Promoting Nitrification in Poultry Processing Wastewater Treatment Using Microalgae and Biochar

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
M.S. Biosystems Engineering

Auburn, Alabama
August 6, 2022

Microalgae, Nitrification, Biochar, Wastewater, Poultry

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Abstract

The exponential growth of the global population poses a need for more sustainable food production and waste remediation tactics. Waste-to-resource food production systems such as aquaponics and "poultry-ponics" may be the solution to this problem. Nitrogen-rich wastewaters from animal production systems can be used as irrigation waters for crop production when treated correctly. Because nitrate is the preferred form of nitrogen for plant growth, and ammonium is toxic to most organisms, nitrification is key to making these wastewaters suitable for plant production.

The presence of algae can promote nitrification in wastewater treatment when ammonium concentrations are non-limiting. The prevailing hypothesis for this promotion is algae's provision of a hyper-oxic environment through photosynthetic oxygenation. Preliminary data from our lab has shown evidence of nitrification promotion even in intensely aerated systems, which poses the question: are there other mechanisms by which algae promote nitrification?

Objective 1 of this master's research project devised and tested additional hypotheses for possible mechanisms. The experiments concluded that algal provision of dissolved oxygen alone is not enough to explain the nitrification promotion effects of algae. Across all experiments, the nitrate level in the supplemental oxygen treatment was never significantly greater than the control ($p > 0.15$). It was observed that algal extracellular excretions may play a role in this promotion. In one experiment, the algal "Spent Medium" produced nitrate concentrations of ~3X the control ($p = 0.002$). More isolated experiments need to be conducted in order to conclude this definitively. Further understanding of the relationship between nitrifying bacteria and microalgae

could directly inform the design of sustainable food production systems such as poultry-ponics and optimize the nutrient transformation in these systems.

The addition of biochar could promote interaction between algae and nitrifying bacteria by providing a solid substrate for colonization. Biochars are very easily “engineered” based on the needs of the user. So, their properties can be optimized for the wastewater treatment process. Objective 2 of this research evaluates the efficiency of two different biochars in terms of nitrification promotion. It was found that the Low Cation Exchange Capacity (CEC) Biochar had the ability to promote nitrification in poultry processing wastewater. The culture containing Low CEC Biochar was capable of producing 10X the amount of nitrate as the control culture ($p = 0.002$). This was true with and without the addition of algae. This research will hopefully inform the future implementation of nutrient transformation systems and encourage the use of biochar as a nitrification-promoting substrate.

Acknowledgments

First of all, I'd like to thank my P.I., Dr. Brendan Higgins. Dr. Higgins has been an incredible boss and mentor to me for the last two years. He is a wonderful teacher, researcher, and manager – which makes him a truly outstanding P.I. Dr. Higgins has gone out of his way to create opportunities for me, and for that I am beyond grateful. Because of him, I have so many workplace and scientific skills that I will be able to bring into the next chapter of my life. I am also thankful to the entire Higgins family for always being welcoming and friendly to me and the rest of the lab.

I would like to thank my committee members Dr. Jeremiah Davis and Dr. David Blersch who have both been instrumental in my success as a graduate student and as a researcher. I would also like to thank Dr. Sushil Adhikari and his lab for being a resource for my research and for being so helpful during my transition to the Biosystems department.

I am extremely grateful to the entire Biosystems department for being a truly kind and welcoming community. I could not have asked for a better department to receive my master's degree from. And, a special Thank You to our incredible administrator, Bionca King, who has been a dear friend of mine since my first week in the department and without whom none of us would be able to function.

I would also like to thank Mr. Tom Devall, who introduced me to Dr. Fasina more than two years ago. I am eternally grateful for all of the guidance Tom continues to provide me as a young professional. I know I would not be as successful without his friendship.

To each of my lab members, Morgan, Peyton, Dillon, Elizabeth, Shima, Wellington, Zach, and Justus: Thank you all so much for being wonderful people and great friends. We are all so lucky to have such a special family we get to see at work every week. And thank you to

Alireza for not only being a great co-worker, but a truly unbeatable friend. I'm so lucky to have met you.

I must reserve a special section for Qichen, though. Dr. Qichen Wang is the best lab leader that anyone could ask for. Along with being a dad, and previously a PhD student, Qichen always made time to answer questions, provide training, or just talk. His interesting insights, sense of humor, and astonishing expertise make him a really exceptional mentor. Qichen, it was so much fun to get to learn from you.

I would like to express my deepest appreciation for my family. To my dad, Scott: thank you for being my inspiration for curiosity and exploration. To my uncle, Beau: thank you for reminding me to always drive fast and take chances. To my brother, Thomas: thank you for believing in me and pushing me to be my best at the same time. And to my mom, Linda: thank you for being the best example of hard work and leadership. You are all the best cheerleaders in the world. I love you so much.

Thank you to my two cats, Dat Kitten and Pinto Bean, for being the best thing to come home to every day.

And, finally, I'd like to thank my boyfriend and life partner John Bryan Holt. Bryan, thank you for everything, really. I love you.

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Introduction

The motivation for this research is based in sustainability: sustainability in wastewater treatment and in agriculture. As the world population continues to grow (estimated 8 billion before 2023 and counting (Worldometer, 2022), it is becoming increasingly necessary for us to create more sustainable practices and infrastructure to support ourselves. The amount of waste being created, and the amount of food consumption required are increasing exponentially. A possible solution is creating waste-to-resources systems that simultaneously mitigate waste and provide resources for consumption.

Furthering sustainability in wastewater treatment creates an avenue for these waste-to-resources systems. When wastewaters go untreated, it can lead to eutrophication and can affect clean water availability. Approximately 1 in 3 people globally are currently facing clean water shortages (WHO, 2019). These shortages are largely in less developed countries (WHO, 2019) where there is not access to traditional wastewater treatment infrastructure. Additionally, eutrophication can have massive environmental implications. It can cause death of aquatic life such as fish, aquatic plants, and microorganisms (Ye et al., 2018). Biodiversity can be destroyed in these eutrophic environments, and the local water quality will also be affected. These effects are significantly increased in areas where extreme droughts are common (Santos, 2021; Zwolsman and van Bokhoven, 2007). It is therefore vital that accessible, sustainable wastewater treatment practices are developed and optimized in order to keep up with the wastewaters being produced by our society.

In the United States, the EPA has specific requirements for wastewaters being discharged into the environment to limit the amount of eutrophication or other environmental effects it may have. However, traditional wastewater treatment methods can consume a significant amount of

energy – approximately 1.04 kWh of electricity per cubic meter of water treated in a municipal wastewater treatment plant (Singh et al., 2012).

Implementing waste-to-resources systems could be the most effective way to combine these two problems: food requires resources to produce, and waste requires energy to treat. Aquaponic systems are great examples of this practice. Aquaponics is an agricultural practice where fish are cultivated in a pond or tank, and the water from this tank is settled and treated and then used as nutrient-dense irrigation for vegetable crops (Ghamkhar et al., 2020). This process can reduce or eliminate the need for the additional fertilizers and potable water often used in traditional crop cultivation; which require significant energy to produce and transport. While this process is being used across the country at a relatively smaller scale, the optimization of this and similar systems is crucial to furthering the waste-to-resources objective in the industry.

In Alabama, the #1 agricultural industry is poultry production (Living, 2017). Poultry processing requires approximately 1.3 gallons of water per pound of meat processed (Northwest), totaling ~12 billion gallons of potable water every year (ADECA-OWR, 2017) in Alabama alone. In order to address this problem, the application of this current work is "Poultry-ponics." This system proposes the use of poultry processing wastewater as high nutrient irrigation for vegetable growth (much like aquaponics). Poultry processing wastewater is rich in nitrogen and phosphorus – two extremely important macronutrients for plant growth. This makes poultry processing wastewater a very valuable resource when treated correctly.

Poultry Processing wastewater is typically high in ammonium (NH_4+). While this large concentration of nitrogen is extremely useful for plant growth, ammonium is the nitrogen form that is most toxic to most organisms – including plants. This nutrient needs to be transformed through nitrification – the microbial process where ammonium is transformed to nitrate. Nitrate

is the preferred form of nitrogen for plant uptake – therefore, nitrification is the most important nutrient transformation to take place in the poultry-ponics system. In order to optimize the poultry-ponics system, it is crucial that the nitrifying bacteria are as healthy as possible, and can perform successfully.

The application of this work is to the optimization of nitrification in poultry processing wastewater. This work proposes that the addition of algae and biochar into a nitrifier culture could increase the fitness of the nitrifiers and therefore create more nitrification withing the system. This research aims to evaluate the specific mechanism by which algae promote nitrification in wastewater, and to evaluate any possible promotion effects provided by biochar. These results could be used to minimize wastes created and resources required in order to optimize a poultry-ponics system.

Chapter 1: Literature Review

1 Waste Nutrients to Resources

Nitrogen and other macronutrients can have extremely damaging ecological effects if they are not removed from wastewaters. Wastewater treatment plants pair nitrification with mechanical separation methods to provide a wholistic treatment that treats both physical and chemical undesirables (Biochemical, 2020). To comply with EPA standards, the effluent must contain the permitted concentrations of ammonium, nitrite, and nitrate in order to be properly discarded (EPA, 2016). Eutrophication, the excessive richness of nutrients in a body of water, is usually caused by run-off of untreated or incompletely treated wastewateres. Eutrophication can cause death of aquatic life such as fish, aquatic plants, and microorganisms (Ye et al., 2018). Biodiversity can be destroyed in these eutrophic environments, and the local water quality will also be affected. These effects are significantly increased in areas where extreme droughts are common (Santos, 2021; Zwolsman and van Bokhoven, 2007).

Nitrogen, among other elements, is an essential nutrient for biological growth (Koch, 2022). In plant or crop growth, it is required for most of the metabolic processes including the production of proteins, nucleic acids, ATP, NAD(P)H, chlorophyll, pigments, secondary metabolites, and hormones (Andrews and Lea, 2013). Many researchers have evaluated the effects of nitrogen starvation on agricultural crops. Rodriguez, et al. reported effects of nitrogen starvation on maize. They observed not only root and leaf growth inhibitions, but also nutrient imbalances in the vegetable; meaning that the reduction of nitrogen within the plant also affected nutrient uptake of other elements – particularly phosphorus (Torres-Rodríguez et al., 2021). Leafy plants, such as lettuce, basil, and even tobacco crops often show symptoms of photosynthesis inhibition in response to nitrogen limitation (Prinsi et al., 2020; Rubio-Wilhelmi et al., 2011). Symptoms include stunted growth, leaf senescence, and chlorophyll loss. This is due to a reduction in the rate

of the Calvin Cycle, which triggers oxidative stress in the crops. These requirements are often supplemented with chemical fertilizers, where the ammonium is produced through the Haber-Bosch process. This is a highly energy-intensive process is controversial because of its high greenhouse gas emissions and global energy consumption (Ghavam et al., 2021; Maloney, 2022; Ritter, 2008; Ye et al., 2018). Additionally, if the compounds in these fertilizers are not taken up by plants, they will eventually be transported through agricultural runoff to an aquatic environment, where they can have the same eutrophication effects as untreated wastewaters (Ye et al., 2018).

As sustainability becomes a bigger area of interest in all fields, agricultural technologies and infrastructure are beginning to be challenged. People and industries are drifting more towards the use of renewable resources; and, consequently, wastewater is becoming an increasingly valued, high nutrient resource (Lin et al., 2016; Ye et al., 2018). It may be apt, in the future, to rename these "resource-waters" instead. Aquaponics, poultry-ponics, and similar nutrient recycling systems are a relatively new form of agricultural crop production in which animal wastewaters (in this example aquaculture waste) are used as high-nutrient irrigation waters for crop growth. These systems not only provide a venue for the use of animal wastewaters, but they can also greatly reduce or even remove the need for chemical fertilizers. Monsees, et al. (2019) evaluated the reduction in chemical fertilizer use in a decoupled aquaponics system. In comparison with their control (a traditional hydroponic system), they found that the aquaponics system reduced the need for supplemental fertilizer by ~69% and fully substituted the need for clean water. Nitrate is the preferred form of nitrogen for plant growth (Hachiya et al., 2012; Ikeda and Tan, 1998), while both ammonium and nitrite are toxic to plants (Lenntech, 1998-2022). Nitrification is, therefore,

one of the most important biological mechanism in aquaponics systems, and complete nitrification is required for optimal transmission of nutrients.

