protothecoides*, and *C. sorokiniana* (UTEX 2714 & 2805).

Species Name	Strain Code	Treatment	Final Neutral Lipid Content (%*‡)	Final Crude Lipid Content (%*‡)	Final Starch Content (%*‡)	Cell Wall Content (%*‡)	Other Biomass Content (%*‡§)
A. protothecoides	UTEX 2341	Chemical medium (N8-NH4)	- b †	26.76 (2.54) a	0.17 (0.01) b	51.03 (3.56) a	22.04 (3.01) b
		Sterile axenic algae	2.15 (0.75) a #	29.89 (3.55) a	0.62 (0.06) b	32.43 (1.21) b	37.06 (4.18) a
		Algae & AD Bacteria	0.53 (0.43) b	22.17 (3.43) a	1.57 (0.56) a	34.52 (3.59) b	41.74 (6.53) a
		AD Bacteria ¶	-	-	-	-	-
C. sorokiniana	UTEX 2714	Chemical medium (N8)	- b †	11.39 (1.65) a	41.87 (5.33) b	26.64 (1.89) a	20.11 (6.97) a
		Sterile axenic algae	0.50 (0.12) a #	16.62 (1.52) a	59.66 (2.67) a	11.83 (0.71) b	11.89 (4.38) a
		Algae & AD Bacteria	0.42 (0.03) a	15.28 (3.91) a	56.99 (4.00) a	13.60 (0.73) b	14.13 (7.08) a
		AD Bacteria ¶	-	-	-	-	-
C. sorokiniana	UTEX 2805	Chemical medium (N8)	0.20 (0.02) c	28.56 (2.47) a	4.72 (1.03) b	48.20 (3.18) a	18.52 (4.60) a
		Sterile axenic algae	2.11 (0.21) b #	20.97 (0.93) b	43.13 (3.83) a	14.48 (1.27) b	21.43 (3.74) a
		Algae & AD Bacteria	3.37 (0.64) a	23.11 (0.32) b	42.13 (2.22) a	23.73 (14.97) b	13.44 (11.76) a
		AD Bacteria ¶	-	-	-	-	-

***** All percentages are on a mass basis.

† Measured but not detectable.

‡ Reported values are averages of the triplicates for each treatment; Standard deviations are in parenthesis.

§ Other Biomass Content is the difference between one and the summation of the final crude lipid, final starch, & cell wall contents. It is principally composed of ash, protein, and nucleic acids.

¶ No lipid, starch, cell wall, or other biomass contents are reported for any of the three batch experiments' AD bacteria treatments

Within an algal strain and column, values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

Since mixed culture treatments were anticipated to experience an increase in lipid content, it was expected that starch content would be lower as a result of higher lipid contents. Increases in algal lipid content are often seen with similar increases in cellular starch content (Tanadul et al., 2014). Experimental starch contents seemed to vary among the different species rather than across each strain. *A. protothecoides* demonstrated that the mixed culture had a significantly larger starch content than that of the axenic culture ($p < 0.0050$); however, the starch contents were, overall, low (Table 4). The *C. sorokiniana* strains both showed similar trends to each other in that there was no significant difference in starch content between the axenic and mixed cultures, ($p > 0.3904$). However, both treatments for the *C. sorokiniana* strains yielded significantly higher starch contents than the observed starch contents in *A, protothecoides* (all were $p < 0.0001$). Additionally, *C. sorokiniana* cultures grown on chemical medium had significantly lower starch content than cultures grown on digestate ($p < 0.0039$) but *A. protothecoides* showed there was no significant difference in starch content between the chemical medium and the axenic cultures ($p > 0.8143$).

The poultry litter digestate is a high-nutrient environment, so nitrogen, phosphorus, and sulfur stress should be absent. What is interesting here is that the starch in the two *C. sorokiniana* species is unexpectedly high in the digestate treatments. *C. sorokiniana* has been reported to have more efficient starch production when grown on anaerobically digested dairy manure, approximately 20-25% intracellular starch after a 24-day growth period (Kobayashi et al., 2013). Kobayashi et al. suggested that the increase in cellular starch storage may be a result of a mechanism within *C. sorokiniana* species which converts organic carbon sources in the AD to starch. Li et al. (2014 & 2015) and Dragone et al. (2011) showed that starch accumulation, in *C. sorokiniana* and *C. vulgaris* species, decreases or stops when medium nitrate concentrations run

out. It is possible that under nitrogen depletion conditions, that available nitrogen is utilized in synthesizing enzymes instead of being converted into protein (Dragone et al., 2011).

It was expected that the addition of the digester microbes would not change the cell wall content, seeing as the amount of microbial cells harvested appeared to be small compared to the amount of algal cells observed. For all experiments, the control cell wall content was significantly larger than both of the treatment cell wall contents ($p < 0.0087$). Overall, cell wall content values between experiments ranged variably but those with higher starch contents yielded lower cell wall contents. Additionally, mixed culture cell wall contents were larger than the axenic culture cell wall contents (Table 4).

The major question raised when analyzing the biomass composition was why there was such a large increase in starch content in the algal species compared to their starch accumulation on a chemical medium? *C. sorokiniana* species have been observed to accumulate lipids under stress conditions, such as a lack of available nitrogen (Li et al., 2015) (Brányiková et al., 2011), high carbon dioxide concentrations (Cheng et al., 2017), high light intensities (Cheng et al., 2017) (Brányiková et al., 2011) (Dragone et al., 2011), and oxidative stress (Burch and Franz, 2016; Yilancioglu et al., 2014). In this experiment, high carbon dioxide concentrations and high light intensities are unlikely to have been the cause of starch accumulation because both conditions were controlled as was listed in the Methods (see section 3.4 “Cultivation Methods”). Limitations in nitrogen and oxidative stress are the most likely causes for the starch accumulation observed. Researchers have recently shown under nitrogen starvation conditions, that specific mRNA expression of enzymes for photosynthesis, carbon dioxide fixation, lipid metabolism, and starch metabolism were lower than they would be in non-stress conditions and coincides with periods of starch accumulation (Cheng et al., 2017). Specifically, it has been discovered that the gene related

to glucose-1-phosphate adenylyltransferase, which converts glucose-1-phosphate to ADP-glucose, is up-regulated during times when an excessive increase in starch is observed (Cheng et al., 2017) (Sturme et al., 2018). However, why algal cells switch between a lipid metabolism and a starch metabolism under similar stress conditions has yet to be explained (Silkina et al., 2017; Vitova et al., 2015). Comparing our data to previous research indicates that increased carbon partitioning into starch is likely a result of overexpression of genes in the starch metabolism, which most likely is a result of oxidative stress rather than nitrogen stress. While oxidative stress was not measured, it is more likely than nitrogen stress, which was not observed. Additionally, digestate have been observed to contain inhibitory molecules which can act as a source of stress (Wang et al., 2018).

4.5 Nutrient Removal from Digestate Effluent

It was hypothesized that nitrogen and phosphorus nutrients would be removed proportionally to any observed algal growth. Monitoring the nutrient concentration throughout the 5-day batch experiments allowed us to determine the nutrient removal rate. As stated earlier, nitrate, nitrite, phosphate, sulfate and ammonium concentrations were the nutrients of interest, due to their potential to cause environmental disruptions (Causapé et al., 2004; Jiao et al., 2012; Klocke et al., 1999; Ljung et al., 2009). Nitrite concentrations were expected to be low in the AD, and this was confirmed by analysis which showed no nitrite at any time throughout the 5-day experiment for any of the three algal strains tested. While nitrate and sulfate concentrations were not zero and concentrations varied across algal strains, the concentration values remained the same over the 5-day experiment in all treatments for each of the three algal strains (see Appendix Figure 1 and Appendix Figure 2 for data). The presence of any sulfate and nitrate is most likely from

either the original digestate or left over from the small amount of media that was inoculated into the cultures with the algae.

The *A. protothecoides* strain removed all phosphate in the mixed and axenic cultures at hour-96 and hour 120, respectively; however, there was no significant difference in the phosphate removal rates between the axenic and mixed cultures ($p > 1.5693$) (Figure 5B). On the other hand, ammonium removal in the mixed culture of *A. protothecoides* was double that of the axenic algae culture (Figure 5A). The fact that nitrite and nitrate levels did not change in these cultures indicates that this additional ammonium removal likely resulted from cellular uptake rather than nitrification carried out by bacteria. *A. protothecoides* observed low values in the removal proportions of phosphate to biomass on a mass basis with proportion values of 0.06, 0.04, and the addition of 0.16 mg PO_4^{3-}/mg biomass in the axenic, mixed, and AD bacteria cultures, respectively. Additionally, *A. protothecoides* observed low values in the removal proportions of ammonium to biomass on a mass basis at values of 0.11, and 0.10 mg NH_4^+/mg biomass and the addition of 2.24 mg NH_4^+/mg biomass in the axenic, mixed and AD bacteria cultures, respectively. In contrast to *A. protothecoides*, the two *C. sorokiniana* species resulted in lower nutrient removal rates overall with the exception of phosphate removal by *C. sorokiniana* (UTEX 2805) (Figure 5B). However, both *C. sorokiniana* strains display similar trends in that there was no significant difference in ammonium ($p > 0.5720$) and phosphate ($p > 1.0402$) removal rates between the axenic and mixed cultures. C. *sorokiniana* (UTEX 2714) also observed low values in the removal proportions of phosphate to biomass - 0.01, 0.01, 0.07 mg PO_4^{3-}/mg biomass – and ammonium to biomass – 0.06, 0.04, and 2.88 mg NH_4^+/mg biomass – all on a mass basis in the axenic, mixed, and AD bacteria cultures, respectively. Lastly, *C. sorokiniana* (UTEX 2805) observed low values in the removal proportions of phosphate to biomass – 0.03, 0.02, and the addition of 0.63 mg PO_4^{3-}/mg biomass

– and ammonium to biomass – 0.04, 0.03, and 1.50 mg NH_4^+/mg biomass – all on a mass basis in the axenic, mixed and AD bacteria cultures respectively. For all algal strains tested, the ammonium and phosphate proportions were un-equal to a one-to-one ratio, as it was originally hypothesized, meaning that co-culturing algae and digester microbes did not significantly increase the portion of ammonium and phosphate removed per mass of biomass ($p > 0.0772$). Thus, ammonium and phosphate removal rates and proportions observed demonstrated that all algal strains tested – regardless of the treatment – removed less ammonium and phosphate that what was proportionally expected for the observed biomass weights recorded.

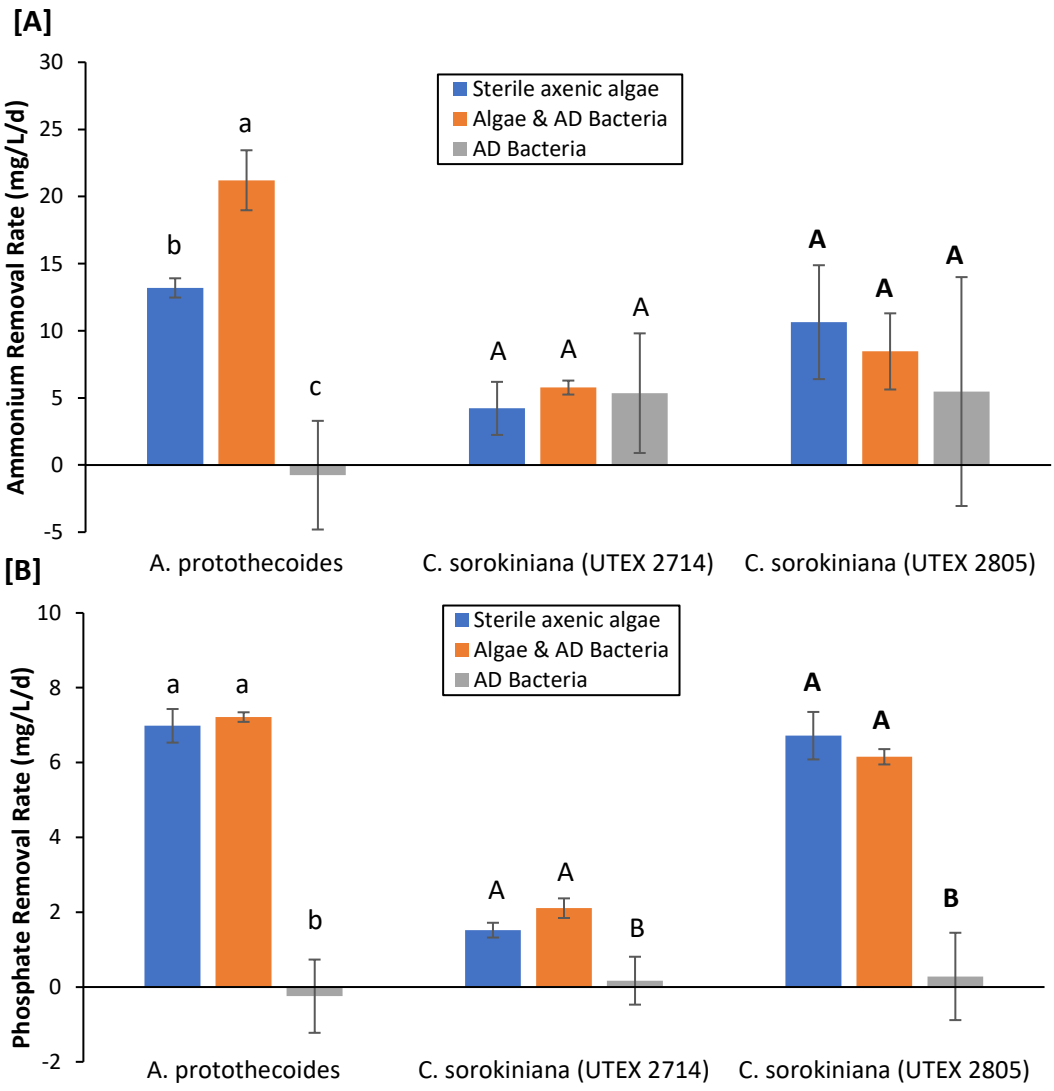


Figure 5: Ammonium and phosphate removal for *A. protothecoides* and *C. sorokiniana* (UTEX2714 & 2805). Error bars for graphs A and B are SD, n=3. Graph A shows the ammonium removal rates among the treatments using *A. protothecoides*, *C. sorokiniana* (UTEX 2714), and *C. sorokiniana* (UTEX 2805). Graph B shows the phosphate removal rates among the treatments using *A. protothecoides*, *C. sorokiniana* (UTEX 2714), and *C. sorokiniana* (UTEX 2805). The reported nutrient removal rates for ammonium and phosphate are the average removal rates per nutrient over 120 hours. Negative values indicate that the nutrient concentration accumulated over time rather than being removed. Within a nutrient type and algal strain, removal rates with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test. Lower case letters indicate significance among the treatments for *A. protothecoides*. Upper case letters indicate significance among the treatments for *C. sorokiniana* (UTEX 2714). Bold upper-case letters indicate significance among the treatments for *C. sorokiniana* (UTEX 2805).