2 Nitrifying Bacteria and Nitrification Enhancement

Nitrification is the enzymatic oxidation of ammonium to nitrite and consequently to nitrate. (Jorgensen and Fath, 2008) Ammonium is toxic to most organisms (Britto and Kronzucker, 2002) and is the highest concentration macronutrient in most wastewaters (Dadrasnia et al., 2021). Animal and agricultural wastewaters are particularly high in ammonium, with high concentrations ranging from 1600 mg/L to 5600 mg/L in some animal wastewaters (Princic et al., 1998). Nitrate, the final form of nitrogen in the nitrification process, is largely innocuous in most environments (LLC, 1998-2022). This process of nitrification is essential to the global nitrogen cycle, and is the primary step in nitrogen removal from wastewater (Ergas and Aponte-Morales, 2014).

The nitrification process is completed by a set of bacteria known as nitrifying bacteria. This group of autotrophic and aerobic microbes are made up of ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (Jorgensen and Fath, 2008). These are inherently sensitive organisms with specific environmental requirements (Urakawa et al., 2019a). Nitrifier sensitivities must be mitigated in order to complete production, notably: total inorganic carbon (TIC) concentrations, dissolved oxygen (DO) levels, and pH values. TIC concentrations affect AOB efficacy, while DO concentrations affect NOB. One study showed that AOB is limited at TIC concentrations lower than 3 mmol C/L (Guisasola et al., 2007). Another study temporarily lowered DO concentrations, and this resulted in the inhibition of NOB. A decrease in DO concentration from 3 to 0.7 mg/L led to effective nitrite accumulation—more than 95% of total oxidized nitrogen was present as nitrite. Additionally, after the DO concentration was returned to

3 mg/L, there were lasting effects from this limitation. Due to a change in the forms of nitrogen represented, the high nitrite accumulation continued for 90 days thereafter (Pacek et al., 2015). Nitrification is also pH-sensitive and rates of nitrification will decline significantly at values below 6.8 (Princic et al., 1998). Because of these limitations, it is extremely important to provide an optimal environment for the nitrifiers to thrive in.

There are many different ways to enhance nitrification in wastewater treatment or nutrient recycling systems. Gonzolas, et al. developed a supervisory pH control system to enhance partial nitrification in municipal wastewater treatment. They used the pH and DO levels in a typical aeration basin to enrich either AOB or NOB populations in order to effectively treat their wastewaters (Ciudad et al., 2007). Another study by Castrillo, et al. evaluated the nitrification potential in a membrane aerated bioreactor (MABR) by optimizing the mass transfer between biofilm and bulk water. In this study, their innovative configuration (involving the use of curved membranes instead of straight membranes) not only increased the nitrification rates inside the system, but reduced the aeration energy consumption by 83.7% (Castrillo et al., 2019). Additionally, powdered activated carbon has also shown a potential to enhance nitrification in activated sludge aeration basins. Ng, et al. 1987, found that the addition of activated carbon enhanced the nitrification of petroleum refinery wastewater, which is typically difficult to nitrify. The hypothesis suggested that the activated carbon adsorbed nitrification-inhibiting compounds from the wastewater, providing a more ideal environment for the nitrifiers to thrive in (Ng et al., 1987).

The addition of algae to nitrification systems has also been shown to be beneficial. As previously stated, nitrifying bacteria have substantial oxygen requirements. Not only do they require oxygen for respiration, but the nitrification process also consumes large amounts of

oxygen as well. Nitrifying bacteria require almost 4.6 mg of dissolved oxygen (DO) per mg of (NH_4^+) to carry out the nitrification process (Fallahi et al., 2021). Algae provide dissolved oxygen through photosynthetic oxygenation, and therefore aid nitrifying bacteria. Algae are also known to mitigate toxins (Tigini et al., 2016), sorb harmful compounds (Higgins, 2019), and protect bacteria from light exposure (Vergara et al., 2016). Bacteria can also benefit algal growth by providing secondary metabolites (Croft Martin et al., 2006) and inorganic carbon sources through respiration (Holmes et al., 2019; Zambrano et al., 2016). For these reasons, algae-bacterial consortiums can be used to promote nitrification in wastewater treatment and reuse systems when their relationship is optimized.

3 Algae in wastewater treatment for nutrient removal

The application of algae in wastewater treatment and nutrient removal systems is not a new phenomenon. The first recorded case of microalgae being added to a municipal wastewater treatment plant was in the early 1900s, where it was noted that algae increased wastewater treatment efficiency by aerating the water and consuming waste (Paddock, 2019). Further, ancient Aztec civilizations (as early as 1300) supplemented algae growth in large above-ground lagoons with manure and other wastes. Notably, Arthrospira (Spirulina), a blue-green algae that is renowned for its nutritional value (Khan et al., 2005; Ross and Dominy, 1990), was grown in these lagoons, fertilized with wastewaters, dried in the sun, and sold at local markets (Michael A. Borowitzka, 2013). Algae have many properties (stated above) that make them an optimal organism for natural biological treatment of undesirables. Algal biomass is also a valuable by-product from this treatment. Algae can be added as a protein supplement to animal feeds (Jia and Yuan, 2018b), in aquaponic systems it can be eaten directly by the fish for added nutrition (Addy et al., 2017), and it can be used as a feedstock for biofuel production (Alireza Fallahi).

Algae are largely used for nutrient removal – especially ammonium and phosphate – because the algae cells uptake these nutrient into their biomass (Fallahi et al., 2020), and then the cells can be filtered out of the wastewater, removing these nutrients with them. In a study completed in Finland in 2020, a research group evaluated the application of microalgae as a post-treatment of hydroponic effluents from Nordic greenhouses. The high-nutrient effluents of hydroponic cucumber production were used as a growth medium for 13 different strains of microalgae – of which 12 strains were capable of proliferating in the effluent. A pilot-scale photo-bioreactor was then operated for 36 days inside the greenhouse, and was capable of removing up to 35% of the Nitrate, and up to 98% of the phosphate in the effluent (Salazar et al., 2021). Additionally, coupled nitrification-denitrification is a sequential process typically used to remove nitrogen from wastewaters, and has been shown to be enhanced by the addition of algae. Zimmo, et al. evaluated the effects of algae vs. duckweed-based waste stabilization ponds. Their in-situ results found an increase of nitrification by 16-42% and an increase of denitrification by 24-43% by the algae ponds. This was mainly attributed to the higher DO percentages and the adsorption of the nitrifiers to available suspended particles and sediments in the algae ponds (Zimmo et al., 2004). Foladori, et al. further enhanced the nitrification-denitrification in their system by taking advantage of the diurnal behavior of algae. In a photo-sequencing bioreactor treating real municipal wastewater, the wastewater was fed during dark phases in order to provide readily biodegradable COD when oxygen was not being produced. This successfully promoted denitrification during the dark phases, while photosynthetic oxygenation promoted nitrification during the light phases (Jia and Yuan, 2018a).

Microalgae are typically used in combination with another biological treatment. They can be used alongside bacterial consortiums due to the natural symbiosis between these two

organism groups. The above instances of enhanced nitrification-denitrification are an example of this behavior, but Ji, et al. evaluated the symbiotic relationship between a non-nitrifying bacteria (*Bacillus licheniformis*) and a microalgae strain for wastewater treatment. The algal-bacterial consortium containing a chlorella strain was capable of 86.55%, 80.28% and 88.95% of sCOD, TDP and TDN respectively in synthetic wastewater. They hypothesized that this was due to the interactions between the algae and the bacterium, through extracellular communication (Ji et al., 2018). Another study compared the biological removal efficiencies of systems with an isolated bacteria consortium (containing many different categories of bacteria including nitrifiers, denitrifiers, and hererotrophs), isolated algae, and the combination of the two. This study showed that the algae-bacterial consortium was the most efficient at removing COD, NH3-N, and TP with efficiencies of 87.3%, 99.2% and 83.9% respectively (Wang et al., 2016).

The addition of microalgae to bacterial communities can reduce the energy consumption of the process – typically through a reduced need for aeration. May studies have evaluated the potential energy reduction in these systems. Steen, et al. saw complete nitrification in their algae-aided system with only occasional surface mixing for aeration (van der Steen et al., 2015). Jia, et al. evaluated the effect of light intensity on a photo-activated system and saw that low-light intensity (1000 Lux) was a sufficient and cost-effective parameter for their system – which could be further enhanced through stabilization of the algae-bacterial relationship over time (Jia and Yuan, 2018a). Foladori, et al. also saw sufficient nutrient removal from municipal wastewater without any external aeration, with DO only supplied by microalgal photosynthesis. They saw a 41% energy savings just by reducing the amount of mixing required (Foladori et al., 2018).

Unfortunately, these microalgae-based wastewater treatment systems have their limitations; notably, overgrowth of algae resulting in self-shading and nutrient competition. One

study noted that the addition of more algal biomass concentration did not increase the nutrient removal efficiencies of the photo-bioreactor. According to the researchers, this was likely due to self-shading (Jia and Yuan, 2018a). This problem becomes particularly important when the application of the system is for nutrient transformation – not nutrient removal. Algae's application in nutrient recycling systems has also not been fully evaluated yet. The efficient design of these systems is extremely important for sustainable agriculture and therefore should be fully investigated. However, the combination of microalgae and bacteria seems to be a promising avenue due to the symbiosis between these two microbes. Optimization of this relationship could be the key to enhancing the effectiveness of poultry-ponics and similar nutrient recycling systems

4 Algal-Bacterial Enhancement of Nitrification for Nutrient Transformation

Waste-to-resource systems require full nitrification to be completely successful. Because agricultural crops prefer nitrate-nitrogen over other forms (Ikeda and Tan, 1998; Lin et al., 2016), and because ammonium and nitrite can both be toxic to these crops (Lenntech, 1998-2022), the enhancement of nitrification and the increased fitness of the nitrifying bacteria becomes ever-more important. It has been concluded that there are many ways in which algae can enhance wastewater treatment systems in order to remove nutrients – but, nutrient transformation is a different process. In order to transform the nutrients in anthropogenic wastewaters into resources for fertilization, the microbial communities must work in harmony. Below, the benefits and limitations of algal addition to these systems will be discussed.

There are many studies that provide evidence of the beneficial relationship between algae and nitrifying bacteria. The natural nitrification-denitrification process in river mouths and other fresh waters has shown to be enhanced by the presence of benthic algae along the riverbed.

Estuarine sediments in Galveston Bay, Texas were observed during daytime and nighttime cycles, revealing that the nitrification rates during the daytime were higher due to the photosynthesis provided by the benthic algae (An and Joye, 2001). Another study, by Chen, et al., evaluated this phenomenon in a eutrophic lake in China. Samples from Lake Taihu were evaluated for nitrification and denitrification parameters, along with qPCR data. Both of these data points revealed a quantitative difference in the bacterial communities as a result of cyanobacteria and algae blooms. The promotion of coupled nitrification-denitrification is so prominent that Lake Taihu is often nitrogen-limited during the summer algae blooms (Chen et al., 2016). Additionally, lab-replicated lake scenarios have also proved the benefit of algae in natural environments. The presence of diatoms in synthetic lake water showed a significant increase in nitrification when illuminated, proving that the addition of photosynthesis aided the nitrifying bacteria in lake sediments (Lorenzen et al., 1998).

This relationship has been further explored in wastewater treatment systems. The focus of nutrient-recycling systems is typically on animal wastewaters. These studies are either completed with field-collected animal wastewaters (i.e., swine anaerobic digestate, poultry processing wastewater, aquaculture pond waters, etc.), or with synthetic medias whose nutrients are devised in order to replicate that of "real" wastewaters.