In addition to nitrogen and phosphorus nutrients, soluble nutrient metals were also analyzed by ion chromatography. It was initially speculated that differences in growth between the mixed and axenic cultures may have been a result of lower magnesium and calcium concentrations rather

than a growth-promoting effect of the digester microbes. Inadvertent loss of magnesium and calcium nutrients during filtration of axenic cultures could have created an experimental artifact. It has been shown that low metal ion concentrations can suppress algae growth (Wong et al., 1978). Additionally, magnesium in particular, can cause phosphate to precipitate out of swine wastewater and possibly other agriculture wastes, which makes phosphate unavailable for algae to utilize and remove from the wastewater (Huang et al., 2015). Similar concentrations of soluble magnesium and calcium ions concentrations in both filtered and unfiltered digestate indicates that loss of bioavailable calcium and magnesium during filtration was negligible (Table 5). There was a significant difference in magnesium ($p < 0.0001$) and calcium ($p < 0.0001$) between treatments for *C. sorokiniana* (UTEX 2714) and the calcium concentration for the axenic cultures in *A. protothecoides* was also significantly lower ($p < 0.0290$) than the other treatments. However, for *A. protothecoides* any significance observed was numerically small. Thus, these results suggest that any significant growth differences between axenic and mixed-cultures are due to the presence of digester microbes living in symbiosis with algae rather than a lack of metal micronutrients.

Table 5: Metal cation concentrations in the four-fold diluted AD used in each treatment.

Species Name	Strain Code	Treatment	Initial Mg Concentration (mg/L†)	Final Mg Concentration (mg/L†)	Initial Ca Concentration (mg/L†)	Final Ca Concentration (mg/L†)
A. protothecoides	UTEX 2341	Sterile axenic algae	2.71 (0.06) a ‡	2.22 (0.26) b	17.67 (0.23) b	18.07 (1.10) a
		Algae & AD Bacteria	2.82 (0.11) a	1.46 (0.11) c	18.54 (0.66) a	19.29 (1.21) a
		AD Bacteria	2.78 (0.03) a	3.19 (0.29) a	18.52 (0.27) a	17.46 (2.54) a
C. sorokiniana	UTEX 2714	Sterile axenic algae	4.54 (0.09) b ‡	4.22 (0.70) b	10.92 (0.38) c	11.43 (0.27) b
		Algae & AD Bacteria	5.64 (0.10) a	6.81 (1.08) ab	13.56 (0.10) b	15.22 (0.75) a
		AD Bacteria	5.82 (0.12) a	7.28 (1.44) a	15.36 (0.68) a	17.20 (1.78) a
C. sorokiniana	UTEX 2805	Sterile axenic algae	4.17 (0.29) a ‡	- a *****	15.12 (1.23) a	7.93 (6.89) a
		Algae & AD Bacteria	4.27 (0.66) a	- a *****	15.99 (1.59) a	9.25 (8.01) a
		AD Bacteria	4.69 (0.70) a	6.42 (5.56) a	17.74 (2.33) a	15.25 (3.95) a

***** Measured but not detectable.

† Reported values are averages of the triplicates for each treatment; Standard deviations are in parenthesis.

‡ Within an algal strain and column, values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

Phosphorus uptake is extremely important when devising new technologies to treat poultry litter, so the increased phosphorus removal in mixed cultures is noteworthy. It should be noted that the lower nutrient removal in both *C. sorokiniana* strains was surprising given the robust growth of *C. sorokiniana*, particularly strain 2805, on the digestate. In general, algae take up nutrients more quickly when they grow quickly. To follow up on this surprising result, we investigated the nitrogen content of the algae species in order to estimate biological uptake of nitrogen during growth.

4.6 TN and Protein in Biomass

Protein analyses showed that across all three experiments, only a small fraction (1.6-5%) of the biomass in both the axenic and mixed cultures was nitrogen (Table 6). The *A. prototheco*ides treatments yielded the highest percentage of nitrogenous biomass at approximately 5% for both the axenic and mixed cultures (Table 6). In contrast, *C. sorokiniana* had nitrogen contents in the range of 1-3% of DW. These findings mirror the nitrogen removal rates by the different algae strains. For all experiments, the mixed cultures had higher percentage of nitrogenous biomass and nitrogenous biomass as crude protein compared to their corresponding axenic cultures, although the differences were not statistically significant, ($p > 0.3303$) and ($p > 0.3303$) respectively. In addition, the increased nitrogen content in the mixed cultures correlated to an increase in nitrogenous uptake rate, regardless of the strain. *A. prototheco*ides' nitrogen uptake rate doubles when the digester microbes are co-cultured with it but the *C. sorokiniana* strain's uptake rate only slightly increases when co-cultured. These results indicate that nitrogen removal is a function of both the growth rate and any change in nitrogen content of the biomass.

The mentioned nitrogenous uptake rates differ from that of the ammonium removal rates that were reported earlier. Nitrogenous uptake refers to the algal strain's ability to assimilate extracellular nitrogen sources, from ammonium or nitrate, into intracellular nitrogen which can be utilized for amino acid synthesis and other biochemical processes. Ammonium removal rate refers to losses in ammonium due to a combination of algal uptake, ammonia volatilization, and nitrification. Lack of nitrite and unchanging nitrate concentrations in all treatments, for any of the three algal strains, indicates that no nitrification occurs. Thus, ammonium removal rates only refer to algal uptake and ammonia volatilization.

Table 6: Nitrogen & protein analyses for *A. protothecoides* and *C. sorokiniana* (UTEX 2714 & 2805).

Species Name	Strain Code	Treatment	Nitrogen in Biomass (%*†)	Nitrogenous Biomass as Crude Protein (%*†‡)	Nitrogen uptake rate (mg/L/d†)
A. protothecoides	UTEX 2341	Sterile axenic algae	5.01 (<0.01) a §	29.78 (0.01) a	4.97 (0.49) b
		Algae & AD Bacteria	5.19 (<0.01) a	30.85 (0.02) a	8.79 (1.06) a
C. sorokiniana	UTEX 2714	Sterile axenic algae	1.60 (<0.01) a §	9.49 (0.04) a	1.93 (0.70) a
		Algae & AD Bacteria	1.99 (<0.01) a	11.85 (<0.01) a	2.45 (0.18) a
C. sorokiniana	UTEX 2805	Sterile axenic algae	2.08 (<0.01) a §	12.39 (<0.01) a	4.32 (0.16) a
		Algae & AD Bacteria	2.29 (<0.01) a	13.64 (0.03) a	4.78 (1.30) a

***** All percentages are on a mass basis.

† Reported values are averages of the triplicates for each treatment; Standard deviations are in parenthesis.

‡ Crude protein was calculated by multiplying the nitrogen in the biomass by a factor of 6.25.

§ Within an algal strain and column, values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

Earlier it was shown that both *C. sorokiniana* species had an unusually high starch content and low lipid content (Table 4). It was therefore unsurprising that the *C. sorokiniana* strains had low protein content when grown on digestate. The overall low protein contents across all three species suggests that the low nutrient uptake rates observed (see Nutrient Removal from Digestate Effluent) are partially the result of the algal species' limited ability to accumulate cellular nitrogen

(Table 6). Collectively, these results indicate that culturing *C. sorokiniana* on digestates led to starch production at the expense of protein production, harming its ability to take up nitrogen.

4.7 COD Removal

It was initially hypothesized that water quality would be improved due to digestate microbes lowering COD, including algal photosynthates secreted. Overall, none of the biological treatments led to large changes (>20%) in COD levels although there were a few trends in the data. Mixed cultures of *A. protothecoides* and both *C. sorokiniana* strains exhibited minor increases in COD over the five-day culture period, less than 8 mg/L (Figure 6A and 6B). These findings conflicted with our initial hypothesis that bacteria in the digestate would consume algal photosynthate, resulting in a decline in COD (Higgins et al., 2018). Moreover, we saw little evidence that photosynthate significantly altered COD as exhibited by nearly unchanged COD levels in the axenic algal axenic cultures over time. Interestingly, *C. sorokiniana* (UTEX 2805) axenic cultures had a significantly larger increase in COD over time ($p > 0.0075$), compared to the mixed cultures (Figure 6C). This suggests that the increase in COD, presumably triggered by release of algal photosynthate, was muted by the presence of wastewater microbes. Acting alone, the digester microbial cultures generally yielded small decreases in COD but the reductions did not meet the threshold of statistical significance ($p > 0.0724$). These results were surprising because they conflicted with results from a similar study carried out on winery wastewater. In that study, the presence of microbes led to 38% reductions in COD ($p < 0.0001$), including consumption of algal photosynthate (Higgins et al., 2018). In that system, an aerobic process was utilized between the digester and algal system which is important distinction from the present study and a possible source of the observed differences.

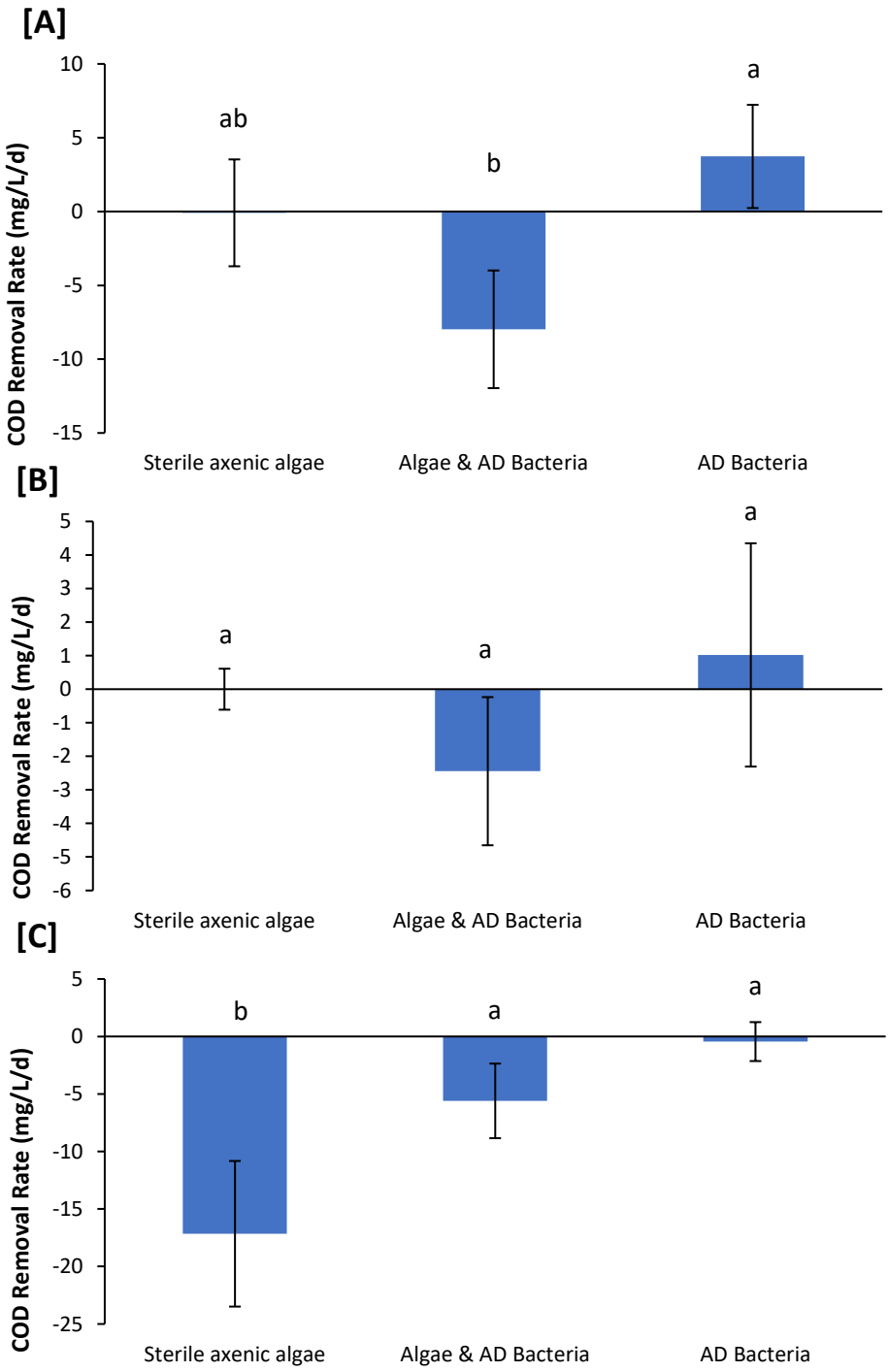


Figure 6: COD removal rates per treatment with *A. protothecoides* and *C. sorokiniana* (UTEX 2714 & 2805). Error bars are SD, n=3. Graph A shows the COD removal rates among the treatments using *A. protothecoides*. Graph B shows the COD removal rates among the treatments using *C. sorokiniana* (UTEX 2714). Graphs C shows the COD removal rates among the treatments using *C. sorokiniana* (UTEX 2805). The reported COD removal rates are the difference between the initial and final COD concentrations at hour-0 and hour-120, divided by the time period (5 days). Within an algal strain, removal rates at hour-120 with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

4.8.0 Activated Sludge Follow-up study

The results of previous experiments resulted in minimal changes in COD or even increases in COD concentration, regardless of the algal species grown with the digestate microbes. These results were contrary to what was hypothesized; thus, the following alternate hypothesis was subsequently tested: Aerobic, rather than anaerobic microbes are primarily responsible for COD removal from digestate.

4.8.1 Impact of Activated Sludge Addition on Algal growth

C. sorokiniana (UTEX 2805) was cultured on poultry litter digestates in the presence and absence of AS that was obtained from the aeration basins of a municipal wastewater treatment plant (Columbus, GA). The AS collected from the aeration basin is expected to include a wide range of aerobic heterotrophic and nitrifying bacteria. *C. sorokiniana* (UTEX 2805) was used to test the impact of aerobic bacteria on COD removal because the mixed culture biomass growth was highest among the three strains tested. This strain was also originally isolated from a municipal wastewater treatment plant (de-Bashan et al., 2008), suggesting a high level of compatibility with wastewater organisms. On their own, AD microbes showed no growth, similar to the findings in the previous experiments (Figure 7). Addition of AS to AD microbes resulted in higher biomass concentrations but still non-detectable culture growth. Both the algae and AD microbe treatment and the algae and AD microbes with AS treatment showed rapid algal growth overtime. Addition of algae to AD microbes yielded a 1.54 g (DW)/L (Figure 7) final biomass concentration which was consistent with what was observed in the previous experiment for *C sorokiniana* (UTEX 2805). When AS was added to the algae and AD microbes, the biomass concentration was observed to significantly increase to 1.91 g (DW)/L ($p < 0.0001$) in comparison to the algae and

AD microbe treatment. This is indicative of aerobic bacteria resulting in changes in biomass overtime rather than anaerobic microbes.

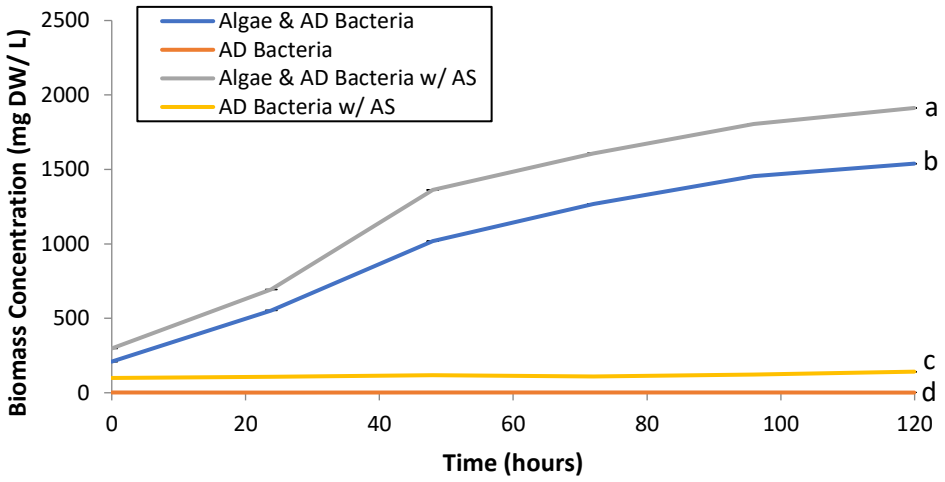


Figure 7: Daily average *C. sorokiniana* (UTEX 2805) DW for AS follow-up study.
Error bars for graph A are SD, n=3. The algae used is *C. sorokiniana* (UTEX 2805). The reported biomass concentrations are the average daily algae dry weight concentrations after the AD ODs have been accounted for. Biomass concentrations at hour-120 with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

Given the apparent growth-promoting effects of adding aerobic bacteria to the mixed culture in *C. sorokiniana* (UTEX 2805), we next examined the fraction of mixed-culture biomass that was bacteria vs. algae. This analysis still showed that a low fraction of biomass in the mixed culture, 2.11% (Table 7). When AS was added to the mixed culture, the bacterial fraction of biomass was also low, 2.91% (Table 7), indicating that most of the culture was algae. There was also no significant difference between the bacterial growth productivity (Table 7) between the mixed culture with AS and the mixed culture. Additionally, adding AS to the algae and AD microbes had no significant effect on the percentage of microbe present in the biomass, 5.19% without AS and 9.40% with AS ($p > 0.2917$). Thus, the addition of AS promoted algal growth.

Table 7: Fraction of *C. sorokiniana* (UTEX 2805) final biomass productivity as bacteria and as other biomass for the AS Follow-up study.

Species Name	Strain Code	Treatment	Total Average Biomass Productivity (mg/L/d)	Fraction of Productivity as Bacteria Growth (mg/L/d)	Fraction of Productivity as Other Biomass Growth (mg/L/d) ‡
C. sorokiniana	UTEX 2805	Algae & AD Bacteria	249.43 (2.29) b †	5.19 (2.29) a	244.24 (13.03) b
		AD Bacteria	0.01 (0.27) c	0.01 (0.27) b	- c *
		Algae & AD Bacteria w/ AS	322.91 (1.82) a §	9.40 (1.82) a §	313.51 (18.91) a §
		AD Bacteria w/ AS	9.11 (2.39) c	9.11 (2.39) a	- c *

***** Measured but not detectable.

† Reported values are averages of the triplicates for each treatment; Standard deviations are in parenthesis.

‡ Other biomass growth includes algal growth and any Archaea growth present.

§ Within a column, values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

4.8.2 Biomass Composition

Addition of AS to cultures of algae had little practical effect on the lipid content of the algae but did significantly decrease the starch content ($p < 0.0391$) (Table 8) When AS was added to the algae and AD microbes, there was approximately a 10% decrease in starch content by the end of the 5-day experimental period. Additionally, the biomass composition analyses show that aerobic bacteria may reduce the starch accumulation potential but that the overall starch content is still higher than what was usually observed with *C. sorokiniana* (UTEX 2805) (Higgins et al., 2018; Kobayashi et al., 2013). As discussed previously, high starch accumulation in *C. sorokiniana* is often driven by stress and it appears that addition of AS microbes may actually help alleviate stress conditions in *C. sorokiniana*. Work by Wang et al. has shown that addition of AS microbes to AD has the potential to greatly alleviate the inhibitory effects of AD (Wang et al., 2018). Viewed within this context, it therefore seems plausible that AS microbes may remove molecules that contribute to stress conditions for *C. sorokiniana*. The outcome is a reduction in starch content. As with previous experiments, it was apparent that stress conditions and alleviation of such conditions

did not stem from nutrient limitation (see section 4.8.3 “Effects on Nutrient Removal from Digestate Effluent”).

As for crude lipid contents, the presence of AS microbes had no significant effect on lipid content ($p > 0.7199$) but AS microbes did significantly decrease neutral lipids ($p < 0.0112$) even though lipid contents were low over all (Table 8).

Table 8: Biomass composition for AS follow-up study with *C. sorokiniana* (UTEX 2805).

Species Name	Strain Code	Treatment	Final Neutral Lipid Content (%*†)	Final Crude Lipid Content (%*†)	Final Starch Content (%*†)	Cell Wall Content (%*†)	Other Biomass Content (%*†‡)
C. sorokiniana	UTEX 2805	Algae & AD Bacteria	0.58 (0.12) a ¶	16.57 (1.85) a ¶	58.66 (1.11) a ¶	13.30 (0.68) b ¶	11.48 (2.27) a ¶
		AD Bacteria §	-	-	-	-	-
		Algae & AD Bacteria w/ AS	0.12 (0.13) b	16.13 (0.60) a	47.68 (6.19) b	19.10 (1.77) a	17.08 (4.01) a
		AD Bacteria w/ AS §	-	-	-	-	-

***** All percentages are on a mass basis.

† Reported values are averages of the triplicates for each treatment; Standard deviations are in parenthesis.

‡ Other Biomass Content is the difference between one and the summation of the final crude lipid, final starch, & cell wall contents. It is principally composed of ash, protein, and nucleic acids.

§ No lipid, starch, cell wall, or other biomass contents are reported for any of the three batch experiments' AD bacteria and AD bacteria w/ AS treatments.

¶ Within a column, values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

4.8.3 Effects on Nutrient Removal from Digestate Effluent

In the previous experiment, it was observed that *C. sorokiniana* (UTEX 2805) resulted in less than 10 mg/L/d ammonium removal and 7 mg/L/d phosphate removal across the treatments and there was no significant difference in the removal rates between the axenic and mixed cultures (ammonium and phosphate, $p > 0.5720$) (Figure 5A and 5B). Cultures with algae, AD microbes, and AS exhibited a significant 68.36% increase in ammonium removal ($p < 0.0026$) and a 20.86% increase in phosphate removal ($p < 0.0016$) compared to just the cultures with algae and AD microbes (Figure 8). One point of interest was that adding AS microbes to the AD cultures resulted in a similar ammonium removal rate to the algae and AD microbe cultures (9.95 mg/L/d vs. 11.16 mg/L/d, respectively). Acting alone, AD microbes removed little ammonium (0.29 mg/L/d). This indicates that even without the presence of algae, aerobic bacteria can facilitate increased ammonium removal that the poultry litter digestate microbial community was unable to do alone.

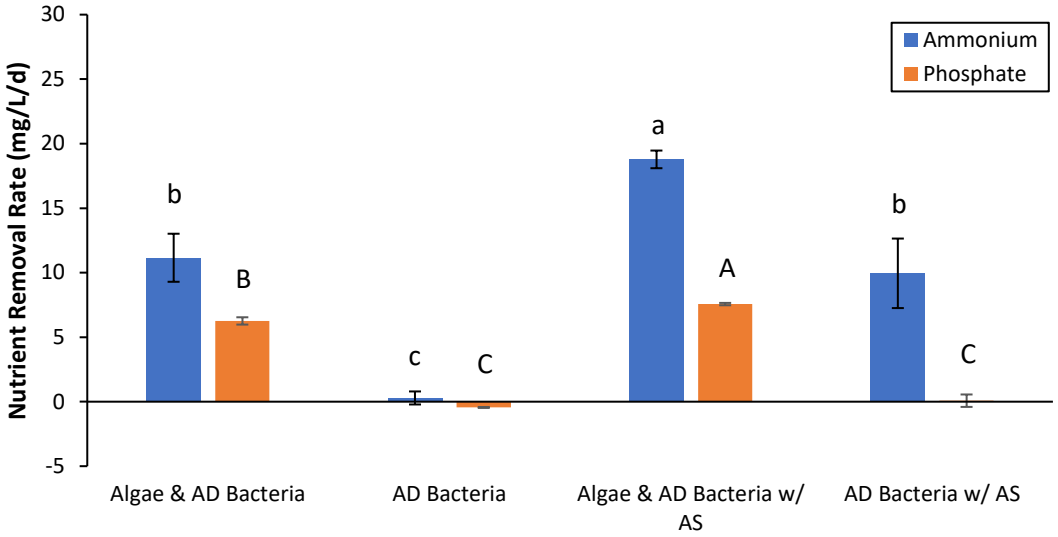


Figure 8: Ammonium and phosphate removal per treatment for the AS follow-up study .
Error bars for graph A are SD, n=3. The algae used is *C. sorokiniana* (UTEX 2805). The reported nutrient removal rates for ammonium and phosphate are the average removal rates per nutrient at hour-120. Initial ammonium concentrations were between 208-212 mg/L, while initial phosphate concentrations were between 103-109 mg/L. Within a nutrient type, removal rates at hour-120 with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test. Lower case letters indicate significance among the treatments for ammonium, while upper case letters indicate significance for phosphate among the treatments.

High pH values (around 8-10) allow for struvite constituted ions (Mg^{2+}, NH_4^+, PO_4^{3-}) to become supersaturated and thus precipitate out of the media, additionally magnesium concentrations influence pH and phosphorus recovery efficiency (Huang et al., 2015). Since all algal and non-algal cultures self-maintained a pH of about 6.8 throughout the 5-day experiment, it can be concluded that phosphate removal remained low (Figure 8) for all treatments due to the low pH values. Additionally, calcium ions react with carbonate anions and phosphate anions during methanogenic reactions occurring in the initial anaerobic digester setup to produce calcium precipitates (Chen et al., 2008; Ei-Mamouni et al., 1995; Keenan et al., 1993). It is possible that the combination of low pH values, and magnesium and calcium ion reactions allow for phosphate to precipitate out and thus contributing to phosphate removal in a chemical way rather than through cellular uptake.

4.8.4 TN and Protein in Biomass

Protein analyses showed that all treatments had low percentages of nitrogen present in the biomass (<2.5%) after the 5-day experimental period (Table 9). The presence of aerobic bacterium significantly increased the percentage of biomass as nitrogen for the algae, AD microbes, and AS cultures ($p > 0.0304$). Additionally, the crude protein as a percentage of biomass was 3.11% larger when aerobic bacteria were added to algae and AD microbes. There was also a significant increase in the nitrogenous uptake rate when AS was added ($p < 0.0052$). This suggests that the addition of aerobic bacteria has an influence on the nitrogenous uptake rate and the ability of *C. sorokiniana* (UTEX 2805) to cellularly accumulate nitrogenous biomass. This finding can also partially be explained by the apparent growth promotion of *C. sorokiniana* by AS microbes. AS microbes also lead to higher protein content ($p < 0.0311$) in *C. sorokiniana*. This corresponds with the lower starch content observed in the presence of AS microbes. Typically, algae have higher protein content under low-stress, high growth conditions (Sialve et al., 2009; Vitova et al., 2015).

Table 9: Percentage of nitrogen & protein in biomass for the AS follow-up study.

Species Name	Strain Code	Treatment	Nitrogen in Biomass (%*†)	Nitrogenous Biomass as Crude Protein (%*†)	Nitrogen uptake rate (mg/L/d†)
C. sorokiniana	UTEX 2805	Algae & AD Bacteria	1.72 (0.11) b ‡	10.74 (0.70) b ‡	4.29 (0.39) b ‡
		Algae & AD Bacteria w AS	2.22 (0.24) a	13.85 (1.49) a	7.15 (0.81) a

***** All percentages are on a mass basis.

† Reported values are averages of the triplicates for each treatment; Standard deviations are in parenthesis.

‡ Within a column, values with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

4.8.5 Impacts on COD Removal

It was initially hypothesized that AD microbes would lower COD concentrations and consume algal photosynthate in digestate when co-cultured with algae. As this was not confirmed by the data, we subsequently hypothesized that aerobic bacteria, rather than anaerobic microbes, are primarily responsible for COD removal from digestate.

It was observed that adding AS microbes to the algae and AD microbe cultures significantly increased the COD removal ($p < 0.0001$) compared to cultures with just algae and AD microbes. In fact, culturing algae with AD microbes actually led to an increase in COD which may have been driven largely by release of algal photosynthate (Figure 9). Higgins et al. previously observed that algae secreted photosynthate that resulted in increased COD in wastewater (Higgins et al., 2018). This demonstrated that adding aerobic bacterium to the algae and AD microbe cultures allowed for the aerobic bacterium to consume COD in the digestate in addition to algal photosynthate, resulting in a net decline in COD. This result aligns with (Higgins et al., 2018). Addition of AS microbes to AD cultures also resulted in faster COD removal ($p < 0.0025$) compared to just the AD microbe cultures (Figure 9). This confirms that aerobic bacteria were able to enhance COD removal in a consortium with anaerobic microbes. Of greater interest, the data suggest that algae have the capacity to enhance COD removal by AS microbes. Addition of algae resulted in 1,177% faster COD removal compared to AS and AD microbes alone ($p = 0.0038$)

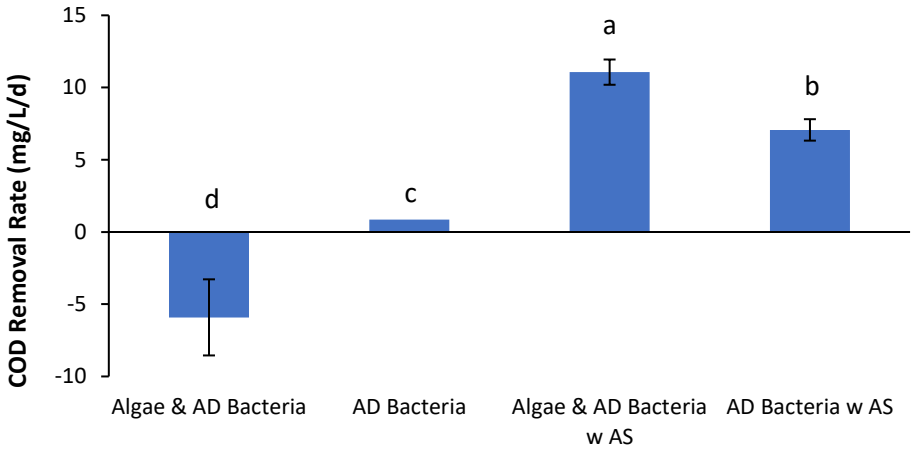


Figure 9: COD removal improvement after AS is added.
Error bars are SD, n=3, except for AD bacteria, which is n=2, and thus no error bar is shown. One data point in AD bacteria was excluded from the graph because one culture had a concentration of 289 mg/L while the other two cultures where in the 260-270 mg/L range. The graph shows the COD removal rates among the treatments using *C. sorokiniana* (UTEX 2805). The reported COD removal rates are the difference between the initial and final COD concentrations at hour-0 and hour-120, divided by the time period (5 days). The average initial COD concentrations for the mixed cultures, AD bacteria, mixed cultures with AS, and AD bacteria with AS were 265, 274, 257, and 271 mg/L, respectively. Within a treatment, removal rates at with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test.