Benchtop studies conducted with synthetic wastewaters have shown the promotion of nitrification by microalgae in many different settings. *Chlorella Sorokiniana* has been shown to promote nitrification in synthetic wastewater treatment. The presence of microalgae showed a promotion of nitrification in photo-bioreactors by mitigating light-intensity inhibition (Vergara et al., 2016). This study observed that at typical outdoor daily light intensities, nitrifying bacteria exhibited a significant sensitivity, but the algae-bacteria consortium overcame the effects of the

light intensity and provided complete nitrification of the mineral wastewater. Sepehri, et al. also completed an in-lab study with *C. Vulgaris* comparing the mixed culture with nitrifier-enriched activated sludge (NAS) to pure cultures of the microalgae all in chemical media. They found that the mixed culture provided the most nitrification promotion, and the highest accumulation of nitrate by the end of the experiment. Another mixed culture with a different microalgae:NAS ratio provided the highest carbon capture, even over the pure microalgae culture (Sepehri et al., 2020). While synthetic wastewaters are valuable because their nutrient compositions can be fine-tuned, they are often less representative of true wastewater treatment systems, because they lack other characteristics or inhibitors that may be present in real wastewaters.

Algal-bacterial consortiums have been used to promote nitrification in many different animal wastewaters. *C. Sorokiniana* has been shown to promote nitrification in poultry litter anaerobic digestate. Bankston, et al. showed that the combination of microalgae and nitrifying bacteria provided the most nitrification when compared to other individual cultures when treating poultry digestate (Bankston et al., 2020). Algae may also have the ability to reduce inhibitory effects of antimicrobials found in poultry processing wastewater (Higgins, 2019). The nitrification capacity in the co-culture was increased when algae was present, even with the presence of peracetic acid, a common antimicrobial used to clean poultry processing facilities. Additionally, algae is known to remove toxins from wastewaters. *C. Vulgaris* showed the ability to reduce the toxicity of anaerobic piggery digestate. A battery of four acute and two chronic ecotoxicity assays were used to evaluate the toxicity of diluted digestate coming from the anaerobic digestion of pig slurry and corn both before and after a twelve-day algae cultivation. Toxicity significantly decreased across all tests, and macronutrients (NH3, TN, and PO4) were largely consumed during the culture growth (Tigini et al., 2016). With the antimicrobial agents

and pathogens removed, the wastewater effluent can be used as organic fertilizer (among other venues), instead of being discarded as unused, high-nutrient waste. This characteristic is another way that the nitrifying bacteria may benefit from algal-bacterial symbiosis.

One of the key mechanisms of this symbiosis is algal provision of a hyper-oxic environment due to photosynthetic oxygenation (Bankston et al., 2020). Nitrifying bacteria have a high oxygen demand (Daigger, 2014), which is why this oxygenation is beneficial to the nitrification process. In one study, nitrification could be induced during dark phases by bulk purging of O_2 gas, which indicated that the stimulation of nitrification in their daytime cultures was due to photosynthetic oxygenation (Lorenzen et al., 1998). In a study evaluating nitrification in dairy waste stabilization ponds, it was seen that aeration greatly increased the productivity of the nitrifiers, but continuous mechanical aeration is expensive and a large consumer of energy. When the researchers switched to night-only aeration, the system produced significantly more nitrate. This was because the algae in the system grew more dense and completed photosynthesis during the day, which allowed for more dissolved oxygen production. The system halved the electricity costs due to the reduction in mechanical aeration (Sukias et al., 2003). Photosynthetic oxygenation even has the ability to completely replace mechanical aeration in some situations. Karya, et al. showed the model microalgae *Auxenochlorella protothecoides* had the ability to provide enough oxygen for full nitrification in synthetic wastewater without any additional aeration (Karya et al., 2013). This photosynthetic oxygenation has even been observed in high-strength agricultural wastewaters. Swine anaerobic digestate with an influent ammonium concentration of ~300 mg/L was still able to be treated when algae was also present. The algae used in this study, a wild consortia consisting of mainly *Chlorella* strains, was able to provide oxygen for complete nitritation during outdoor light periods (Wang et al., 2015). Electric energy

reduction can be a huge contributor to costs and greenhouse gas emissions from wastewater treatment systems. Kwon, et al. evaluated six different microalgae strains on their ability to supplement forced aeration, and four strains (*C. vulgaris*, *S quadricauda*, *D. communism* and *C. emersonii*) were able to produce complete nitrification in synthetic wastewater without forced aeration. This resulted in a significant reduction in kWh consumed, including the light energy required for algal growth (Kwon et al., 2019).

While the evidence of this promotion through oxygen provision is clear, each of these studies compares the algae-aided system with a low-oxygen alternative, whether that's in un-aerated cultures, non-mixing systems, or benthic and sedimentary microbial communities. These systems are prime examples of ways that algae can aid nitrifying bacteria, but they are not the only examples. Other data presented earlier (Bankston et al., 2020), as well as preliminary data from our lab (more info found in Section 2.4) has shown a promotion of nitrification in algae-aided systems even in highly aerated systems. This phenomenon presents an avenue for further investigation: are there any other mechanisms by which algae promote nitrification? As stated previously, algae have the ability to mitigate toxins in wastewater (Franchino et al., 2016; Higgins, 2019), and to sorb nutrients such as ammonium (Fallahi et al., 2021). Algae also produce extracellular molecules or secretions that have been known to have particular properties that may aid in the fitness of nitrifying bacteria. The term 'Phycosphere' was coined in 1972 by Bell and Mitchell, and it refers to a chemically enriched zone where "tight interactions between algae and other organisms are controlled by exuded chemicals" (Cirri and Pohnert, 2019). These metabolic hotspots are where we can observe the behavior of interest. In a documented relationship between *Kordia algicida* and *Skeletonema costatum*, a protease produced by the bacterium causes the alga to lyse and the accompanying release of organic compounds sustains

the bacteria's growth (Paul and Pohnert, 2011). However, *Kordia algicida* are not nitrifying bacteria, which are the focus of this work. The most commonly studied nitrifying bacteria are proteobacteria (Hovanec and DeLong, 1996), which makes them specifically susceptible to a relationship with algae, as noted by Cirri & Pohnert: "Tight associations between microalgae and bacteria, mostly Proteobacteria, have resulted in the evolution of a complex network of cross-kingdom interactions and a fine specialization of different organisms" (Cirri and Pohnert, 2019). Ji, et al. noted extracellular communication between *Chlorella vulgaris* and *Bacillus licheniformis*. They concluded that the production of extracellular organic matter by the algae-bacterial consortia was received by *Bacillus licheniformis*. This then created changes in the growth of the bacteria which were beneficial to the chlorophyll metabolism of the *C. Vulgaris* cells (Ji et al., 2018).

Outside of extracellular secretions, remediation of toxins, and provision of dissolved oxygen, there are other possible pathways through which algae may promote nitrification. As stated before, Vergara, et al. exhibited a sunscreen-like quality in HRAP systems colonized by nitrifying bacteria. It was concluded that the presence of algae at concentrations typically found in HRAP reactors can mitigate the photoinhibition of nitrifying bacteria. Therefore, the inhibition of nitrification capacity is not to be expected in photobioreactors due to daily light intensity (Vergara et al., 2016). Microbial community makeup can also affect the nitrification capacity of a culture. In Lake Taihu, China, it was observed that the microbial community (specifically nitrifying and denitrifying bacteria) changed over the course of an algal bloom. It is possible that the microbial change in response to the addition of algae may increase the fitness of the nitrifying community. This, in turn, could have led to the increase in nitrification-denitrification seen in this study (Chen et al., 2016).

To our knowledge, however, no research has been completed to isolate these possible mechanisms and evaluate the effect they may have on nitrifying bacteria. The optimization of this relationship is crucial in understanding how nutrient recycling systems can be efficient uses for wastes. However, there is also some evidence of algae having a negative effect on nitrification. This evidence can provide further insight into the relationship between these two organisms.

Risgaard-Petersen, et al. reported estuarian sediments colonized by microalgae had significantly less coupled nitrification-denitrification than those without. When this scenario was recreated in a benchtop study, the same results were seen, but the nitrification-denitrification of the cultures could be increased by adding supplemental ammonium to the system (Risgaard-Petersen, 2003). Choi, et al. also reported an inhibition of nitrification by the presence of microalgae. It was also seen that the nitrifying bacterial community did not show any changes, which led to the conclusion that the inhibition was due to nutrient competition between the two species (Choi et al., 2010). Both of these reports, however, are evaluating algal-bacterial interactions under low nutrient conditions (<60 mg/L NH_4). These values are lower than those often seen in wastewaters – especially animal wastewaters which are relevant to this present work. In high NH_4-loaded wastewater treatment or reuse systems, an equilibrium often exists between microalgae and AOB. When ammonium is not present in excess, microalgae and AOB reduce the NH_4 concentration of the culture, limiting their activities (González-Camejo et al., 2022). This competition can affect both organisms as well. González-Camejo, et al. reported an inhibition of micro-algae growth in the presence of nitrite accumulation in a membrane photobioreactor (González-Camejo et al., 2020). However, there is sufficient research to prove

that as long as ammonium is not limiting, this competition does not occur, and the two organisms only benefit from each other's presence (An and Joye, 2001).

5 Preliminary Data

Some preliminary data in our lab provides further evidence of algal support of nitrifying bacteria. We have observed this promotion in a variety of contexts (in the presence of multiple inhibitors), and also in different wastewaters, namely: anaerobic digestate, municipal wastewater, and poultry processing wastewater. Evaluation of this phenomenon through ion reduction or accumulation, as well as genetic qPCR data will be presented here. Note that all of this data presents motivation for the current study by providing support for the hypothesis that there are additional mechanisms of promotion besides provision of dissolved oxygen through photosynthesis. This hypothesis was developed due to the apparent benefit to the cultures containing algae, even though all cultures (including the control) are intensely aerated.

Previously, it was discussed that algae may have an ability to mitigate toxins in wastewater treatment. Figure 1 is data from two batch studies, one with Peracetic acid (PAA) as the added inhibitor, and one with cetylperidinium chloride (CPC) as the added inhibitor. Both PAA and CPC are common antimicrobials used in poultry processing and are therefore often common in the wastewaters discharged from these processes. Nitrifying bacteria tend to be sensitive to chemical toxins. Antimicrobial agents found in poultry processing wastewater have low EC50 values for nitrifying bacteria. PAA has an EC50 toward nitrite-oxidizing bacteria of 9 mg/L (Pavlostathis, 2019) whereas CPC has an EC50 of only 1.5 mg/L (Box and Higgins 2020). Moreover, our tests with diluted poultry litter anaerobic digestate showed apparent toxicity toward both ammonia-oxidizing and nitrite-oxidizing bacteria.

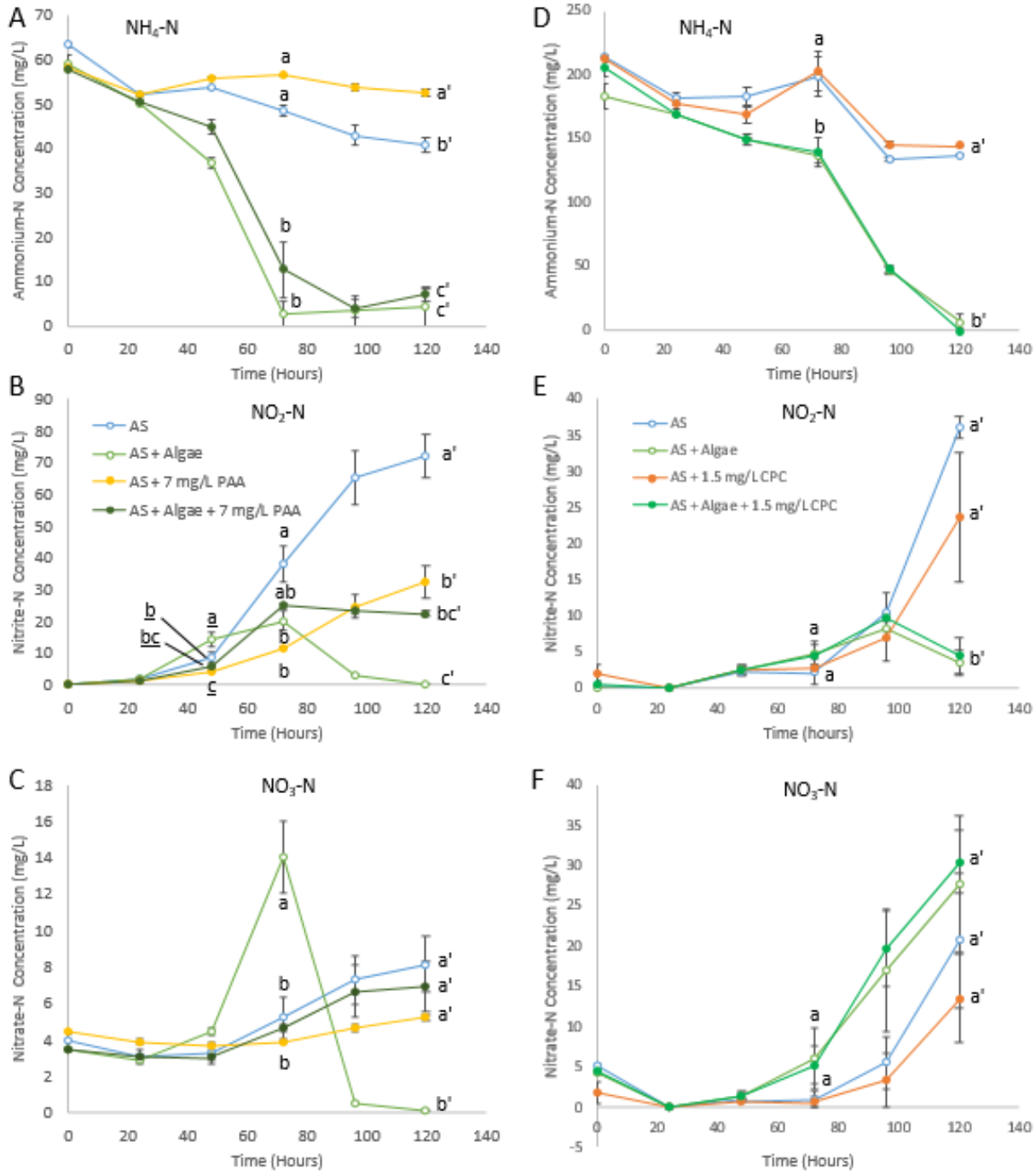

Figure 1: Nitrification parameters from preliminary experiments using Chlorella sorokiniana. Comparison between PAA treated and non-treated wastewaters: A = ammonium-nitrogen removal, B = nitrite-nitrogen accumulation, C = nitrate-nitrogen accumulation. Comparison between CPC treated and non-treated wastewaters: D = ammonium-nitrogen removal, E = nitrite-nitrogen accumulation, F = nitrate-nitrogen accumulation.

Each study presented in Figure 1 was a 120-hour batch culture, where four treatments were all run in triplicate, totaling 12 bioreactors. The treatments can be seen in the legends of each figure, but in short: the green data points are cultures that contain algae. The algae used in these experiments was *Chlorella sorokiniana,* and the NH_4, NO_2, and NO_3 concentrations were evaluated with HPLC Ion Chromatography.

It is clear that algae promote ammonium removal in these studies – whether through assimilation, or through enhanced nitrification. The presence of algae resulted in an initial increase in nitrite accumulation, followed by a decline. However, since nitrite and ammonium are both toxic, the data of interest is the nitrate concentration. In the PAA study, there is a clear and substantial proportion of nitrate accumulation in the algae treatment up to 72 hours, and then followed by a sharp decline. This is likely because of algal consumption of nitrate as a nitrogen source, in the absence of ammonium. A follow-up study confirmed this *C. sorokiniana's* ability to consume ammonium, nitrite, and nitrate and that it preferentially consumes them in that order. Unfortunately, this algae's consumption of oxidized forms of nitrogen (Nitrite and Nitrate) cloud the results of these studies and confound the conclusions. In order to make a more informed conclusion, both of these studies were conducted again, with an algae strain that is known to only consume ammonium-nitrogen: *Auxenochlorella protothecoides.*

Two similar studies were consequently conducted and can be seen in Figure 2. These experiments were identical to the previous experimental design, aside from the use of a new algae strain.

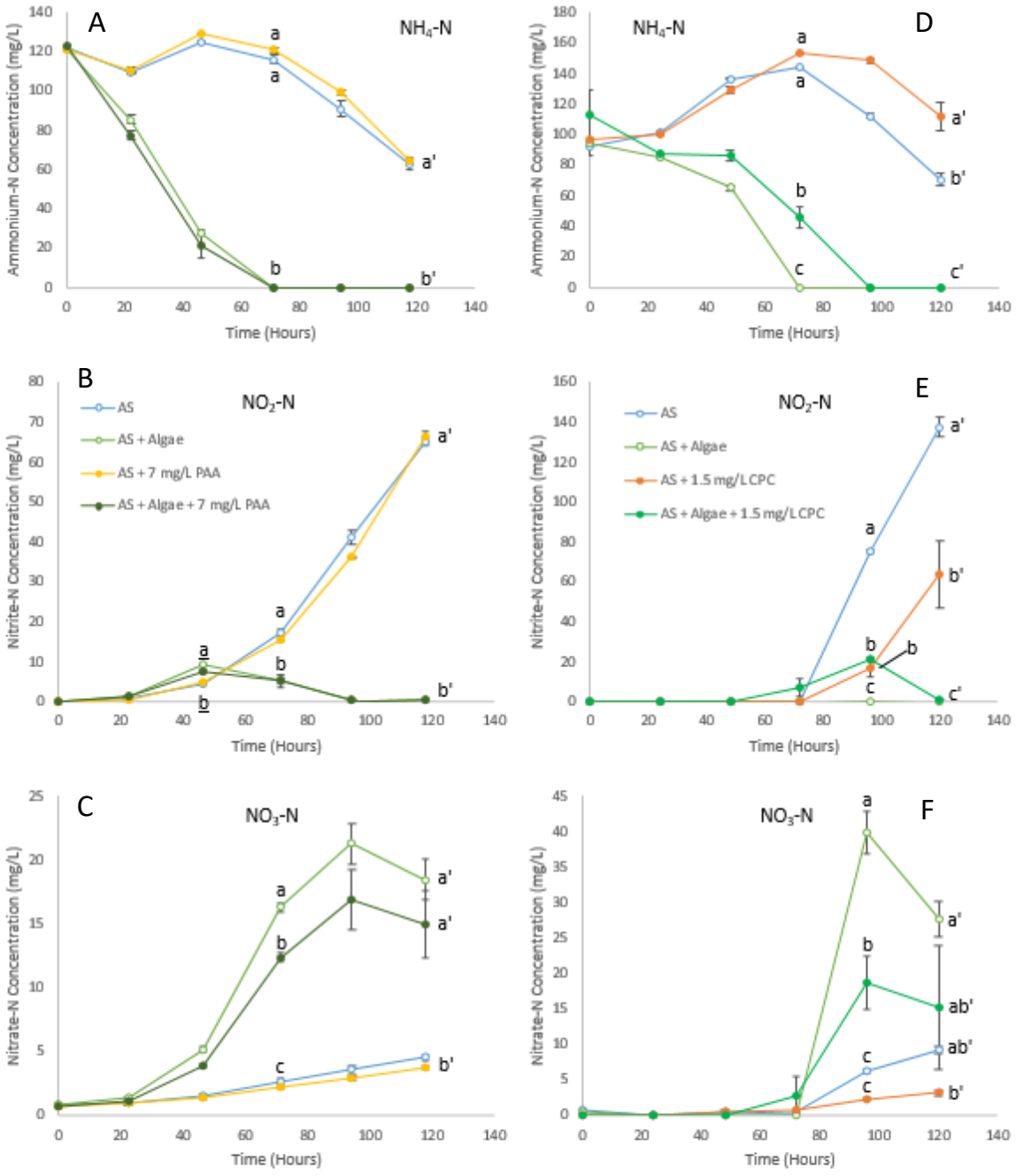

Figure 2: Nitrification parameters from preliminary experiments using Auxenochlorella protothecoides. Comparison between PAA treated and non-treated wastewaters: A = ammonium-nitrogen removal, B = nitrite-nitrogen accumulation, C = nitrate-nitrogen accumulation. Comparison between CPC treated and non-treated wastewaters: D = ammonium-nitrogen removal, E = nitrite-nitrogen accumulation, F = nitrate-nitrogen accumulation.

In these studies, the same patterns of increased ammonium consumption by algae cultures were observed. Additionally, there was an apparent increase in the ammonium concentration of the non-algae cultures. This was likely driven by mineralization of organic N which is prevalent in poultry processing wastewater. There is a clear suppression of ammonium oxidation where CPC is present, but not in nitrogen mineralization. With the consumption of oxidized forms nitrogen, it is clear that the nitrogen promotion potential of algae was dimmed in the previous studies. These studies provide a clear conclusion of the support of nitrification by algae. The final nitrate concentrations of the algae-aided treatments were 3-10x higher than those of the bacteria-only cultures. Both antimicrobial inhibitors were also visibly mitigated by the presence of algae. The results from this study presented further questions for our research group. The literature clearly suggests a promotion of nitrification through photosynthetic oxygenation due to nitrifying bacteria's high oxygen demand, but only when algae-aided cultures are compared to anoxic or oxygen-limited environments. However, these studies show further promotion even when the control cultures are highly aerated. It follows that there could be other mechanisms at play.

6 Promotion of Nitrification by Biochar or Activated Carbon for Nutrient Recycling

Enhancing nitrification can be done through many different pathways – only one of which is the addition of algae. Another supplement that is used to enhance nitrification in wastewater treatment is activated carbon. Activated carbon is a dark, powdered substance that is typically created by a feedstock (most commonly coconut shells, coal, or wood) being superheated at temperatures from 600-1200 degrees Celsius under low oxygen conditions (General Carbon, 2022). It is usually presented in a powdered form, and has an extraordinarily large internal surface area and pore volume (Frederick S. Baker, 2000). These characteristics are

responsible for its unique and impressive adsorptive properties, which can aid in toxin sorption (Mosa et al., 2016).

In wastewater treatment or recycling systems, activated carbon can provide an opportunity for adsorption of potential toxins which could be inhibiting nitrification. This is a phenomenon that has been studied and benefited from for decades. A paper from 1987 by Ng, et al. evaluated the toxin-mitigation abilities of powdered activated carbon (PAC) in an activated sludge basin. The results showed that there was an increase in nitrification as the concentration of PAC increased. It was also seen that each inhibitor (Ethanol, Cyanide, or Analine) had an optimal concentration of PAC at which the nitrification inhibition was mitigated up to 97%. They concluded that this increase in nitrification efficiency was due solely to toxin mitigation, and not through enhanced growth of nitrifiers via the PAC surfaces (Ng and Stenstrom, 1987). Another study completed in 2001 by Aktas, et al. also observed enhanced nitrification in activated sludge systems by the presence of PAC. The wastewater treated in this study was a combination of traditional municipal wastewater and landfill leachate, which has high levels of nitrification inhibitors and Free Ammonia (FA). When the percentage leachate was kept to a minimum (<20%), the PAC showed a capacity to mediate the toxicity and increase the nitrification efficiency by adsorbing compounds inhibitory to *Nitrosomonas* and *Nitrobacter*. They also noted some additional advantages of PAC: better sludge settling and increased sludge dewaterability (Aktas and Çeçen, 2001).

In the same vein as nutrient recycling systems, sometimes it is better to use a by-product or "waste" as a resource in order to create sustainable processes. Biochar can be used as our resource in this sense. Biochar is very similar to activated carbon in many ways including surface area, pore size, and creation processes. Biochar is also made by superheating (or "heat treating")

carbon sources, specifically biological feedstocks such as wood or grasses. The difference is that it is created as a valuable by-product of biofuel production (Qian et al., 2015). Due to its similar characteristics, it is hypothesized that biochar could enhance a wastewater treatment or nutrient recycling system in the same way that activated carbon does.