4.8.6 Observed Nitrification with Presence of Activated Sludge

Unlike previously, nitrate and nitrite concentrations reveled interesting and distinct trends through the data, but sulfate concentrations while above zero, were static for all treatments over the 5-day experiment (see Appendix Figure 3 for data). At the end of the experimental period, the AD microbes with AS treatment experienced a moderate and significant increase in nitrate ($p < 0.0008$) concentration in comparison to concentrations in the AD microbe treatment (Figure 10A). Moreover, the AD microbes and AS treatment was the only set of cultures to have any and significant increases in nitrite ($p < 0.0001$) (Figure 10B). By the end of the experimental period, the AD microbes and AS treatment had accumulated 80.12 mg/L of nitrite (Figure 10B). Cultures containing algae, AD microbes, and AS yielded the highest final nitrate concentration at 62 mg/L (Figure 10A). In fact, adding AS to algae and AD microbes increased nitrate concentrations by 351% ($p < 0.0001$). Moreover, the addition of AS had no affect on nitrite concentration when

added to algae and AD microbe cultures (Figure 10B). The combination of the large nitrite increase, and moderate nitrate accumulation in the presence of AS with AD microbes indicates that the AS community resulted in at least partial nitrification of ammonium present in the digestate. Additionally, adding algae to the aerobic and anaerobic consortium leads to complete nitrification which indicates a symbiotic relationship between algae and nitrifying organisms in the AS. The presence of a symbiotic interaction between the AS and algae suggests that adding AS introduces the presence of nitrifying bacteria, which are known to help convert ammonium to nitrite (ammonia oxidizing bacteria) and then to nitrate (nitrite oxidizing bacteria).

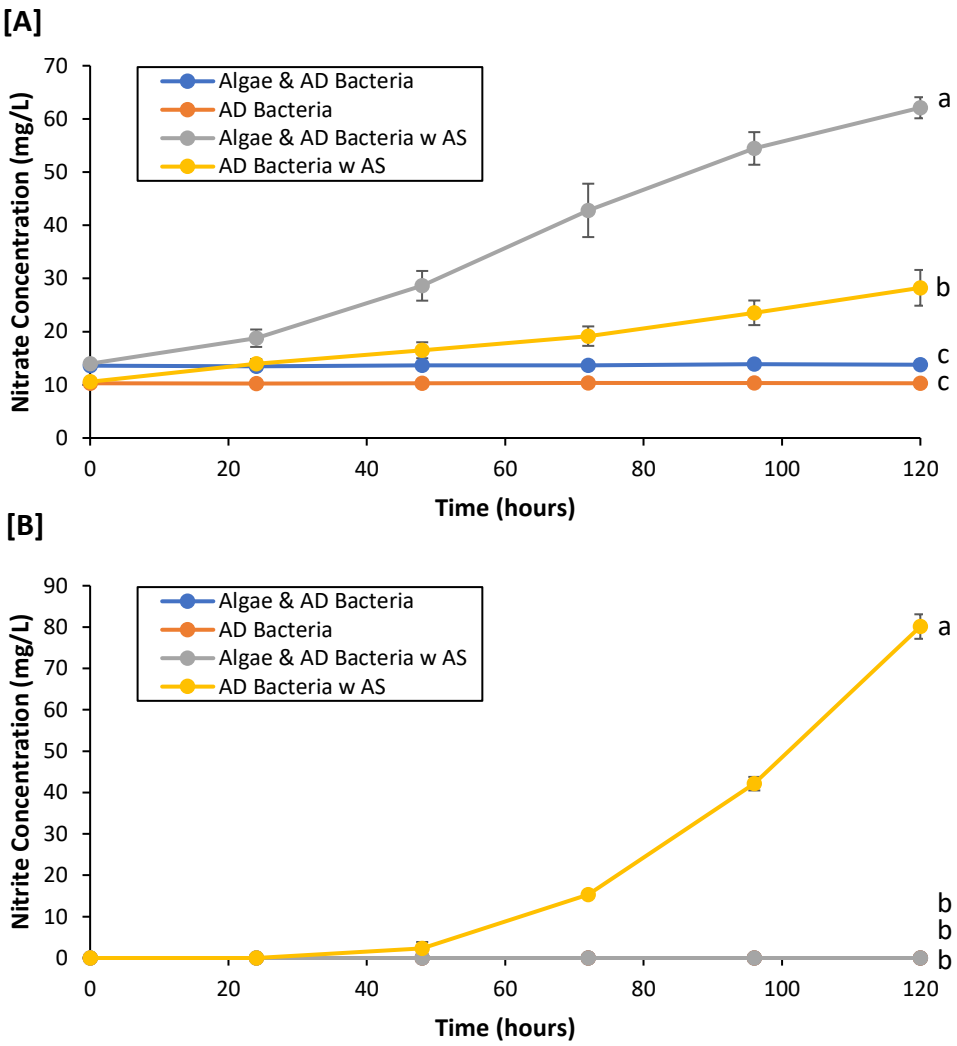


Figure 10: Nitrate and nitrite concentrations over the 5-day time period for the AS follow-up study. Error bars for graphs A and B are SD, n=3. Graph A shows the nitrate concentrations among the treatments using *C. sorokiniana* (UTEX 2805). Graph B shows the nitrite concentrations among the treatments using *C. sorokiniana* (UTEX 2805). The reported nutrient concentrations for nitrate and nitrite are the average concentration within a treatment per nutrient at hour-120. Within a nutrient type, concentrations at hour-120 with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test. Lower case letters indicate significance among the treatments.

4.8.7 Impacts of C. sorokiniana on the Microbial Community

Facilitated COD removal in the presence of algae with aerobic bacteria indicates that algae impact the live aerobic community in a way that allows for the consumption of algal photosynthate. Additionally, the observation of complete nitrification when algae are cultured with AS and partial nitrification when AD microbes were cultured with AS indicates that the presence of algae impacts the microbial community in some way that the AD microbes cannot. The genera *Nitrosomonas* and *Nitrobacter* are the most known nitrifying bacteria, which belong to the *Proteobacteria* phylum (Reece et al., 2014). The nitrate data suggested that adding AS to cultures introduces ammonia-oxidizing bacteria and nitrite-oxidizing bacteria into the community. Moreover, when the algae, AD microbes, and AS are co-cultured large nitrate concentrations and no nitrite is observed, which suggests similarly a symbiotic relationship where *C. sorokiniana* increases ammonium to nitrite conversion by nitrite-oxidizing bacterium.

To determine if the nitrifying bacteria were introduced into the cultures with the AS inoculum or already present in the poultry litter digestate community, 16S rRNA tag sequencing was employed. From the sequencing results, phylum level and genus level data were obtained, however, over 500 genera were identified from the samples thus phylum level data was used to determine relative abundance in the samples. Sequencing results identified 41 phyla, all with varying dominance between the 6 treatments tested. A percent relative abundance analysis showed that the dominate phylum detected in the AD inoculum community was *Proteobacteria* (22.0%), followed by *Bacteroidetes* (20.9%), *Spirochaetes* (13.8%), *Firmicutes* (12.9%), and 9 other phylum categories (30.4%) (Figure 11). Percent relative abundance also showed that growing the AD microbes in an aerobic environment for 5 days significantly increased *Proteobacteria* abundance ($p < 0.0001$) and drastically decreased *Firmicutes* and *Bacteroidetes* abundance. Co-

culturing algae and AD microbes for 5 days showed a shift in the relative abundance of *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* with a large percent increase in the number of unassigned sequences observed but, overall, there was no addition of new phyla or an increase in abundance of interesting phyla (Figure 11). Percent relative abundance of *Bacteroidetes* significantly decreased along with other small abundance microorganisms that were observed in the AD inoculum ($p < 0.0001$). It appears that co-culturing algae and AD microbes causes a significant rise in unassigned sequences ($p < 0.0001$), which is likely a result of chimeric sequences that were not adequately filtered out. The above results correspond with the lack of COD removal observed and indicate that the *Proteobacteria* organisms present from AD were not nitrifying bacteria, like *Nitrosomonas* and *Nitrobacter*.

It was observed that the AS inoculum had several phyla that were not previously observed in the AD inoculum: *Acideobacteria*, *Actinobacteria*, *Armatimonadetes*, *Chlamydiae*, *Chlorobi*, *Elusimicrobia*, *Nitrospirae*, *Plantomycetes*, and TM7 (Figure 11). When the AS was cultured with AD microbes for 5 days the following changes were observed to the microbial community: *Proteobacteria* abundance significantly increased by 61.73% while *Bacteroidetes* and *Chloroflexi* abundance significantly decreased by 26.24% and 45.10% respectively ($p < 0.0001$). Adding algae resulted in similar changes to the microbial community: *Proteobacteria* significantly increase by 29.61% and *Bacteroidetes* and *Chloroflexi* abundance significantly decreased by 35.08% and 76.47% respectively ($p < 0.0001$).

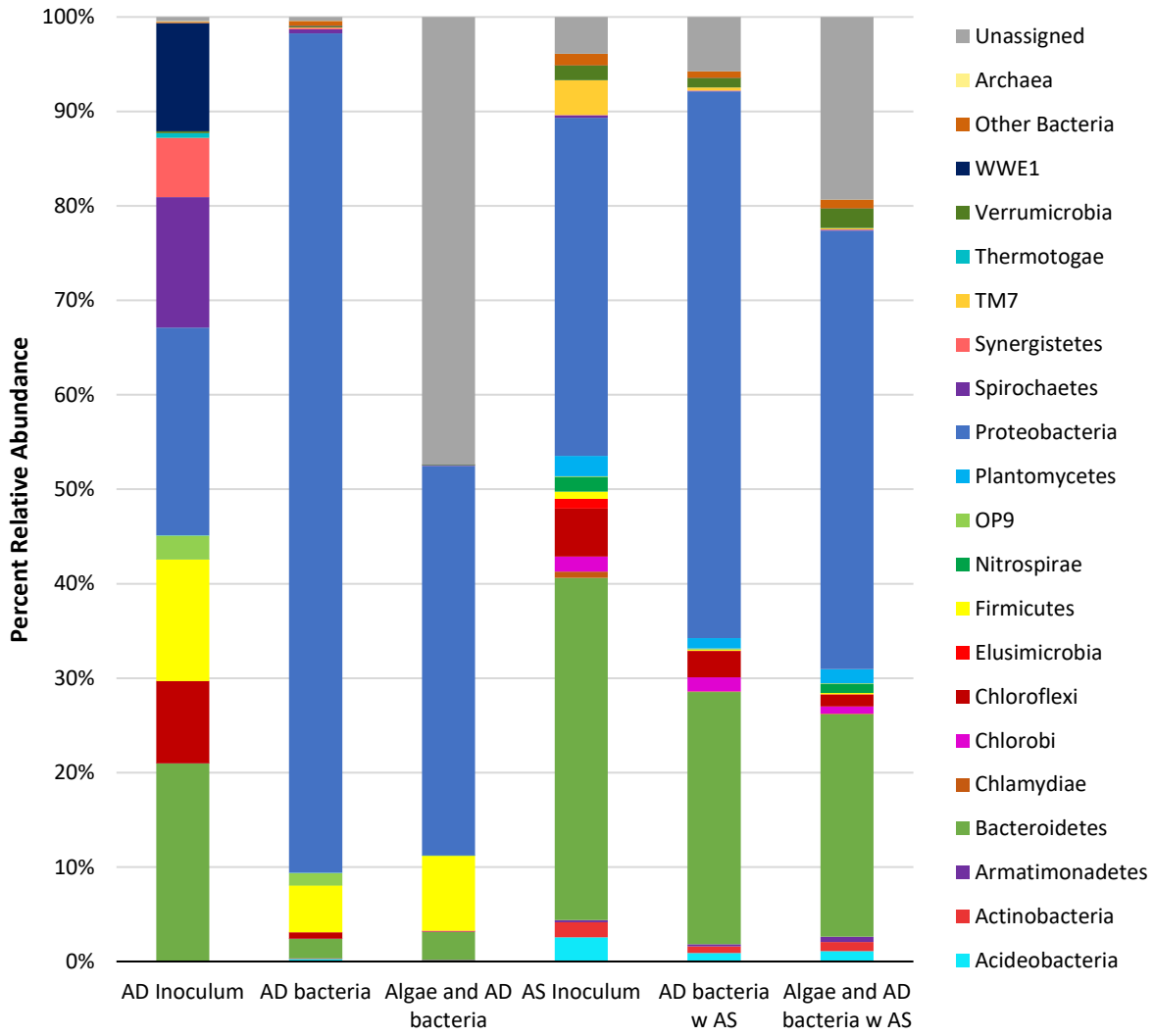


Figure 11: Percentage of phylums present in AS follow-up study treatments and inoculums. No Error bars are shown but n=3 for each treatment. The graph shows the percent relative abundance among the treatments and inoculums used. The reported relative abundances are the average abundance per treatment at hour-120.

When AS was added to algae and to AD microbes, the *Firmicutes* and OP9 phyla were found to be significantly reduced to 0.1% each ($p < 0.0286$) (Figure 11). On the other hand, the *Spirochaete* phylum was dominantly abundant in the AD inoculum but significantly reduced when grown in oxic conditions regardless of treatment ($p < 0.0001$). The *Synergistete* and WWE1 phyla were also found in the AD inoculum, however, both were completely repressed in all treatments but the AD microbe treatment. This suggests that the *Spirochaete*, *Synergistete*, and WWE1 phyla

present in the digestate were unable to propagate when grown in oxic conditions regardless of the presence of *C. sorokiniana* or aerobic bacteria from the AS.

The addition of AS to both the algae and AD microbe cultures and the AD microbe cultures introduced the following phyla into the microbial community, which were not previously present in the AD inoculum (Figure 11): *Armatimonadetes*, *Chlamydiae*, *Chlorobi*, *Elusimicrobia*, *Nitrospirae*, *Plantomycetes*, and TM7. Most known nitrite-oxidizing bacteria belong in either the *Proteobacteria* phylum or the *Nitrospirae* phylum; however the phylum *Chloroflexi* also contains a known nitrifying bacterium (Sorokin et al., 2012). A look at the *Nitrospirae* phylum abundance shows that *Nitrospirae* bacterium were introduced into the bioreactors through the addition of AS (Figure 12).

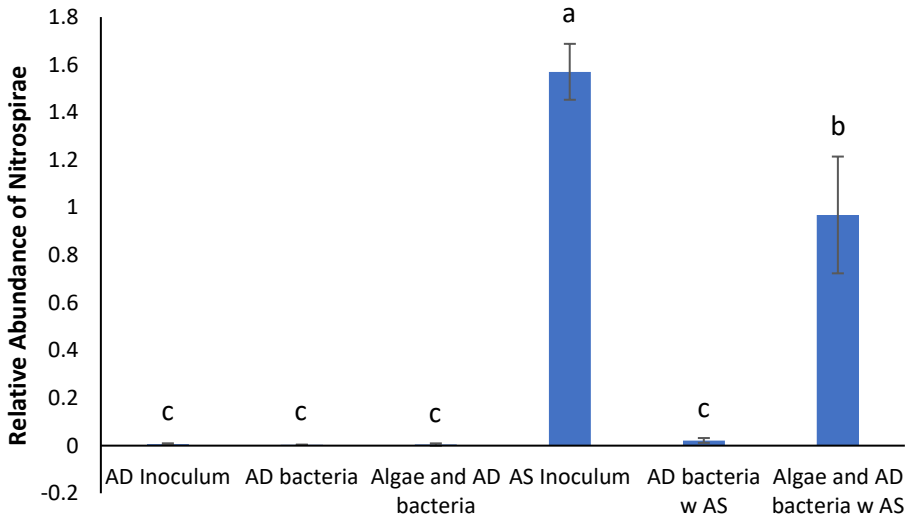


Figure 12: Relative abundance of *Nitrospirae* in AS follow-up study treatments and inoculums. Error bars for graph A are SD, n=3. The graph shows the percent relative abundance among the treatments and inoculums. The reported relative abundances are the average abundance per treatment at hour-120. Within a treatment, abundances with the same letter are not significantly different at the 0.05 level based on Tukey's HSD test. Lower case letters indicate significance among the treatments.