Biochar has been seen to enhance nitrification in solid, organic environments such as soil (Prommer et al., 2014) and composts (Zainudin et al., 2020), to enhance the nutritional status of hydroponic vegetables (Awad et al., 2017), and to reduce the toxic effects of both heavy metals (Mosa et al., 2016) and ammonia in hydroponic and aquaponic (Su et al., 2020) systems. In wastewater treatment, nitrifying bacteria benefit from the increased surface area available for biofilm formation and the suspended particles allow for more attachment as opposed to stationary walls or sediments (Akizuki et al., 2021). Ideally, the combination of these qualities would make it a perfect additive to nutrient recycling systems – provided that the biochar being used is capable of enhancing nitrification, providing surface area for biofilm formation, and mitigating toxins all at once.

Luckily, biochar is "engineer-able," meaning that its qualities can be enhanced depending on the feedstock and the process used to create it. This offers an extreme advantage to biochar as a system supplement because it can be tuned to a system in order to further optimize it. Biochars have been known to be specifically engineered for nutrient removal. Shakoor, et al. evaluated many different biochars that have been tuned for Nitrogen and Phosphorus removal from wastewaters (Shakoor et al., 2021). They can also be engineered to enhance soil nutritional properties, agricultural carbon sequestration, and pollution remediation (Kazemi Shariat Panahi et al., 2020).

Nitrifying bacteria are not the only microorganisms who benefit from available surface area for biofilm formation; many algae species do as well (Gross et al., 2015). For this reason, it is hypothesized that the interaction between nitrifying bacteria and algae may be further enhanced by the presence of Biochar. Wang, et al. evaluated the effect of an algal-bacterial consortium combined with a biologically activated carbon (BAC) column at the removal of COD from black odorous river sediment wastewaters. The results showed that the combined treatment achieved COD removal of ~83%, which was the best removal efficiency of all columns tested. The combined impact of both sufficient DO percentages and biofilm production was attributed to the column's capacity for nutrient removal (Wang et al., 2021b). Aside from this report, there is very little research being conducted about the interaction between these three constituents in the context of nutrient recycling systems. However, the optimization of the relationship between them could be very beneficial to the enhancement of sustainable agriculture.

7 Applications in Nutrient Recycling Systems

The goal of this present work is to evaluate the interactions of nitrifying bacteria, algae, and biochar. Improving nitrogen utilization efficiency of poultry-ponics by introducing algal-bacterial consortia could be the key to furthering sustainable crop production. Currently, aquaponic and hydroponic crop production is not comparable to commercial agriculture due to the limits of undeveloped technology. The optimization of these systems is crucial to implementing sustainable processes and promoting waste-to-resources systems.

Increasing nitrate concentration in wastewaters makes them better as plant fertilizers, more sustainable for discharging, and capable of being reused for irrigation. Optimal use of microalgae and/or biochar could not only enhance nitrification (thereby increasing nitrate concentrations), but also aid in antimicrobial mitigation, reduce aeration energy consumption, and increase sludge

settleability and dewaterability. Understanding the mechanisms behind these microbial interactions is central to optimizing wastewater reuse and creating a path for sustainable agricultural practices.

Chapter 2: Determining the Mechanism by which Algae Promote Nitrification in Wastewater Treatment

Introduction

The implementation of algae has been shown to increase the efficiency of wastewater treatment (Higgins, 2019). Nitrification can be greatly increased in these systems provided the ammonium concentrations are not limiting (An and Joye, 2001; Lorenzen et al., 1998). Nitrification is the oxidation of nitrogen from its most toxic form (ammonium), to its much more innocuous form (nitrate). This is done by a group of bacteria known as nitrifying bacteria, who are inherently sensitive organisms (Urakawa et al., 2019b). Therefore, providing ideal environments for them to thrive in is extremely important to the wastewater treatment process.

Waste-to-resource systems (i.e., aquaponics, poultry-ponics, etc.) utilize "wastes" produced by organisms as nutrient-rich fertilizers for other organisms. Nitrification is perhaps the most important biological process in wastewater treatment for use in plant cultivation (Hachiya et al., 2012). Ammonium is toxic to most organisms, especially the concentrations present in most wastewaters; but, nitrate is the preferred form of nitrogen for plant growth (Hachiya et al., 2012; Ikeda and Tan, 1998). By adding algae to these systems, we can potentially increase the nitrification efficiency in the treatment, which increases the plant production stability.

Algae-Bacterial interactions are a known phenomenon and can be mutualistic – meaning that it is beneficial for both parties. One of the key mechanisms of this symbiosis is algal provision of a hyper-oxic environment due to photosynthetic oxygenation (Bankston et al., 2020). Nitrifying bacteria have a high oxygen demand (Daigger, 2014), which is why this oxygenation is beneficial to the nitrification process. Quantitative research has shown that the addition of algae to nitrifying bacterial communities increases their capacity for treatment of wastewaters (Fallahi et al., 2021; Higgins, 2019, 2020). The presence of algae can also aid

nitrifying bacteria in overcoming the antimicrobial agents found in poultry processing wastewater (Higgins, 2019), specifically PAA, which is the less toxic, but wider-used antimicrobial. With the antimicrobial agents and pathogens removed, the wastewater effluent can be used as organic fertilizer (among other applications), instead of being discarded as unused, high-nutrient waste. There is also some evidence of algae having a negative effect on nitrification, due to nutrient competition (Risgaard-Petersen, 2003). However, there is sufficient research to suggest that as long as ammonium is not limiting, this does not occur (An and Joye, 2001).

The prevailing hypothesis for algal promotion of nitrification is the provision of dissolved oxygen (DO) through photosynthesis. However, our research group has seen promotion of nitrification even in intensely aerated cultures where oxygen was likely not limited. It follows that provision of DO may not be the only mechanism by which algae aid nitrifying bacteria. Unfortunately, there is very limited research currently on this topic due to algae and bacteria's complicated relationship.

The mechanisms by which algae promote nitrification is not well explored in the scientific community. This research project aims to evaluate additional mechanisms by which algae promote nitrification. Two hypotheses were evaluated through the experimental design. The hypotheses are as follows:

1. Algae provide necessary oxygen to nitrifying bacteria through photosynthesis.
2. Algae secrete molecules that increase the fitness of nitrifying bacteria to overcome toxicity in wastewater.

Other possible mechanisms that could be responsible for this promotion are: 3) that algae restructure the microbial community in ways that lead to more rapid elimination of toxic

compounds and 4) that algae sorb toxic molecules in wastewater. These mechanisms were also evaluated as hypotheses, but due to time constraints, they were not evaluated in the current work. However, the understanding of the relationship between algae and nitrifying bacteria may inform the development of waste-to-resource systems like aquaponics and hydroponics by optimizing the use of nutrients and minimizing the need for outside fertilizers and supplements.

Materials and Methods

1 Resource acquisition

1.1 Poultry Processing Wastewater

The poultry processing wastewater (PPWW) used in these experiments was obtained from the scald tank of a poultry processing plant in Georgia. This water is the dirtiest water in the processing plant and the scald tank lies upstream of any addition of antimicrobial agents which might interfere with nitrification. The same wastewater was used for each treatment. The wastewater was sent in gallon jugs via express mail and kept in a cold room at ~ 4°C until use. The ammonium concentration of each bottle was enumerated using HPLC Ion Chromatography via standard methods – further outlined in Analytical Methods.

Before the experiment, the PPWW was then filtered to 0.2 um using a vacuum filter to remove any large sediments or microbes present. This was also done to mimic the primary treatment or settleability done in most wastewater treatment systems. The filtered PPWW was then diluted using DI water to approximate the nitrogen concentration (~100-150 mg/L) of final plant effluent which is typically discharged to the sewer system. The PPWW was then pH adjusted to ~7.5 using 3M HCl as the acid and 3M NaOH as the base. 185 mL were added to each of the nine hybrid tube bioreactors for treatments 1-3; and a portion was used to grow the algae stock for the Spent Medium in Treatment 4.

A more detailed description of all analyzed ions contained in the wastewater is outlined in Appendix 1.

1.2 Nitrifying Bacteria

The nitrifying bacteria source for these experiments was field-collected activated sludge. Activated sludge (AS) was collected from an aeration basin at a municipal wastewater treatment plant in Columbus, GA – Columbus Water Works. This AS was collected in 500 mL bottles and kept in the refrigerator until needed for use.

Before the experiment, the AS was settled, and the concentrated sludge consortia was centrifuged at 5,000 rpm for 5 minutes. The supernatant was removed, and the sludge was resuspended in the same PPWW used in the experiment, to minimize any additional compounds being added to the cultures. 2 mL (~0.093 g/L) of this concentrated sludge was added to each of the twelve hybrid tubes as a nitrifier source.

1.3 Algae

There were two strains of microalgae used for these experiments: *Auxenochlorella protothecoides* (UTEX 2341) and *Chlorella sorokiniana* (UTEX 2805). Both strains have previously been shown to promote nitrification in poultry processing wastewater. *A. protothecoides* only consumes ammonium-nitrogen and not nitrite or nitrate. *C. sorokiniana* consumes all forms of nitrogen. This characteristic makes the nitrification potential of the treatments more difficult to track, so each experiment was run with both UTEX 2341 and UTEX 2805 to more clearly conclude the nitrification patterns.

Before each experiment, the algae were grown in a stock bottle using N8-NH_4 media to exponential growth (OD 550 ~ 0.4). The algae was then concentrated by centrifuge at 5,000 rpm for 5 minutes, and the supernatant was removed. The algae was then resuspended in the same

PPWW used in the bioreactors, in order to minimize any additional nutrients that could be added through the media. A small concentration of this algae was added to the spent medium stock bottle before the experiment was run; and 3 mL of the concentrated stock were added to each hybrid tube in Treatment 2 of the experiment.

2 Experimental Design

This project consisted of multiple runs of the same or similar experiments. Multiple experiments were designed, but only the first two of the hypotheses were able to be tested in the time allotted for this master's research. Each experiment was a 5-day (120 hour) batch culture with four different treatments each run in triplicate – totaling 12 bioreactors. The bioreactors used were "hybrid tubes," which are tall, thin cylinders containing approximately 200 mL of mixed culture each. A graphical representation of the bioreactors and treatments can be seen in Figure 3. The treatments were designed to test the hypotheses that enhanced dissolved oxygen (Hypothesis 1) and algal secretions (Hypothesis 2) can promote nitrification. The treatments were as follows:

Table 1: Objective 1 experiments treatment outline

Treatment 1	Activated sludge + aeration @ 0.5 vvm
Treatment 2	Activated sludge + algae + aeration @ 0.5 vvm
Treatment 3	Activated sludge + aeration with 35% DO @ 0.5 vvm
Treatment 4	Activated sludge + spent medium + aeration @ 0.5 vvm

Treatments 1-3 also contained poultry processing wastewater. Each treatment was also aerated at 0.5 vvm to simulate wastewater treatment.

Treatment 1 was the control treatment – with only activated sludge and wastewater present. The activated sludge acts as the nitrifier source, and the wastewater provides the ammonium nitrogen and other nutrients. This treatment provided insight into the nitrifier activity without any interference from other additives. Treatment 2 was the algae-aided treatment. This treatment contained one of two algae strains along with the nitrifiers and will show any possible promotion of nitrification by the addition of algae. The purpose of Treatment 3 was to test Hypothesis 1: algal promotion of nitrification through provision of dissolved oxygen. To do this, the DO levels in this culture needed to match those in the algae-aided culture. A healthy algae culture can typically reach a DO level of up to 40%, so the DO% in Treatment 3 was kept at roughly 35% by adding additional pure oxygen through the aeration tubes. Treatment 4 was a test of Hypothesis 2: algae secrete molecules that increase the fitness of nitrifying bacteria to overcome toxicity in wastewater. In order to test this hypothesis, the secretions provided through algae growth needed to be separated from the live algae cells and added to the culture – so that the nitrifiers could not be influenced by either the physical presence of the algae cells, or the photosynthetic oxygen that they provide. To do so, algae cells were grown in the filtered and diluted wastewater for 48 hours, which would give them enough time to be in exponential growth. Then, the algae cells were centrifuged, and filtered to 0.2um. The remaining "Spent Medium" was then supplemented with ammonium and phosphate to replicate the nutrient levels of the original wastewater. The Spent medium was then sterile filtered to be sure no algae cells remained in the culture. For the experiment, activated sludge was added directly to the spent medium, which was aerated the same as the rest of the cultures. Ideally, this treatment should provide insight into the role of algal secretions, also known as algal photosynthate, in enhancing nitrification.