The data shows that *Nitrospirae* bacteria die out over time when cultured in the bioreactors with other AD microbes. As mentioned earlier, most known nitrifiers belong to the *Nitrospirae* and *Proteobacteria* phyla but *Nitrospirae* is most known for its nitrite-oxidizing bacteria (Koch et

al., 2015). The presence but slow die out of *Nitrospirae* in the cultures with AD microbes, and AS, can explain the high nitrite accumulation in these reactors. The die out over time of *Nitrospirae* could be a result of toxicity effects of the high ammonium concentrations found in the digestate. The relative abundance results show that the presence of algae reduce the abundance of *Nitrospirae* which also yields more complete nitrification. These results indicate that algae create a more favorable environment for *Nitrospirae* to inhabit. This could be in part due to algae providing excess dissolved oxygen or due to algae remediating possible toxicity effects of ammonium.

4.9 Mass Balance of Nitrogen and Phosphorus

Coupling anaerobic digestion followed by algal cultivation on the digestate liquid fraction can remove a large fraction of the COD remaining in the effluent as well as lowering dissolved nitrogen and phosphorus concentrations (de-Bashan et al., 2002; García et al., 2000; Higgins et al., 2018; Su et al., 2011). The results of this thesis also agree that combining these processes can increase removal of soluble nitrogen and phosphorus, but that COD removal only increases with the presence of AS in the photo-bioreactor. The process diagram shows that adding the photo-bioreactor step can reduce nitrogen and phosphorus content, on average, by 27% and 45% respectively, when using any of the algal strains tested without the addition of AS into the photo-bioreactor (Figure 13). Additionally, adding AS into the photo-bioreactor when growing *C. sorokiniana* (UTEX 2805) seems to aid in removing more nitrogen and phosphorus from the system, seeing as nitrogen reduction increased from 19% to 37% while phosphorus reduction increased from 26% to 35%. Without the addition of AS into the photo-bioreactor when growing *C. sorokiniana* (UTEX 2805), COD content increased 10% but when AS is added COD content is reduced by 22% (Figure 13).

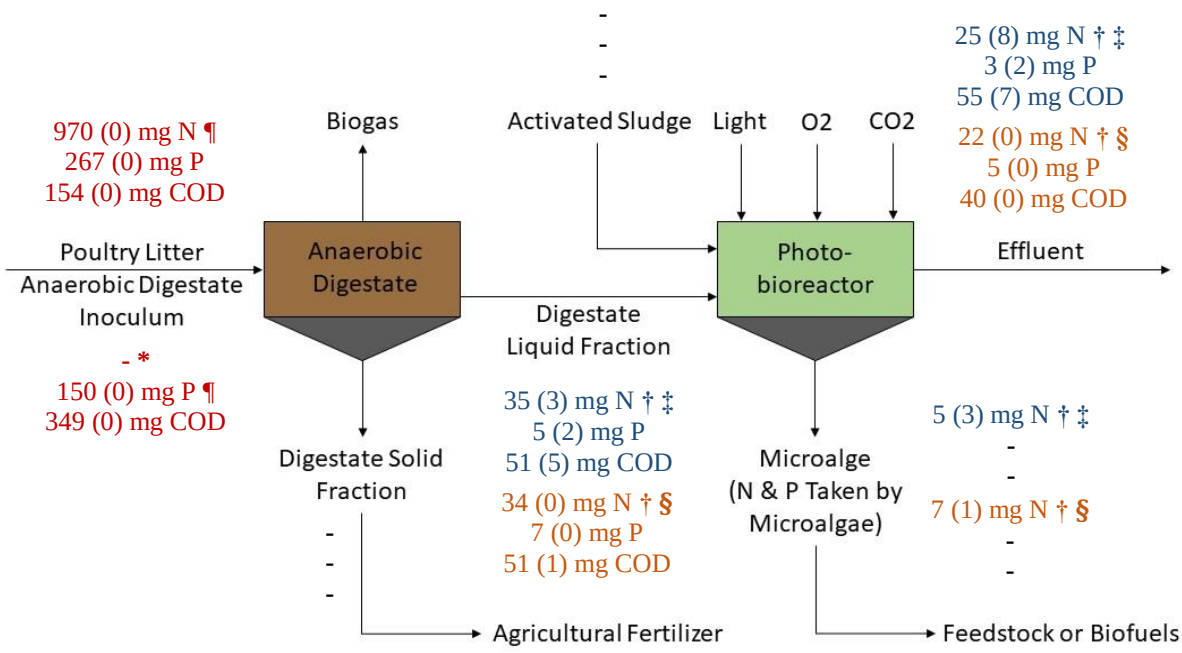


Figure 13: Mass balance of N, P, and COD when coupling of anaerobic digestion & algae cultivation to treat poultry litter waste.

N represents nitrogen while P equates to phosphorus. The dashes with a * indicate undetected values, black dashes without a * indicate that a value was not measured for that constituent. Masses indicated with † are based on experimental masses and volumes used. N masses were calculated by multiplying observed ion concentrations of nitrite, nitrate, and ammonium by the bioreactor volume and then summing these ion masses together after all values are converted to a nitrogen basis. P masses were calculated by multiplying observed ion concentrations of phosphate by the bioreactor volume after converting to a phosphorus basis. COD masses were calculated by multiplying observed COD concentrations by the bioreactor volume. Only the algal cultures with AD bacteria were used to calculate N, P, and COD masses for each experiment. Reported values with ‡ are averages of the triplicates for all three experiments without the addition of AS; Standard deviations are in parenthesis. Reported values with § are averages of the triplicates for the experiment with the addition of AS; Standard deviations are in parenthesis. Red reported values with ¶ are N, P, and COD masses using experimental weights added to the digestate and the percentage of N, P, and COD found in the litter and inoculum based on test results from Chaump et al. 2018. Blue reported values indicate calculated N, P, and COD masses for all experiments conducted without the addition of AS with *A. protothecoides* and *C. sorokiniana* (UTEX 2714 & 2805). Orange reported values indicate calculate N, P, and COD masses for the experiment conducted with the addition of AS to the bioreactor with *C. sorokiniana* (UTEX 2805).

Overall, from the introduction of poultry litter and inoculum into the digester to the effluent at the end of the photo-bioreactor step without AS addition, nitrogen and phosphorus contents are reduced by 97 and 98%, respectively, while COD is reduced by 89%. When AS is added to the photo-bioreactor, overall nitrogen, phosphorus, and COD content is reduced by 98, 97, and 92%, respectively. The process schematic shows that coupling AD and algal cultivation can reduce the

high N, P, and COD concentrations resulting from poultry litter but adding AS into this process can further improve COD removal, and nitrogen recovery and removal. However, low cellular N observed in algae, regardless of AS addition, indicates that there is a need to further improve nitrogen and phosphorus uptake by algal. Adding AS into the photo-bioreactor helped to facilitate better nitrogen and COD removal but more research is needed to determine how algae stimulate protein production during this coupling process.

5.0 Conclusions

It was hypothesized that growing algae in the presence of poultry litter digestate microbes would have minimal impact on the algal biomass growth and composition for all three strains tested. This hypothesis was partially disproven by this research. AD microbes promoted *A. protothecoides* growth and increased starch accumulation in two *C. sorokiniana* strains. Growth promotion was likely due to AD bacteria providing hormones or cofactors, like thiamine, to *A. protothecoides.* It is also possible that the AD community was responsible for causing oxidative stress in both *C. sorokiniana* strains which promoted an excess of starch mechanism enzymes.

It was also hypothesized that algae would reduce nitrogen and phosphorus concentrations in the digestate proportional to algal growth. This hypothesis was also disproved. Ammonium removal was improved with the presence of AD bacteria, but phosphate was only completely removed with *A. protothecoides*. Complete phosphate removal was most likely due to AD bacteria symbiosis with *A. protothecoides* providing need cofactors, which enhanced growth and thus nutrient uptake.

It was thought that water quality would be improved due to digestate microbes lowering COD, including any algal photosynthates secreted; however, results showed to the contrary. There were no significant changes in COD despite growth promotion and nutrient removal enhancement in *A. protothecoides.* Both *C. sorokiniana* strains also displayed no significant change in COD

removal. This leads us to believe that aerobic bacteria are responsible for COD removal rather than anaerobes.

Adding AS enhanced algal growth, nutrient removal, and cellular uptake of nitrogen in *C. sorokiniana* but reduced starch accumulation potential. This was likely due to AS alleviating the stress of *C. sorokiniana* growing in digestate. Aerobic bacterium also consumed COD in the digestate, when present with algae, resulting in a net decline in COD. Indicating that adding aerobic bacteria consumed algal photosynthate which native AD microbes were unable to do.

The AS inoculum enhanced the presence of some phyla already present in the digestate and introduced a variety of organisms into the bioreactors which were not native to the digestate community. AS was specifically shown to introduce a group of nitrifying bacteria from the *Nitrospirae* phylum. The presence of *Nitrospirae* coincided with full nitrification in cultures with algae. Without the presence of algae, *Nitrospirae* died out over time and reduced to only partial nitrification in those bioreactors, indicated by large nitrite increases. When *Nitrospirae* and algae were co-cultured, full nitrification was observed through large increases in nitrate, no nitrite, and reductions in ammonium.

The mass balance of nitrogen, phosphorus, and COD showed that coupling AD and algal cultivation has high potential for treating high organic wastes such as poultry litter. The addition of AS to this process also helped to facilitate nitrogen and COD removal and recovery. However, more research is needed to determine how much nitrogen, phosphorus, and COD is removed with the digestate solid fraction, and to determine how algae recover such nutrients and stimulate protein production during this process.

5.1 Future Work

It was interesting to find that *Nitrospirae* was unable to survive without the presence of *C. sorokiniana.* Thus, it would be beneficial to determine why *Nitrospirae* died out without the presence of algae. In this thesis we theorized that algae aided in *Nitrospirae* survival by either providing excess dissolved oxygen or by mitigating ammonium toxicity effects from the digestate. I propose conducting several bioreactor experiments to determine this. The effects of ammonium toxicity could be tested with several sets of dose response experiments where AS and *C. sorokiniana* are cultured in media with known concentrations of ammonium. After which qPCR and sequencing can be used to determine which ammonium concentrations had the highest and lowest abundance of *Nitrospirae* and additionally which concentrations saw full nitrification and partial nitrification.

6.0 References

Abdel-Hamid, A.M., Solbiati, J.O., Cann, I.K.O., 2013. Insights into Lignin Degradation and its Potential Industrial Applications. Adv. Appl. Microbiol. 82, 1–28. https://doi.org/10.1016/B978-0-12-407679-2.00001-6

Abdel-Raouf, N., Al-Homaidan, A.A., Ibraheem, I.B.M., 2012. Microalgae and wastewater treatment. Saudi J. Biol. Sci. 19, 257–275. https://doi.org/10.1016/J.SJBS.2012.04.005

Ahammad, S.Z., Sreekrishnan, T.R., 2016. Energy from Wastewater Treatment, in: Bioremediation and Bioeconomy. Elsevier, pp. 523–536. https://doi.org/10.1016/B978-0-12-802830-8.00020-4

ALFA Farmers, Alabama Farmers Federation, 2015. Alabama Farm Facts. Montgomery.

American Biogas Council, 2018. Biogas Market Snapshot | American Biogas Council [WWW Document]. Am. Biogas Counc. URL https://americanbiogascouncil.org/biogas-market-snapshot/ (accessed 5.25.19).

Ansa, E.D.O., Lubberding, H.J., Gijzen, H.J., 2012. The effect of algal biomass on the removal of faecal coliform from domestic wastewater. Appl. Water Sci. 2, 87–94. https://doi.org/10.1007/s13201-011-0025-y

Arkhipchenko, I.A., Shaposhnikov, A.I., Kravchenko, L.V., 2006. Tryptophan concentration of animal wastes and organic fertilizers. Appl. Soil Ecol. 34, 62–64. https://doi.org/10.1016/J.APSOIL.2005.12.008

Barakat, M.A., 2011. New trends in removing heavy metals from industrial wastewater. Arab. J. Chem. 4, 361–377. https://doi.org/10.1016/J.ARABJC.2010.07.019

Barry Hilton, B.L., Oleszkiewicz, J.A., 1988. SULFIDE-INDUCED INHIBITION OF ANAEROBIC DIGESTION. J. Environ. Eng. 114, 1377–1391. https://doi.org/https://doi.org/10.1061/(ASCE)0733-9372(1988)114:6(1377)

Benner, R., Maccubbin, A.E., Hodson, R.E., 1984. Anaerobic biodegradation of the lignin and polysaccharide components of lignocellulose and synthetic lignin by sediment microflora. Appl. Environ. Microbiol. 47, 998–1004.

Bernet, N., Delgenes, N., Akunna, J.C., Delgenes, J.P., Moletta, R., 2000. Combined anaerobic–aerobic SBR for the treatment of piggery wastewater. Water Res. 34, 611–619. https://doi.org/10.1016/S0043-1354(99)00170-0

Bjornsson, W.J., Nicol, R.W., Dickinson, K.E., Mcginn, P.J., 2013. Anaerobic digestates are useful nutrient sources for microalgae cultivation: functional coupling of energy and biomass production. J. Appl. Phycol. 25, 1523–1528. https://doi.org/10.1007/s10811-012-

9968-0

Bolan, N.S., Szogi, A.A., Chuasavathi, T., Seshadri, B., Rothrock, M.J., Panneerselvam, P., 2010. Uses and management of poultry litter. Worlds. Poult. Sci. J. 66, 673–698. https://doi.org/10.1017/S0043933910000656

Bolzonella, D., Pavan, P., Battistoni, P., Cecchi, F., 2005. Mesophilic anaerobic digestion of waste activated sludge: influence of the solid retention time in the wastewater treatment process. Process Biochem. 40, 1453–1460. https://doi.org/10.1016/j.procbio.2004.06.036

Borowski, S., Weatherley, L., 2013. Co-digestion of solid poultry manure with municipal sewage sludge. Bioresour. Technol. 142, 345–352. https://doi.org/10.1016/j.biortech.2013.05.047

Brányiková, I., Maršálková, B., Doucha, J., Brányik, T., Bišová, K., Zachleder, V., Vítová, M., 2011. Microalgae-novel highly efficient starch producers. Biotechnol. Bioeng. 108, 766–776. https://doi.org/10.1002/bit.23016

Brown, D., Li, Y., 2013. Solid state anaerobic co-digestion of yard waste and food waste for biogas production. Bioresour. Technol. 127, 275–280. https://doi.org/10.1016/J.BIORTECH.2012.09.081

Bujoczek, G., Oleszkiewicz, J., Sparling, R., Cenkowski, S., 2000. High Solid Anaerobic Digestion of Chicken Manure. J. Agric. Eng. Res. 76, 51–60. https://doi.org/10.1006/JAER.2000.0529

Burch, A.R., Franz, A.K., 2016. Combined nitrogen limitation and hydrogen peroxide treatment enhances neutral lipid accumulation in the marine diatom Phaeodactylum tricornutum. Bioresour. Technol. 219, 559–565. https://doi.org/10.1016/J.BIORTECH.2016.08.010

Cai, T., Park, S.Y., Li, Y., 2013. Nutrient recovery from wastewater streams by microalgae: Status and prospects. Renew. Sustain. Energy Rev. 19, 360–369. https://doi.org/10.1016/J.RSER.2012.11.030

Causapé, J., Quílez, D., Aragüés, R., 2004. Assessment of irrigation and environmental quality at the hydrological basin level: II. Salt and nitrate loads in irrigation return flows. Agric. Water Manag. 70, 211–228. https://doi.org/10.1016/J.AGWAT.2004.06.006

Chaump, K., Preisser, M., Shanmugam, S.R., Prasad, R., Adhikari, S., Higgins, B.T., 2019. Leaching and anaerobic digestion of poultry litter for biogas production and nutrient transformation. Waste Manag. 84, 413–422. https://doi.org/10.1016/j.wasman.2018.11.024

Chen, Y., Cheng, J.J., Creamer, K.S., 2008. Inhibition of anaerobic digestion process: A review. Bioresour. Technol. 99, 4044–4064. https://doi.org/10.1016/J.BIORTECH.2007.01.057

Cheng, D., Li, D., Yuan, Y., Zhou, L., Li, X., Wu, T., Wang, L., Zhao, Q., Wei, W., Sun, Y., 2017. Improving carbohydrate and starch accumulation in Chlorella sp. AE10 by a novel two-stage process with cell dilution. Biotechnol. Biofuels 10, 75. https://doi.org/10.1186/s13068-017-0753-9

Chisti, Y., 2007. Biodiesel from microalgae. Biotechnol. Adv. 25, 294–306. https://doi.org/10.1016/J.BIOTECHADV.2007.02.001

Cho, S., Lee, N., Park, S., Yu, J., Luong, T.T., Oh, Y.-K., Lee, T., 2013. Microalgae cultivation for bioenergy production using wastewaters from a municipal WWTP as nutritional sources.

Bioresour. Technol. 131, 515–520. https://doi.org/10.1016/J.BIORTECH.2012.12.176

Clijsters, I.I., Van Assche, F., 1985. Inhibition of photosynthesis by heavy metals. Photosynth. Res. 7, 31–40.

Coker, C., 2017. Poultry Litter Digestion. Biocycle vol 58 58, 1.

Conley, D.J., Paerl, H.W., Howarth, R.W., Boesch, D., Seitzinger, S.P., Havens, karl E., Lancelot, C., Likens, G.E., 2009. Controling Eutrophication: Nitrogen and Phosphorus. Science (80-.). 323, 1014–1015. https://doi.org/10.1126/science.1167755

Craggs, R.J., McAuley, P.J., Smith, V.J., 1997. Wastewater nutrient removal by marine microalgae grown on a corrugated raceway. Water Res. 31, 1701–1707. https://doi.org/10.1016/S0043-1354(96)00093-0

Davis, R., Aden, A., Pienkos, P.T., 2011. Techno-economic analysis of autotrophic microalgae for fuel production. Appl. Energy 88, 3524–3531. https://doi.org/10.1016/J.APENERGY.2011.04.018

de-Bashan, L.E., Moreno, M., Hernandez, J.-P., Bashan, Y., 2002. Removal of ammonium and phosphorus ions from synthetic wastewater by the microalgae Chlorella vulgaris coimmobilized in alginate beads with the microalgae growth-promoting bacterium Azospirillum brasilense. Water Res. 36, 2941–2948. https://doi.org/10.1016/S0043-1354(01)00522-X

de-Bashan, L.E., Trejo, A., Huss, V.A.R., Hernandez, J.-P., Bashan, Y., 2008. Chlorella sorokiniana UTEX 2805, a heat and intense, sunlight-tolerant microalga with potential for removing ammonium from wastewater. Bioresour. Technol. 99, 4980–4989. https://doi.org/10.1016/J.BIORTECH.2007.09.065

De La Noie, J., Lalibert6, G., Proulx, D., 1992. Algae and waste water. J. Appl. Phycol. 4, 247–254.

Dragone, G., Fernandes, B., Vicente, A.A., Teixeira, J.A., 2010. Third generation biofuels from microalgae, in: Current Research, Technology and Education Topics in Applied Microbial Biotechnology. Formatex Research Center, Braga, Portugal, pp. 1355–1366.

Dragone, G., Fernandes, B.D., Abreu, A.P., Vicente, A.A., Teixeira, J.A., 2011. Nutrient limitation as a strategy for increasing starch accumulation in microalgae. Appl. Energy 88, 3331–3335. https://doi.org/10.1016/J.APENERGY.2011.03.012

Edwards, D.R., Daniel, T.C., 1993. Effects of Poultry Litter Application Rate and Rainfall Intensity on Quality of Runoff from Fescuegrass Plots. J. Environ. Qual. 22, 361. https://doi.org/10.2134/jeq1993.00472425002200020017x

Ei-Mamouni, R., Guiot, S.R., Mercier, P., Sail, B., Samson, R., E1-Mamouni, R., 1995. Liming impact on granules activity of the reactor (MPAR) treating whey permeate multiplate anaerobic, Bioprocess Engineering lz. Springer-Verlag.

Elwell, D.L., Keener, H.M., Carey, D.S., Schlak, P.P., 1998. Composting Unamended Chicken Manure. Compost Sci. Util. 6, 22–35. https://doi.org/10.1080/1065657X.1998.10701918

Franchino, M., Tigini, V., Varese, G.C., Mussat Sartor, R., Bona, F., 2016. Microalgae treatment removes nutrients and reduces ecotoxicity of diluted piggery digestate. Sci. Total Environ.

569–570, 40–45. https://doi.org/10.1016/J.SCITOTENV.2016.06.100

García, J., Mujeriego, R., Hernández-Mariné, & M., 2000. High rate algal pond operating strategies for urban wastewater nitrogen removal, Journal of Applied Phycology.

Gonzalez, L.E., Bashan, Y., 2000. Increased growth of the microalga Chlorella vulgaris when coimmobilized and cocultured in alginate beads with the plant-growth-promoting bacterium Azospirillum brasilense. Appl. Environ. Microbiol. 66, 1527–31. https://doi.org/10.1128/AEM.66.4.1527-1531.2000

Guieysse, B., Borde, X., Muñoz, R., Hatti-Kaul, R., Nugier-Chauvin, C., Patin, H., Mattiasson, B., 2002. Influence of the initial composition of algal-bacterial microcosms on the degradation of salicylate in a fed-batch culture. Biotechnol. Lett. 24, 531–538. https://doi.org/10.1023/A:1014847616212

Gupta, S.K., Ansari, F.A., Shriwastav, A., Sahoo, N.K., Rawat, I., Bux, F., 2016. Dual role of Chlorella sorokiniana and Scenedesmus obliquus for comprehensive wastewater treatment and biomass production for bio-fuels. J. Clean. Prod. 115, 255–264. https://doi.org/10.1016/J.JCLEPRO.2015.12.040

Hanshew, A.S., Mason, C.J., Raffa, K.F., Currie, C.R., 2013. Minimization of chloroplast contamination in 16S rRNA gene pyrosequencing of insect herbivore bacterial communities. J. Microbiol. Methods 95, 149–155. https://doi.org/10.1016/J.MIMET.2013.08.007

Hartmann, H., Ahring, B.K., 2005. Anaerobic digestion of the organic fraction of municipal solid waste: Influence of co-digestion with manure. Water Res. 39, 1543–1552. https://doi.org/10.1016/j.watres.2005.02.001

Higgins, B.T., Gennity, I., Fitzgerald, P.S., Ceballos, S.J., Fiehn, O., VanderGheynst, J.S., 2018. Algal-bacterial synergy in treatment of winery wastewater. Nat. Partn. Journals 1, 6. https://doi.org/10.1038/s41545-018-0005-y

Higgins, B.T., Gennity, I., Samra, S., Kind, T., Fiehn, O., VanderGheynst, J.S., 2016. Cofactor symbiosis for enhanced algal growth, biofuel production, and wastewater treatment. Algal Res. 17, 308–315. https://doi.org/10.1016/J.ALGAL.2016.05.024

Higgins, B.T., Labavitch, J.M., VanderGheynst, J.S., 2015. Co-culturing Chlorella minutissima with Escherichia coli can increase neutral lipid production and improve biodiesel quality. Biotechnol. Bioeng. 112, 1801–1809. https://doi.org/10.1002/bit.25609

Higgins, B.T., VanderGheynst, J.S., 2014. Effects of Escherichia coli on Mixotrophic Growth of Chlorella minutissima and Production of Biofuel Precursors. PLoS One 9, e96807. https://doi.org/10.1371/journal.pone.0096807

Hogenhout, S.A., Loria, R., 2008. Virulence mechanisms of Gram-positive plant pathogenic bacteria. Curr. Opin. Plant Biol. 11, 449–456. https://doi.org/10.1016/J.PBI.2008.05.007

Hogenhout, S.A., Oshima, K., Ammar, E.-D., Kakizawa, S., Kingdom, H.N., Namba, S., 2008. Phytoplasmas: bacteria that manipulate plants and insects. Mol. Plant Pathol. 9, 403–423. https://doi.org/10.1111/j.1364-3703.2008.00472.x

Huang, H., Liu, J., Jiang, Y., 2015. Crystallization and precipitation of phosphate from swine wastewater by magnesium metal corrosion. Sci. Rep. 5, 1–13.

https://doi.org/10.1038/srep16601

Ishida, M., Haga, R., Odawara, Y., 1980. Anaerobic Digestion Process. 4,213,857.

Jenkins, A.H., Schyns, G., Potot, S., Sun, G., Begley, T.P., 2007. A new thiamin salvage pathway. Nat. Chem. Biol. 3, 492–497. https://doi.org/10.1038/nchembio.2007.13

Jiao, W., Chen, W., Chang, A.C., Page, A.L., 2012. Environmental risks of trace elements associated with long-term phosphate fertilizers applications: A review. Environ. Pollut. 168, 44–53. https://doi.org/10.1016/j.envpol.2012.03.052

Jumpertz, R., Le, D.S., Turnbaugh, P.J., Trinidad, C., Bogardus, C., Gordon, J.I., Krakoff, J., 2011. Energy-balance studies reveal associations between gut microbes, caloric load, and nutrient absorption in humans. Am. J. Clin. Nutr. 94, 58–65. https://doi.org/10.3945/ajcn.110.010132

Kassaveti, A., 2008. Dairy Waste Management: Treatment Methods and Potential Uses of Treated Waste, in: Waste Management for the Food Industries. Academic Press, pp. 801–860. https://doi.org/10.1016/B978-012373654-3.50016-X

Kearney, T.E., Larkin, M.J., Frost, J.P., Levett, P.N., 1993. Survival of pathogenic bacteria during mesophilic anaerobic digestion of animal waste. J. Appl. Bacteriol. 75, 215–219. https://doi.org/10.1111/j.1365-2672.1993.tb02768.x

Keenan, P.J., Iza, J., Switzenbaum, M.S., Leachate, W.L., Keenan, P.J., Iza, J., Switzenbaum, M.S., 1993. Inorganic Solids Development in a Pilot-Scale Anaerobic Reactor Treating Municipal Solid, Source: Water Environment Research.

Kelleher, B.., Leahy, J.., Henihan, A.., O'Dwyer, T.., Sutton, D., Leahy, M.., 2002. Advances in poultry litter disposal technology – a review. Bioresour. Technol. 83, 27–36. https://doi.org/10.1016/S0960-8524(01)00133-X

Keplinger, K.O., Hauck, L.M., 2006. The Economics of Manure Utilization: Model and Application. J. Agric. Resour. Econ. 31, 414–440.

Khalid, A., Arshad, M., Anjum, M., Mahmood, T., Dawson, L., 2011. The anaerobic digestion of solid organic waste. Waste Manag. 31, 1737–1744. https://doi.org/10.1016/j.wasman.2011.03.021

Kithome, M., Paul, J.W., Bomke, A.A., 1999. Reducing Nitrogen Losses during Simulated Composting of Poultry Manure using Adsorbents or Chemical Amendments. J. Environ. Qual. 28, 194–201. https://doi.org/10.2134/jeq1999.00472425002800010023x

Klocke, N.L., Watts, D.G., Schneekloth, J.P., Davison, D.R., Todd, R.W., 1999. Nitrate Leaching in Irrigated Corn and Soybean in a Semi-Arid Climate. Am. Soc. Agric. Eng. 42, 1621–1630.

Kobayashi, N., Noel, E.A., Barnes, A., Watson, A., Rosenberg, J.N., Erickson, G., Oyler, G.A., 2013. Characterization of three Chlorella sorokiniana strains in anaerobic digested effluent from cattle manure. Bioresour. Technol. 150, 377–386. https://doi.org/10.1016/J.BIORTECH.2013.10.032

Koch, H., Lücker, S., Albertsen, M., Kitzinger, K., Herbold, C., Spieck, E., Nielsen, P.H., Wagner, M., Daims, H., 2015. Expanded metabolic versatility of ubiquitous nitrite-

oxidizing bacteria from the genus Nitrospira. Proc. Natl. Acad. Sci. U. S. A. 112, 11371–6. https://doi.org/10.1073/pnas.1506533112

Kostic, A.D., Howitt, M.R., Garrett, W.S., 2013. Exploring host-microbiota interactions in animal models and humans. Genes Dev. 27, 701–718. https://doi.org/10.1101/gad.212522.112

Lange, I.G., Daxenberger, A., Schiffer, B., Witters, H., Ibarreta, D., Meyer, H.H.., 2002. Sex hormones originating from different livestock production systems: fate and potential disrupting activity in the environment. Anal. Chim. Acta 473, 27–37. https://doi.org/10.1016/S0003-2670(02)00748-1

Li, T., Gargouri, M., Feng, J., Park, J.-J., Gao, D., Miao, C., Dong, T., Gang, D.R., Chen, S., 2015. Regulation of starch and lipid accumulation in a microalga Chlorella sorokiniana. Biosource Technol. 180, 250–257. https://doi.org/10.1016/j.biortech.2015.01.005

Li, T., Zheng, Y., Yu, L., Chen, S., 2014. Mixotrophic cultivation of a Chlorella sorokiniana strain for enhanced biomass and lipid production. Biomass and Bioenergy 66, 204–213. https://doi.org/10.1016/J.BIOMBIOE.2014.04.010

Liverani, M., Waage, J., Barnett, T., Pfeiffer, D.U., Rushton, J., Rudge, J.W., Loevinsohn, M.E., Scoones, I., Smith, R.D., Cooper, B.S., White, L.J., Goh, S., Horby, P., Wren, B., Gundogdu, O., Woods, A., Coker, R.J., 2013. Understanding and Managing Zoonotic Risk in the New Livestock Industries. Environ. Health Perspect. 121, 873–877. https://doi.org/10.1289/ehp.1206001

Ljung, K., Maley, F., Cook, A., Weinstein, P., 2009. Acid sulfate soils and human health-A Millennium Ecosystem Assessment. Environ. Int. 35, 1234–1242. https://doi.org/10.1016/j.envint.2009.07.002

Logan, T.J., 1993. Agricultural best management practices for water pollution control: current issues. Agric. Ecosyst. Environ. 46, 223–231. https://doi.org/10.1016/0167-8809(93)90026-L

Lorenzen, A., Hendel, J.G., Conn, K.L., Bittman, S., Kwabiah, A.B., Lazarovitz, G., Massé, D., McAllister, T.A., Topp, E., 2004. Survey of hormone activities in municipal biosolids and animal manures. Environ. Toxicol. 19, 216–225. https://doi.org/10.1002/tox.20014

Lundquist, T.J., Woertz, I.C., Quinn, N.W.T., Benemann, J.R., 2010. A Realistic Technology and Engineering Assessment of Algae Biofuel Production. Berkeley, CA.