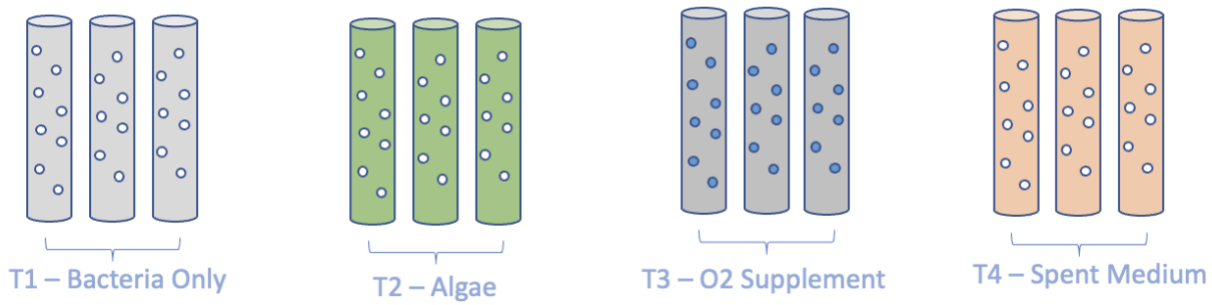

Figure 3: Graphical representation of Objective 1 Experimental Treatments

4 Analytical Methods

During the 5-day experiment, daily samples were taken of each of the twelve cultures. On days 0 and 5, two 2-mL samples were taken from each of the twelve cultures. One of the two samples was measured for Optical Density (OD) at wavelengths 550 and 680 using a spectrophotometer. These values were recorded, then all 24 samples were centrifuged at 12,000 rpm for five minutes. A biomass pellet was saved from the full 2-mL sample not used for OD measurements and frozen. The remaining supernatant from both samples was syringe filtered (0.2 um), and frozen until further use. On days 1, 2, 3, and 4, a single 2-mL sample was taken from the cultures and measured for OD 550 and 680. These measurements were recorded, and all 12 samples were centrifuged at 12,000 rpm for five minutes. The supernatant from these samples was syringe filtered (0.2 um), and frozen for later use in Ion Chromatography. The pellet from these samples was discarded.

Additionally, the DO% of treatments 1-3 were read each day with a DO probe and recorded. Oxygen flow to treatment 3 was adjusted in order to keep ~35% DO. DO time-graphs were then created to show the difference in the control, algae, and oxygen aided cultures throughout the experiment.

On day 5, 150 mL of each culture were centrifuged down and the pellet was freeze dried. These pellets were used to calculate final biomass concentration in each culture.

The daily samples collected throughout both experiments were analyzed via HPLC Ion Chromatography. Standard methods were used to evaluate the cation and anion concentrations in the cultures (Wang et al., 2019). Nitrification potential was evaluated through the time-graphs of ammonium, nitrite, and nitrate concentrations. Additionally, growth curves of all treatments were calculated though OD and dry weight data collection (Wang et al., 2021a).

5 Data Analysis

Experiments were all conducted in biological triplicate. Statistical analyses (ANOVA and Turkey's HSD test) were conducted in R with the 'car' package and 'agricolae' package. Significance was evaluated at $\alpha = 0.05$. Standard deviations were calculated in Microsoft Excel.

Results & Discussion

The following results are from four different experiments, all with almost identical experimental designs (seen in Table 1 above). Any differences between the experiments are outlined in the sections below.

Pilot Experiments 1&2

These pilot experiments were the first experiments to be completed. The experimental design of the "Follow-Up Experiments" was informed by the results of the pilot experiments.

Figure 4 illustrates results from "Pilot Experiment 1." This experiment was conducted using *Chlorella sorokiniana*. There are two datapoints of interest. First, the peak and subsequent decline of nitrate concentration in the algae treatment in this experiment display *C. sorokiniana's* tendency to consume nitrate. The significant ($p < 0.001$) and complete consumption of Ammonium could be attributed to direct consumption by the algae or nitrification. Because the algae also consume nitrate, the nitrification potential cannot be assessed easily. Pilot Experiment #2 was completed after this experiment with *Auxenochlorella protothecoides* to eliminate this

problem. The other important takeaway from this experiment was that significant promotion of nitrification by the oxygen-aided treatment was not observed. This was determined by the nitrate concentration in the oxygen-aided treatment not being significantly different than the control treatment ($p = 0.15$). This data point was surprising because the prevailing theory for algal promotion of nitrification is the provision of dissolved oxygen.

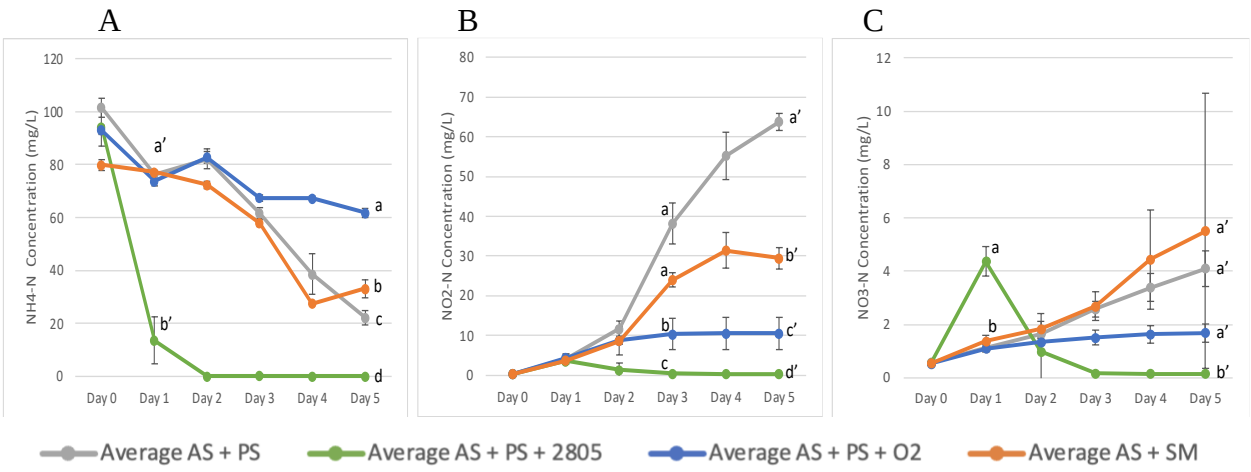

Figure 4: Nitrification parameter data for Pilot Experiment 1. A = ammonium-nitrogen removal, B = nitrite-nitrogen accumulation, C = nitrate-nitrogen accumulation. AS: Activated Sludge, PS: Poultry Scald Wastewater, SM: Spent Medium.

Figure 5 illustrates the data from "Pilot Experiment 2." *Auxenochlorella protothecoides* was used for this experiment. These data show two primary features of interest. First, there is significant ($p < 0.001$) promotion of nitrification from Treatment 2 (the treatment containing algae). This data point proves definitively that we can see algal promotion of nitrification in our system. Secondly, we continue to not see promotion of nitrification by Treatment 3. Nitrite and nitrate production in the supplemental oxygen condition were initially below those of the control culture, but the final results were not statistically significant ($p = 0.99$). After this experiment, however, it was hypothesized that the industrial grade oxygen being used for both pilot experiments could contain toxins that might suppress the nitrifiers. Because of this, the following experiments were conducted with medical grade oxygen. This was done to remove any potential

confounding effects of contaminants in the oxygen supply. There were also no DO measurements taken during this experiment or Pilot Experiment 1. To ensure that the oxygen was flowing and was at the correct percentage, daily DO measurements were taken during both follow-up experiments. Additionally, we did not see significant promotion of nitrification from Treatment 4 in this experiment or in Pilot Experiment 1. Final nitrate concentrations in this treatment were not statistically significant from the control in either experiment ($p > 0.1$).

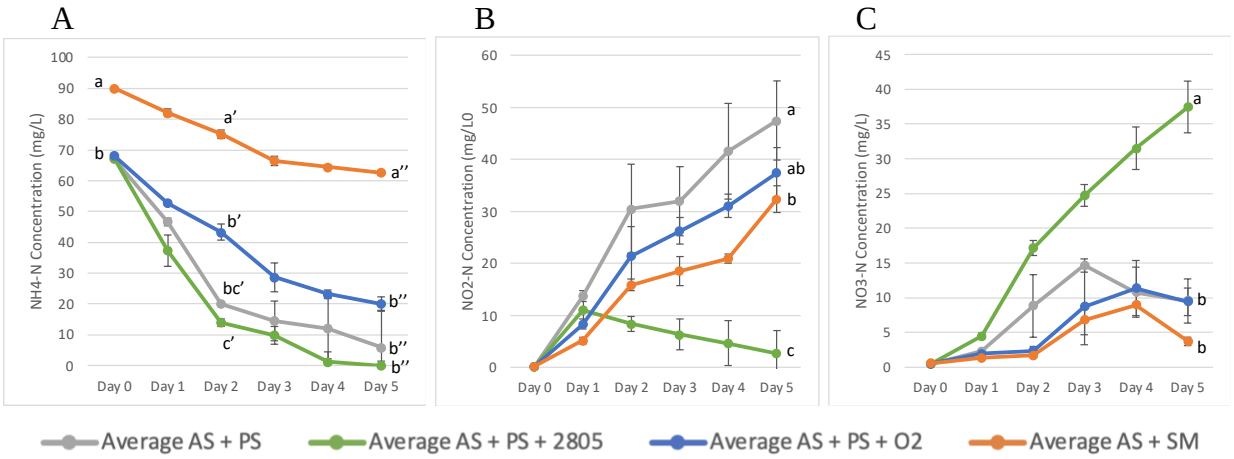

Figure 5: Nitrification parameter data for Pilot Experiment 2. A = ammonium-nitrogen removal, B = nitrite-nitrogen accumulation, C = nitrate-nitrogen accumulation. AS: Activated Sludge, PS: Poultry Scald Wastewater, SM: Spent Medium.

Follow-Up Experiments 3 & 4

The pilot experiments informed the following two experiments. These experiments were exact replicas of each other except that a different strain of algae was used in each. Again, these experiments used medical grade oxygen to minimize possible toxic effects.

Figure 6 represents the nitrification parameter data for "Follow-Up experiment 3." The experiment was competed using *Chlorella sorokiniana.* As a reminder, this is the algae that consumes nitrite and nitrate. Unfortunately, as is evident by the ammonium consumption graph, there was a large discrepancy between the ammonium concentration in the Spent Medium (Treatment 4) and the rest of the treatments. This was due to a new batch of poultry processing

wastewater that arrived between the running of the spent medium stock and the actual experiment. This did, however, provide insight into the large range of ammonium concentrations possible in poultry processing wastewater, and informed the process of checking the ammonium concentrations before the experiments are run. Because of this constituent, no spent medium information can be devised from this experiment. However, the other treatments can still be analyzed, as they all had similar ammonium inputs.

Again, in this experiment, there is no apparent promotion of nitrification by the supplemental oxygen alone, even with pure, medical grade oxygen. The DO readings seen in Figure 7 show that the oxygen was flowing and was kept at a similar concentration to the algae treatment. This further supports the claim that there are other mechanisms at play between the algae and nitrifying bacteria. Finally, there is no visible sign of nitrification promotion in the algae treatment (Treatment 2) of this experiment, but this is likely due to this algae's consumption of nitrite-nitrogen and nitrate-nitrogen.