Mallin, M.A., Cahoon, L.B., 2003. Industrialized Animal Production-A Major Source of Nutrient and Microbial Pollution to Aquatic Ecosystems. Popul. Environ. 24, 369–385.

Manivasagan, P., Venkatesan, J., Sivakumar, K., Kim, S.-K., 2014. Pharmaceutically active secondary metabolites of marine actinobacteria. Microbiol. Res. 169, 262–278. https://doi.org/10.1016/J.MICRES.2013.07.014

Manyi-Loh, C., Mamphweli, S., Meyer, E., Makaka, G., Simon, M., Okoh, A., Manyi-Loh, C.E., Mamphweli, S.N., Meyer, E.L., Makaka, G., Simon, M., Okoh, A.I., 2016. An Overview of the Control of Bacterial Pathogens in Cattle Manure. Int. J. Environ. Res. Public Health 13, 843. https://doi.org/10.3390/ijerph13090843

Marcilhac, C., Sialve, B., Pourcher, A.-M., Ziebal, C., Bernet, N., Béline, F., 2014. Digestate

color and light intensity affect nutrient removal and competition phenomena in a microalgal-bacterial ecosystem. Water Res. 64, 278–287. https://doi.org/10.1016/J.WATRES.2014.07.012

Marshall, B.M., Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. Clin. Microbiol. Rev. 24, 718–33. https://doi.org/10.1128/CMR.00002-11

Mendes, R., Garbeva, P., Raaijmakers, J.M., 2013. The rhizosphere microbiome: significance of plant beneficial, plant pathogenic, and human pathogenic microorganisms. FEMS Microbiol. Rev. 37, 634–663. https://doi.org/10.1111/1574-6976.12028

Millner, P.D., 2009. Bioaerosols associated with animal production operations. Bioresour. Technol. 100, 5379–5385. https://doi.org/10.1016/J.BIORTECH.2009.03.026

Moore, P.A.J., Daniel, T.C., Sharpley, A.N., Wood, C.W., 1995. Poultry manure management: Environmentally sound options. J. Soil Water Conserv. 50, 321–327.

Mouget, J.-L., Dakhama, A., Lavoie, M.C., De La Notie, J., 1995. Algal growth enhancement by bacteria: Is consumption of photosynthetic oxygen involved?, FEMS Microbiology Ecology. https://doi.org/10.1111/j.1574-6941.1995.tb00159.x

Muñoz, R., Guieysse, B., 2006. Algal–bacterial processes for the treatment of hazardous contaminants: A review. Water Res. 40, 2799–2815. https://doi.org/10.1016/J.WATRES.2006.06.011

National Chicken Council, 2019. Broiler Chicken Industry Key Facts 2019 [WWW Document]. URL https://www.nationalchickencouncil.org/about-the-industry/statistics/broiler-chicken-industry-key-facts/ (accessed 5.25.19).

Nicholson, F.A., Groves, S.J., Chambers, B.J., 2005. Pathogen survival during livestock manure storage and following land application. Bioresour. Technol. 96, 135–143. https://doi.org/10.1016/J.BIORTECH.2004.02.030

NSTATE LLC, 2017. Alabama Economy [WWW Document]. Netstate.com. URL http://www.netstate.com/economy/al_economy.htm (accessed 4.29.19).

Oyewo, O.A., Agboola, O., Onyango, M.S., Popoola, P., Bobape, M.F., 2018. Current Methods for the Remediation of Acid Mine Drainage Including Continuous Removal of Metals From Wastewater and Mine Dump, in: Bio-Geotechnologies for Mine Site Rehabilitation. Elsevier, pp. 103–114. https://doi.org/10.1016/B978-0-12-812986-9.00006-3

Park, J., Jin, H.-F., Lim, B.-R., Park, K.-Y., Lee, K., 2010. Ammonia removal from anaerobic digestion effluent of livestock waste using green alga Scenedesmus sp. Bioresour. Technol. 101, 8649–8657. https://doi.org/10.1016/J.BIORTECH.2010.06.142

Perdue, S., Hamer, H., 2019. 2017 Census of Agriculture - Alabama State and County Data.

Pienkos, P.T., Laurens, L., Aden, A., 2011. Making Biofuel from Microalgae. Am. Sci. 99, 474. https://doi.org/10.1511/2011.93.474

Pires, J.C.M., Alvim-Ferraz, M.C.M., Martins, F.G., Simões, M., 2013. Wastewater treatment to enhance the economic viability of microalgae culture. Environ. Sci. Pollut. Res. 20, 5096–5105. https://doi.org/10.1007/s11356-013-1791-x

Pittman, J.K., Dean, A.P., Osundeko, O., 2011. The potential of sustainable algal biofuel

production using wastewater resources. Bioresour. Technol. 102, 17–25. https://doi.org/10.1016/J.BIORTECH.2010.06.035

Power, J.F., Dick, W.A., Sims, J.T., Pierzynski, G.M., 2000. Assessing the Impacts of Agricultural, Municipal, and Industrial By-Products on Soil Quality, in: Land Application of Agricultural, Industrial, and Municipal By-Products. Soil Science Society of America, pp. 237–261. https://doi.org/10.2136/sssabookser6.c7

Prandini, J.M., da Silva, M.L.B., Mezzari, M.P., Pirolli, M., Michelon, W., Soares, H.M., 2016. Enhancement of nutrient removal from swine wastewater digestate coupled to biogas purification by microalgae Scenedesmus spp. Bioresour. Technol. 202, 67–75. https://doi.org/10.1016/J.BIORTECH.2015.11.082

Preisser, M., 2018. Anaerobic Digestion Case Study: Identifying Viable Feedstock Sources for Small Scale Biogas Production at Auburn University. 2018 NCUR 0.

Preusch, P.L.L., Adler, P.R.R., Sikora, L.J.J., Tworkoksi, T.J., Tworkoski, T.J., 2002. Nitrogen and Phosphorus Availability in Composted and Uncomposted Poultry Litter. J. Environ. Qual. - Waste Manag. 31, 2051–2057. https://doi.org/10.2134/jeq2002.2051

Ramírez-García, R., Gohil, N., Singh, V., 2019. Recent Advances, Challenges, and Opportunities in Bioremediation of Hazardous Materials, in: Phytomanagement of Polluted Sites. Elsevier, pp. 517–568. https://doi.org/10.1016/B978-0-12-813912-7.00021-1

Ranade, V. V., Bhandari, V.M., Tantak, N., Chandan, N., Raina, P., 2014. An Introduction to Biological Treatment and Successful Application of the Aqua EMBR System in Treating Effluent Generated from a Chemical Manufacturing Unit: A Case Study, in: Industrial Wastewater Treatment, Recycling and Reuse. Butterworth-Heinemann, pp. 369–397. https://doi.org/10.1016/B978-0-08-099968-5.00009-X

Rawat, I., Ranjith Kumar, R., Mutanda, T., Bux, F., 2011. Dual role of microalgae: Phycoremediation of domestic wastewater and biomass production for sustainable biofuels production. Appl. Energy 88, 3411–3424. https://doi.org/10.1016/J.APENERGY.2010.11.025

Reece, J.B., Urry, L.A., Cain, M.L., Wasserman, S.A., Minorsky, P. V., Jackson, R.B., 2014. Bacteria and Archaea, in: Campbell Biology. Pearson, pp. 578, 582–583.

Resende, J.A., Silva, V.L., de Oliveira, T.L.R., de Oliveira Fortunato, S., da Costa Carneiro, J., Otenio, M.H., Diniz, C.G., 2014. Prevalence and persistence of potentially pathogenic and antibiotic resistant bacteria during anaerobic digestion treatment of cattle manure. Bioresour. Technol. 153, 284–291. https://doi.org/10.1016/J.BIORTECH.2013.12.007

Sánchez, A., Artola, A., Font, • Xavier, Gea, T., Barrena, R., Gabriel, D., Ngel Sánchez-Monedero, M.A.´, Roig, • Asunción, María, •, Cayuela, L., Mondini, • Claudio, 2015. Greenhouse gas emissions from organic waste composting. Environ. Chem. Lett. 13, 223–238. https://doi.org/10.1007/s10311-015-0507-5

Santos Dalólio, F., da Silva, J.N., Carneiro de Oliveira, A.C., Ferreira Tinôco, I. de F., Christiam Barbosa, R., Resende, M. de O., Teixeira Albino, L.F., Teixeira Coelho, S., 2017. Poultry litter as biomass energy: A review and future perspectives. Renew. Sustain. Energy Rev. 76, 941–949. https://doi.org/10.1016/J.RSER.2017.03.104

Saunders, O., Harrison, J., Fortuna, A.M., Whitefield, E., Bary, A., 2012. Effect of Anaerobic Digestion and Application Method on the Presence and Survivability of E. coli and Fecal Coliforms in Dairy Waste Applied to Soil. Water, Air, Soil Pollut. 223, 1055–1063. https://doi.org/10.1007/s11270-011-0923-5

Sialve, B., Bernet, N., Bernard, O., 2009. Anaerobic digestion of microalgae as a necessary step to make microalgal biodiesel sustainable. Biotechnol. Adv. 27, 409–416.

Silbergeld, E.K., Graham, J., Price, L.B., 2008. Industrial Food Animal Production, Antimicrobial Resistance, and Human Health. Annu. Rev. Public Heal. 29, 151–169. https://doi.org/10.1146/annurev.publhealth.29.020907.090904

Silkina, A., Zacharof, M.-P., Hery, G., Nouvel, T., Lovitt, R.W., 2017. Formulation and utilisation of spent anaerobic digestate fluids for the growth and product formation of single cell algal cultures in heterotrophic and autotrophic conditions. Bioresour. Technol. 244, 1445–1455. https://doi.org/10.1016/J.BIORTECH.2017.05.133

Sims, J.T., Edwards, A.C., Schoumans, O.F., Simard, R.R., 2000. Integrating Soil Phosphorus Testing into Environmentally Based Agricultural Management Practices. J. Environ. Qual. 29, 60. https://doi.org/10.2134/jeq2000.00472425002900010008x

Singh, K., Lee, K., Worley, J., Risse, L.M., Das, K.C., 2010. Anaerobic Digestion of Poultry Litter: A Review. Appl. Eng. Agric. 26, 677–688.

Singh, M., Reynolds, D.L., Das, K.C., 2011. Microalgal system for treatment of effluent from poultry litter anaerobic digestion. Bioresour. Technol. 102, 10841–10848. https://doi.org/10.1016/j.biortech.2011.09.037

Smillie, C.S., Smith, M.B., Friedman, J., Cordero, O.X., David, L.A., Alm, E.J., 2011. Ecology drives a global network of gene exchange connecting the human microbiome. Nature 480, 241–244. https://doi.org/10.1038/nature10571

Smith, A.M., Sharma, D., Lappin-Scott, H., Burton, S., Huber, D.H., 2014. Microbial community structure of a pilot-scale thermophilic anaerobic digester treating poultry litter. Appl. Microbiol. Biotechnol. 98, 2321–2334. https://doi.org/10.1007/s00253-013-5144-y

Smith, S.R., Lang, N.L., Cheung, K.H.M., Spanoudaki, K., 2005. Factors controlling pathogen destruction during anaerobic digestion of biowastes. Waste Manag. 25, 417–425. https://doi.org/10.1016/J.WASMAN.2005.02.010

Snaidr, J., Amann, R., Huber, I., Ludwig, W., Schleifer, K.-H., 1997. Phylogenetic Analysis and In Situ Identification of Bacteria in Activated Sludge, APPLIED AND ENVIRONMENTAL MICROBIOLOGY.

Sorokin, D.Y., Lücker, S., Vejmelkova, D., Kostrikina, N.A., Kleerebezem, R., Rijpstra, W.I.C., Damsté, J.S.S., Le Paslier, D., Muyzer, G., Wagner, M., van Loosdrecht, M.C.M., Daims, H., 2012. Nitrification expanded: discovery, physiology and genomics of a nitrite-oxidizing bacterium from the phylum Chloroflexi. ISME J. 6, 2245–2256. https://doi.org/10.1038/ismej.2012.70

Spiehs, M., Goyal, S., 2007. Best ManageMent Practices for Pathogen control in Manure ManageMent systeMs.

Sturme, M.H.J., Gong, Y., Heinrich, J.M., Klok, A.J., Eggink, G., Wang, D., Xu, J., Wijffels,

R.H., 2018. Transcriptome analysis reveals the genetic foundation for the dynamics of starch and lipid production in Ettlia oleoabundans. Algal Res. 33, 142–155. https://doi.org/10.1016/J.ALGAL.2018.05.004

Su, Y., Mennerich, A., Urban, B., 2011. Municipal wastewater treatment and biomass accumulation with a wastewater-born and settleable algal-bacterial culture. Water Res. 45, 3351–3358. https://doi.org/10.1016/J.WATRES.2011.03.046

Tanadul, O., Vandergheynst, J., Beckles, D., Powell, A., Labavitch, J., 2014. The impact of elevated CO2 concentration on quality of algal starch as a potential biofuel feedstock. Biotechnol. Bioeng. 111, 1323–1331. https://doi.org/10.1002/bit.25203

Uggetti, E., Sialve, B., Latrille, E., Steyer, J.-P., 2014. Anaerobic digestate as substrate for microalgae culture: The role of ammonium concentration on the microalgae productivity. Bioresour. Technol. 152, 437–443. https://doi.org/10.1016/J.BIORTECH.2013.11.036

US Poultry and Egg Association, 2019. Industry Economic Data, Consumption, Exports, Processing, Production: uspoultry.org [WWW Document]. USDA. URL https://www.uspoultry.org/economic_data/ (accessed 5.25.19).

Větrovský, T., Steffen, K.T., Baldrian, P., 2014. Potential of Cometabolic Transformation of Polysaccharides and Lignin in Lignocellulose by Soil Actinobacteria. PLoS One 9, e89108. https://doi.org/10.1371/journal.pone.0089108

Vitova, M., Bisova, K., Kawano, S., Zachleder, V., 2015. Accumulation of energy reserves in algae: From cell cycles to biotechnological applications. Biotechnol. Adv. 33, 1204–1218. https://doi.org/10.1016/J.BIOTECHADV.2015.04.012

Wang, Haijun, Wang, Hongzhu, 2009. Mitigation of lake eutrophication: Loosen nitrogen control and focus on phosphorus abatement. Prog. Nat. Sci. 19, 1445–1451. https://doi.org/10.1016/J.PNSC.2009.03.009

Wang, Q., Higgins, B., Ji, H., Zhao, D., 2018. Improved microalgae biomass production and wastewater treatment: Pre-treating municipal anaerobic digestate for algae cultivation Written for presentation at the 2018 ASABE Annual International Meeting Sponsored by ASABE Detroit, Michigan. 2018 ASABE Annu. Int. Meet. 2–9. https://doi.org/10.13031/aim.201801333

Wang, X., Yang, G., Feng, Y., Ren, G., Han, X., 2012. Optimizing feeding composition and carbon-nitrogen ratios for improved methane yield during anaerobic co-digestion of dairy, chicken manure and wheat straw. Bioresour. Technol. 120, 78–83. https://doi.org/10.1016/j.biortech.2012.06.058

Ward, A.J., Hobbs, P.J., Holliman, P.J., Jones, D.L., 2008. Optimisation of the anaerobic digestion of agricultural resources. Bioresour. Technol. 99, 7928–7940. https://doi.org/10.1016/j.biortech.2008.02.044

Webb, A.R., Hawkest, F.R., 1985. The Anaerobic Digestion of Poultry Manure: Variation of Gas Yield With Influent Concentration and Ammonium-Nitrogen Levels, Agricultural Wastes.