The data in figure 7 is to verify that the DO% was kept in the expected range, and that the algae did grow to a high concentration of nearly 1.2 g/L during this experiment.

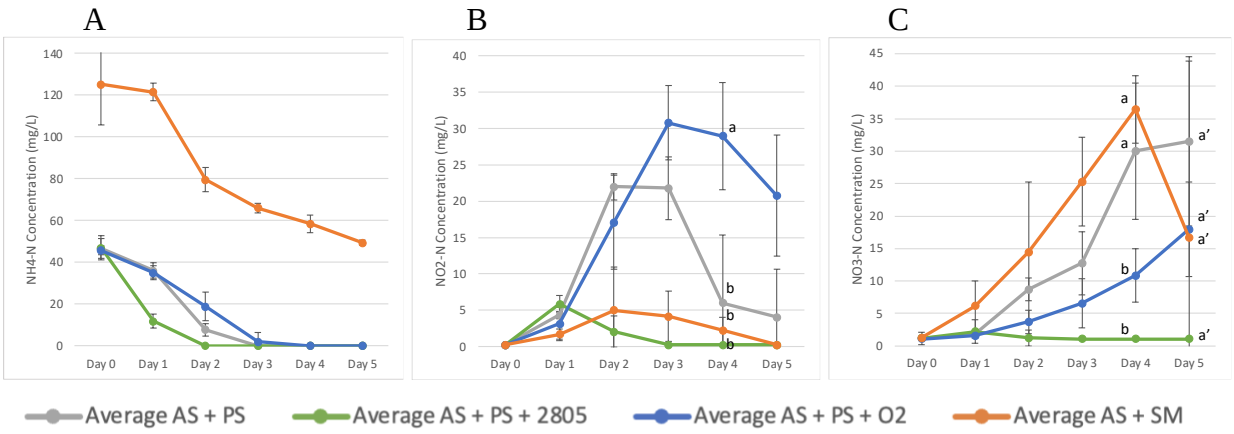

Figure 6: Nitrification parameter data for Follow-Up Experiment 3. A = ammonium-nitrogen removal, B = nitrite-nitrogen accumulation, C = nitrate-nitrogen accumulation. AS: Activated Sludge, PS: Poultry Scald Wastewater, SM: Spent Medium.

A B

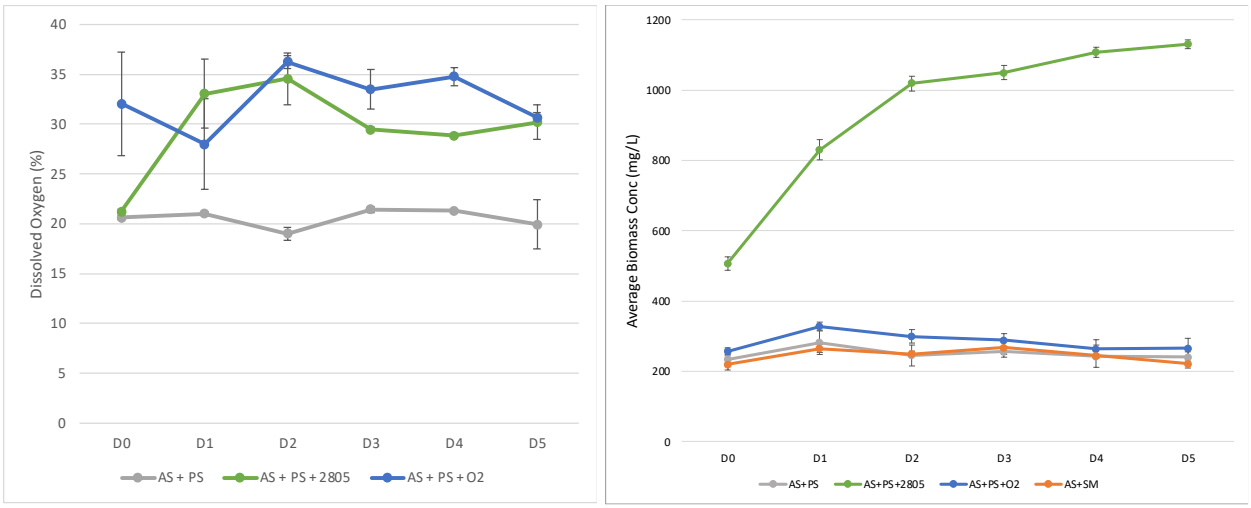

Figure 7: DO% time-graph and Biomass Concentration/Growth Curve for Follow-Up Experiment 3. A = Dissolved Oxygen, B = Biomass Accumulation. AS: Activated Sludge, PS: Poultry Scald Wastewater, SM: Spent Medium.

Figure 8 illustrates the nitrification data for Follow-Up Experiment 4. This experiment is an exact replica of Follow-Up Experiment 3, but it was completed with *Auxenochlorella protothecoides.* As a reminder, this is the algae strain that does not consume nitrite-N or nitrate-N. The first data point to take note of in this experiment is the concentration of ammonium on Day 0 of the cultures. Unfortunately, this concentration of ~60 mg/L is in the limitation zone for algae and bacteria interactions. This means that ammonium has become the limiting nutrient in this culture, and the algae and nitrifying bacteria must compete for the nitrogen. The fact that nitrification was not promoted in this case was somewhat surprising and might be explained by an unhealthy algae culture. Comparing to Figure 7, the algae in this experiment grew much more slowly, and totaled almost a 3rd of the previous experiment's growth. According to some literature, limiting ammonium concentrations are the main reason for reported negative interactions between algae and nitrifying bacteria (Choi et al., 2010; Nils, 2003). Since there was no live algae in the other treatments, conclusions can still be drawn about their behavior.

Figure 9 illustrates the DO% and the biomass concentration of the treatments in Follow-Up Experiment 4. It is shown here that the DO% was kept in the expected range, but due to low

growth, the algae culture (Treatment 2) did not produce as much dissolved oxygen as is usual in a healthy algae culture.

The Spent Medium (Treatment 4) provided a large and significant ($p = 0.002$) promotion of nitrification in this experiment. This is a very interesting result. In the future, it would be necessary to evaluate the spent medium closer and try to define what is the optimal way to provide these algal secretions to the nitrifiers, or possibly which secretions are beneficial, in order to further optimize our system.

Finally, in this last experiment, there was again no significant promotion of nitrification by the supplementary oxygen treatment. While this is an important discovery because it does not align with the prevailing hypothesis in the field, it is also important because it provides insight into the other possible mechanisms of promotion. After four experiments, it can be concluded that there is no evidence to prove that the provision of dissolved oxygen through photosynthesis fully explains algal promotion of nitrification in wastewater treatment. This opens the door for many possibilities of mechanisms that may support the symbiosis between algae and nitrifying bacteria.

Lorenzen, et al. induced nitrification in a freshwater benthic culture by purging the bulk water with O_2 gas. This differs from the current work because it is comparing the O_2 purged water to an anoxic or oxygen limited environment (Lorenzen et al., 1998). The basis for the current hypothesis is that there is additional promotion provided by microalgae even in highly aerated cultures. This difference is extremely important, because the application of this work is to systems that already have aeration, so evaluating the algae in a non-oxygen-limited environment is crucial.

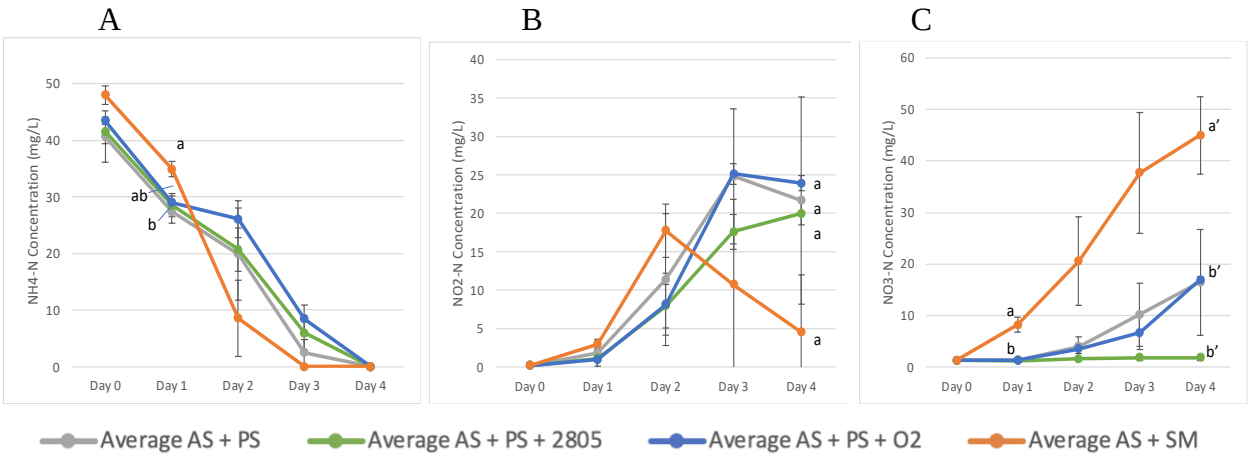

Figure 8: Nitrification parameter data for Follow-Up Experiment 4. A = ammonium-nitrogen removal, B = nitrite-nitrogen accumulation, C = nitrate-nitrogen accumulation. AS: Activated Sludge, PS: Poultry Scald Wastewater, SM: Spent Medium.

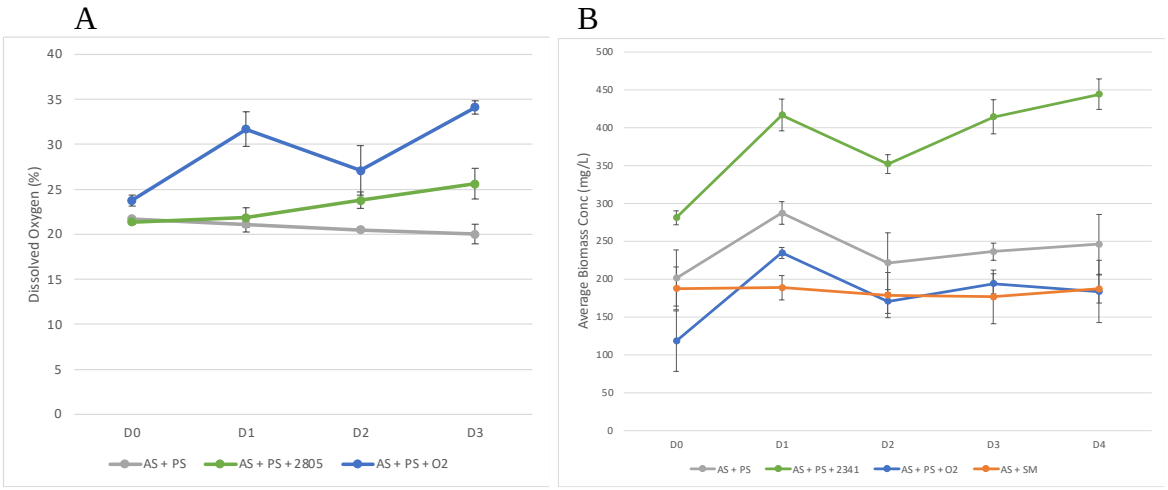

Figure 9: DO% time-graph and Biomass Concentration/Growth Curve for Follow-Up Experiment 4. A = Dissolved Oxygen, B = Biomass Accumulation. AS: Activated Sludge, PS: Poultry Scald Wastewater, SM: Spent Medium.

Conclusions

Further understanding the mechanism behind algal promotion of nitrification is crucial to optimizing waste-to-resource systems and creating sustainable agriculture processes. In this research, it was observed that the prevailing theory of promotion is incomplete. The data showed no significant promotion of nitrification by the addition of pure dissolved oxygen. Therefore, other mechanisms are likely responsible for the additional promotion of nitrification seen by the

algae-aided cultures. Algal secretions may be a promising avenue of further research. This research showed at least one strain of algae's ability to promote nitrification through its "Spent Medium." More research will need to be conducted to further isolate the benefit of algal secretions. Additionally, algae's sorption and genetic community influences also need to be evaluated to fully address the range of mechanisms that could be providing nitrification promotion in wastewater treatment. By defining these mechanisms, sustainable agriculture systems can be optimized, and the industry's wastes can be more efficiently used for crop growth.