Westfall, P.H., Tobias, R.D., Wolfinger, R.D., 2011. Multiple Comparisons and Multiple Tests Using SAS - Peter H. Westfall, Randall D. Tobias, Russell D. Wolfinger - Google Books, in: Multiple Comparisons and Multiple Tests Using SAS. SAS Institute Inc., Cary, NC, pp.

213–245.

Woertz, I., Feffer, ; A, Lundquist, ; T, Nelson, Y., 2009. Algae Grown on Dairy and Municipal Wastewater for Simultaneous Nutrient Removal and Lipid Production for Biofuel Feedstock. J. Environ. Eng. 135, 1115–1122. https://doi.org/10.1061/ASCEEE.1943-7870.0000129

Wong, P.T.., Chau, Y.K., Luxon, P.L., 1978. Toxicity of a Mixture of Metals on Freshwater Algae. Can. J. Fish. Aquat. Sci. 35, 479–481. https://doi.org/https://doi.org/10.1139/f78-084

Woolhouse, M.E.J., Gowtage-Sequeria, S., 2005. Host range and emerging and reemerging pathogens. Emerg. Infect. Dis. 11, 1842–7. https://doi.org/10.3201/eid1112.050997

Wright, A.L., Provin, T.L., Hons, F.M., Zuberer, D.A., White, R.H., 2008. Compost impacts on dissolved organic carbon and available nitrogen and phosphorus in turfgrass soil. Waste Manag. 28, 1057–1063. https://doi.org/10.1016/J.WASMAN.2007.04.003

Yao, Y., 2016. Cronicon O P E N A C C E S S BACTERIOLOGY AND VIROLOGY RESEARCH Effect of Illumination on the Metabolic Pathways in Anaerobic Digestion.

Yilancioglu, K., Cokol, M., Pastirmaci, I., Erman, B., Cetiner, S., 2014. Oxidative Stress Is a Mediator for Increased Lipid Accumulation in a Newly Isolated Dunaliella salina Strain. PLoS One 9, e91957. https://doi.org/10.1371/journal.pone.0091957

Zhang, T., Shao, M.-F., Ye, L., 2011. 454 Pyrosequencing reveals bacterial diversity of activated sludge from 14 sewage treatment plants. ISME J. 6, 1137–1147. https://doi.org/10.1038/ismej.2011.188

Zhang, Y., Zamudio Cañas, E.M., Zhu, Z., Linville, J.L., Chen, S., He, Q., 2011. Robustness of archaeal populations in anaerobic co-digestion of dairy and poultry wastes. Bioresour. Technol. 102, 779–785. https://doi.org/10.1016/J.BIORTECH.2010.08.104

Zhao, C., Brück, T., Lercher, J.A., 2013. Catalytic deoxygenation of microalgae oil to green hydrocarbons. Green Chem. 15, 1720. https://doi.org/10.1039/c3gc40558c

Ziganshina, E.E., Belostotskiy, D.E., Ilinskaya, O.N., Boulygina, E.A., Grigoryeva, T. V., Ziganshin, A.M., 2015. Effect of the Organic Loading Rate Increase and the Presence of Zeolite on Microbial Community Composition and Process Stability During Anaerobic Digestion of Chicken Wastes. Microb. Ecol. 70, 948–960. https://doi.org/10.1007/s00248-015-0635-2

7.0 Appendix

7.1 Appendix Table 1

Appendix Table 1: Control media recipes used for *A. protothecoides and* C. sorokiniana (UTEX 2714 and 2805).

	Compounds	N8 Media *§	N8-NH4 Media *‡
10x Macronutrients	KNO3 (g)	10	- †
	NH4Cl (g)	- †	7.64
	KH2PO4 (g)	7.4	9.75
	Na2HPO4	2.07	- †
	CaCl2*2H2O (g)	0.13	0.13
	FeNa EDTA (g)	0.1	0.1
	MgSO4 (g)	0.25	0.25
	DH2O volume (mL)	1000	1000
Micronutrients	Al2(SO4)3*18H2O (g/L)	3.58	3.58
	MnCl2*4H2O (g/L)	12.98	12.98
	CuSO4*5H2O (g/L)	1.83	1.83
	ZnSO4*7H2O (g/L)	3.2	3.2
Nutrient ratios for each media	10x Macronutrients (mL)	100	100
	Micronutrients (mL)	1.0	1.0
	DH2O (mL)	699.0	699.0

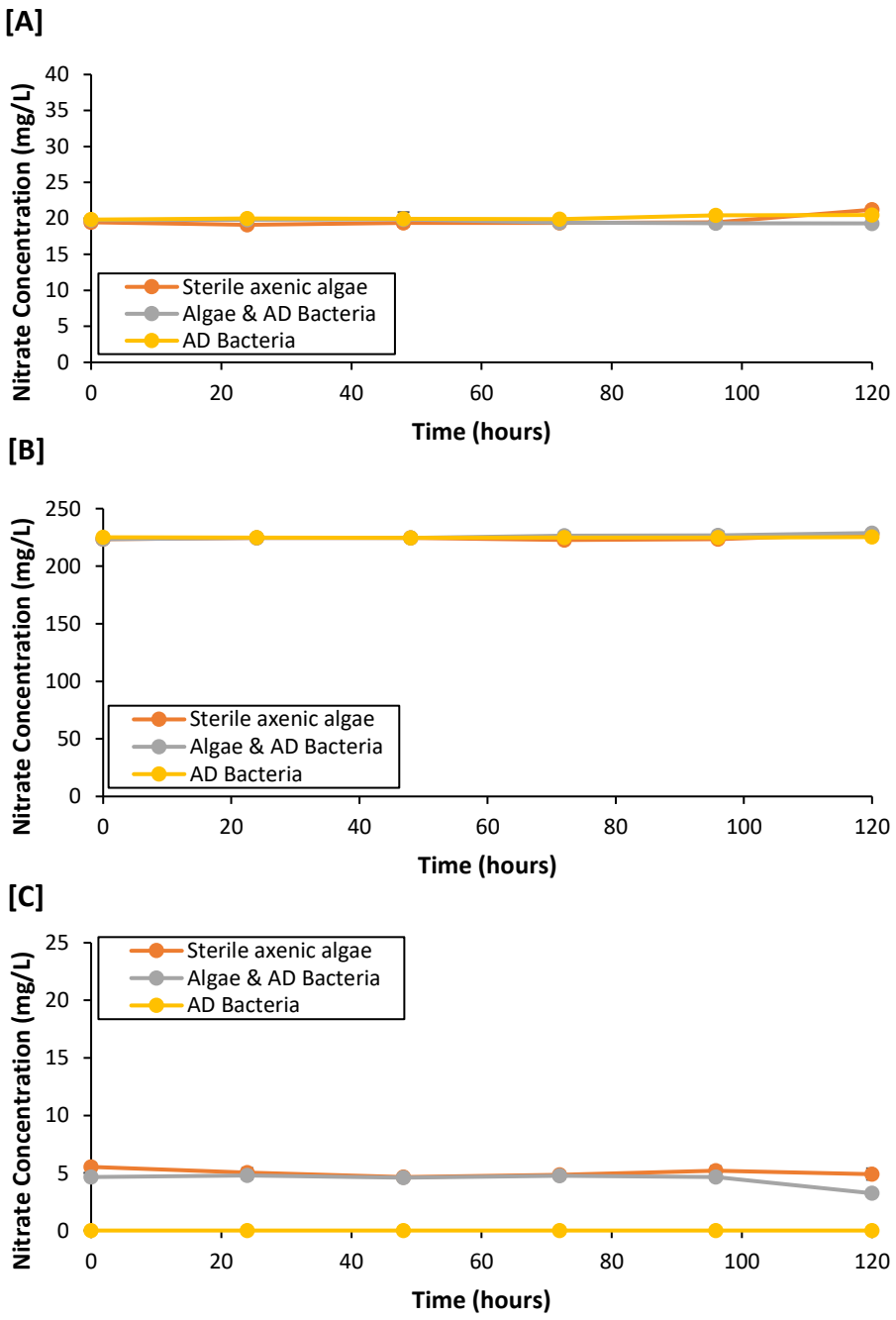
***** All media pH's were adjusted to 7.2 with NaOH

† Indicates that compound is not used in the specified minimal media.

‡ Reported recipe comes from Higgins et al. (2014).

§ Reported recipe comes from Tanadul et al. (2014).

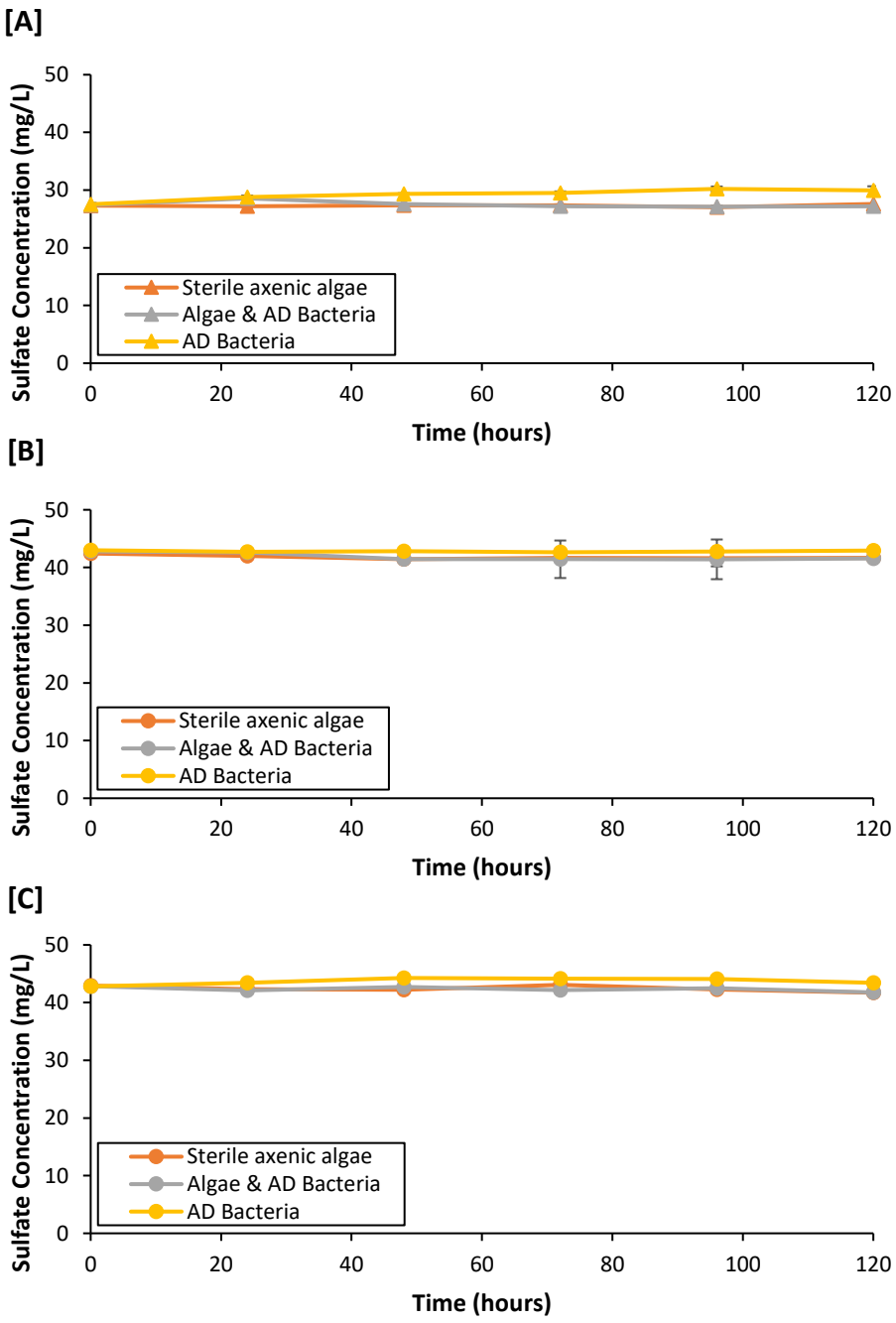
7.2 Appendix Figure 1



Appendix Figure 1: Nitrate concentration during the 5-day experiment for *A. protothecoides* and *C. sorokiniana* (UTEX 2714 & 2805).

Error bars for graphs A, B, and C are SD, n=3. Graph A shows the nitrate concentrations among the treatments using A. protothecoides. Graph B shows the nitrate concentrations among the treatments using C. sorokiniana (UTEX 2714). Graph C shows the nitrate concentrations among the treatments using C. sorokiniana (UTEX 2805). The reported nitrate concentrations are the average concentration per treatment at hour-120.

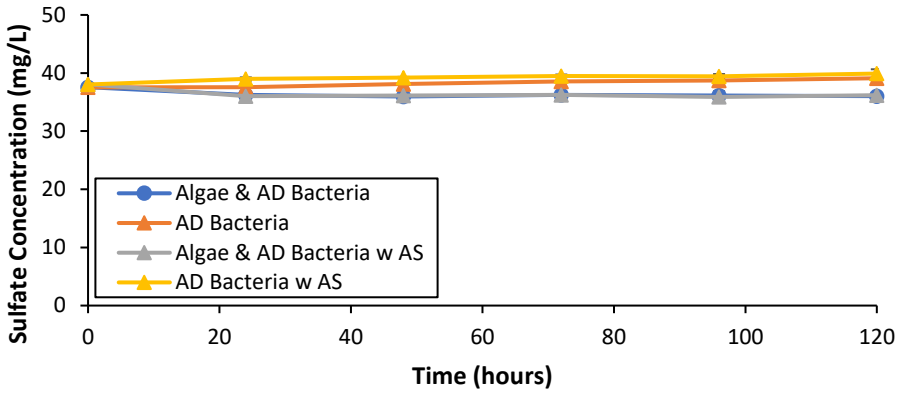
7.3 Appendix Figure 2



Appendix Figure 2: Sulfate concentration during the 5-day experiment for *A. protothecoides* and *C. sorokiniana* (UTEX 2714 & 2805).

Error bars for graphs A, B, and C are SD, n=3. Graph A shows the sulfate concentrations among the treatments using A. protothecoides. Graph B shows the sulfate concentrations among the treatments using C. sorokiniana (UTEX 2714). Graph C shows the sulfate concentrations among the treatments using C. sorokiniana (UTEX 2805). The reported sulfate concentrations are the average concentration per treatment at hour-120.

7.4 Appendix Figure 3



Appendix Figure 3: Sulfate concentration for *C. sorokiniana* (UTEX 2805) during the 5-day experiment for the AS follow-up study.
Error bars for graph A are SD, n=3. Graph A shows the sulfate concentrations among the treatments using C. sorokiniana (UTEX 2805). The reported sulfate concentrations are the average concentration per treatment at hour-120.