Chapter 3: Evaluating biochar as a nitrification promotion additive in Algae-aided wastewater treatment for nutrient recycling

Introduction

The implementation of efficient waste-recycling systems is imperative to the future of our growing population. Aquaponics and similar "ponics" systems are often promoted as the future of food cultivation in our society. Our growing population as well as exponentially increasing agricultural waste products presents a specific need for nutrient recycling systems. The optimization of these systems is therefore crucial for these systems to compete with traditional agricultural cultivation methods and for the industry's wastes to be utilized to their fullest potential.

Nutrient recycling is the use of "waste" nutrients from one system as resource for another production system. Aquaponics is a perfect example of this idea. Aquaponics is a system where fish are grown to be harvested for food, and the water they are grown in becomes extremely rich in nutrients due to their waste production. The nutrient-dense water is then used to irrigate hydroponic plant production. This nutrient recycling typically reduces or negates the need for chemical fertilizers and other additives typically required in traditional crop growth (Benke and Tomkins, 2017). While the research completed herein does not represent traditional aquaponics systems, the application of this work is to a similar nutrient recycling system dubbed "poultry-ponics," where treated poultry processing wastewaters are used to grow hydroponic crops.

In aquaponics and other similar systems, nitrification is the most important microbial mechanism for treating the wastewaters (Hachiya et al., 2012; Ikeda and Tan, 1998). Nitrification is the oxidation of nitrogen from its most toxic form, ammonium, to nitrite and subsequently to nitrate (Jorgensen and Fath, 2008). Both ammonium and nitrite are toxic to plants and most other organisms, but nitrate is largely innocuous and is the preferred form of nitrogen for plant growth (Hachiya et al., 2012). This necessary transformation of nitrogen is

completed by a group of bacteria called nitrifying bacteria. These bacteria are inherently sensitive organisms (Urakawa et al., 2019b) and providing an ideal environment for them is extremely important in order for nitrification to be completed successfully.

Microalgae have been shown to promote nitrification in wastewater treatment under certain conditions (An and Joye, 2001; Lorenzen et al., 1998). One of the hypothesized mechanisms of this symbiosis is algal provision of a hyper-oxic environment due to photosynthetic oxygenation (Bankston et al., 2020). Nitrifying bacteria have a high oxygen demand (Daigger, 2014), which is why this oxygenation is beneficial to the nitrification process. Quantitative research shows that the addition of algae to nitrifying bacterial communities increases their capacity for treatment of wastewaters (Fallahi et al., 2021; Higgins, 2019).

Another way to promote nitrification is through the addition of activated carbon. Activated carbon is a dark, powdered substance that is typically created by a feedstock (most commonly coconut shells, coal, or wood) being superheated at temperatures from 600-1200 degrees Celsius under low oxygen conditions and treated with choice chemicals (General Carbon, 2022). It is usually presented in a powdered form, and has an extraordinarily large internal surface area and pore volume (Frederick S. Baker, 2000). In wastewater treatment or recycling systems, activated carbon can provide an opportunity for adsorption of potential toxins which could be inhibiting nitrification. The addition of powdered activated carbon (PAC) has been shown to enhance nitrification in multiple scenarios (Aktas and Çeçen, 2001; Ng and Stenstrom, 1987).

Biochar is similar to activated carbon in many ways including surface area, pore size, and creation processes. The difference is that it is created as a valuable by-product of biofuel production (Qian et al., 2015). Biochar has been seen to enhance nitrification in solid, organic

environments such as soil (Prommer et al., 2014) and composts (Zainudin et al., 2020), to enhance the nutritional status of hydroponic vegetables (Awad et al., 2017), and to reduce the toxic effects of both heavy metals (Mosa et al., 2016) and ammonia in hydroponic and aquaponic (Su et al., 2020) systems.

It is not known if biochar can further improve the symbiosis between algae and nitrifying bacteria, but the hypothesis driving the current research is that biochar may have favorable properties that could enrich the system and create a more optimal environment for the algal-bacterial interactions to take place. Biochar is black carbon produced from biomass sources [i.e., wood chips, plant residues, manure or other agricultural waste products] for the purpose of transforming the biomass carbon into a more stable form (carbon sequestration) (USDA, 2020). Biochar is known to alter microbial populations by providing sorption sites for organisms, facilitating interspecies electron transport (Wang et al., 2020), sorption and desorption of nutrients (Awad et al., 2017), and sorption of toxins (Mosa et al., 2016). Additionally, biochars are highly "engineer-able" – meaning that their properties can be tuned to the needs of the system. Therefore, if there is a characteristic that promotes nitrification potential, that property can be enhanced to further the efficiency of the microbial community.

The interaction of algae, nitrifying bacteria, and biochar in treating poultry processing wastewater for the purpose of nutrient recycling has, to our knowledge, not be evaluated before. The objective of this research is to evaluate the effectiveness of biochar as a wastewater treatment additive to further promote nitrification in nutrient recycling systems. Two experiments were conducted to complete this objective. The first experiment, Experiment 1, will determine which between two provided biochars is more effective. The second experiment,

Experiment 2, will determine whether the biochar itself can promote nitrification in poultry processing wastewater without the presence of algae.

Materials and Methods

1 Material Sourcing

The material sourcing and usages described below apply to both Experiment 1 and Experiment 2.

1.1 Poultry Processing Wastewater

The poultry processing wastewater (PPWW) used in these experiments was supplied by a poultry processing plant in Georgia. The wastewater was sent in gallon jugs via express mail and kept in a cold room at ~ 4°C until use. The ammonium concentration of each bottle was measured using ion chromatography using previously-published methods (Wang et al., 2019).

Before the experiment, the PPWW was then filtered to 0.2 um using a vacuum filter to remove any large sediments or microbes present. This was done to mimic the primary treatment commonly used to remove solids from poultry processing wastewater. The filtered PPWW was then diluted using DI water to create a final ammonium concentration of ~150 mg/L NH_4. The PPWW was then pH adjusted to ~7.5, and 185 mL was added to each of the twelve hybrid tube bioreactors.

A more detailed description of all analyzed ions contained in the wastewater is outlined in Appendix 2.

1.2 Nitrifying Bacteria

The nitrifying bacteria source for these experiments was field-collected nitrifying activated sludge (AS) collected from an aeration basin at a municipal wastewater treatment plant in

Columbus, GA – Columbus Water Works. This AS was collected in 500 mL bottles and kept in the refrigerator until use.

Before the experiment, the AS was settled, and the concentrated sludge consortia was centrifuged at 5,000 rpm for 5 minutes. The supernatant was removed, and the sludge was resuspended in the same PPWW used in the experiment, to minimize any additional compounds being added to the cultures. 2 mL (~0.093 g/L) of this concentrated sludge was added to each of the twelve hybrid tubes as a nitrifier source.

1.3 Algae

The microalgae used for these experiments was *Auxenochlorella protothecoides*. This algae strain only consumes ammonium-nitrogen and not nitrite or nitrate (Higgins et al., 2018). This characteristic makes the nitrification potential of the treatments easier to track. Also, *A. protothecoides* has previously been shown to promote nitrification in poultry processing wastewater.

Before each experiment, the algae were grown in a stock bottle using N8-NH_4 media to exponential growth (OD 550 ~ 0.4). The algae was then concentrated by centrifuge at 5,000 rpm for 5 minutes, and the supernatant was removed. The algae was then resuspended in the same PPWW used in the bioreactors, in order to minimize any additional nutrients that might be added through the media. 3 mL of the concentrated algae suspension were added to treatments 2, 3, and 4 of Experiment 1; and, to treatments 2 and 3 for Experiment 2.

1.4 Biochar

Two different biochars were used in Experiment 1. Both biochars were supplied by Dr. Sushil Adhikari's lab at Auburn University. These two biochars were characterized by their large difference in CEC – Cation Exchange Rate. The "Low CEC Biochar" had a CEC of ~97; the

"High CEC Biochar" had a CEC of 490.2. The Low CEC Biochar is a wood-based biochar that was made by pyrolysis and was purchased from Amazon. The High CEC Biochar is a pine-based biochar that was made via gasification at the Forest Products Lab at Auburn University. For Experiment 1, 200 mg of the Low CEC Biochar were added to each replicate of treatment 3, and 200 mg of the High CEC Biochar were added to each replicate of treatment 4 (~1g/L for both biochars). For Experiment 2, Low CEC Biochar was added to each replicate of both treatment 3 and treatment 4 in a concentration of 1 g/L.

2 Experimental Design

The present work contains two experiments. The purpose of the first experiment (Experiment 1) was to evaluate the effectiveness of the two different biochars at promoting nitrification within an algae-bacterial consortium. After the first experiment was conducted, a follow-up experiment (Experiment 2) was conducted to evaluate if the biochar alone could promote nitrification in the absence of algae. Each experiment had four treatments, each run in triplicate, for a total of twelve (12) bioreactors. The bioreactors used in this study were 300 ml bubble column reactors filled to 200 ml with the mixed culture and their operation has been previously described (Wang et al. 2019). The bubble column reactors were suspended in a water bath at ~25 °C to minimize any changes in temperature. All treatments were illuminated with fluorescent grow lights for 16 hours/day. Both experiments also use the same wastewater, nitrifying bacteria source, and microalgae described in Material Sourcing.

2.1 Experiment 1

A graphical outline of the experimental design for Experiment 1 can be found in Figure 10. This experiment contains four (4) treatments, defined below in Table 2.

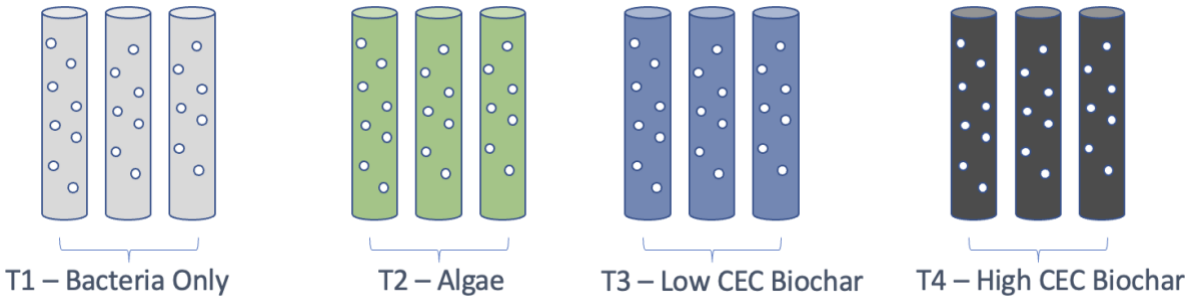

Figure 10: Graphical outline of Experiment 1 bioreactors

Table 2: Experiment 1 Treatment Outline. PPWW = Poultry Processing Wastewater, AS = Activated Sludge.

Treatment 1	PPWW + AS
Treatment 2	PPWW + AS + Algae
Treatment 3	PPWW +AS + Algae + Low CEC Biochar
Treatment 4	PPWW + AS + Algae + High CEC Biochar

Experiment 1 was conducted for 20 days using semi-continuous reactor operation. Every 5 days, the culture was settled for 1 hour, 50% of the liquid supernatant was removed using a vacuum filter and replaced with fresh wastewater. This fresh wastewater was filtered and diluted in the same fashion as the original wastewater from inoculation (Day 0), outlined in *Material Sourcing.* Each day, a single 2-mL sample was taken from the cultures and measured for Optical Density (OD) at wavelengths 550 and 680 using a spectrophotometer. These measurements were recorded, and all 12 samples were centrifuged at 12,000 rpm for five minutes. The supernatant from these samples was syringe filtered (0.2 um), and frozen until later use. The pellet from these samples was discarded. Additionally, on days 0, 5, 10, 15, and 20, a second 2-mL sample was taken from each of the twelve cultures. After the optical density reading, all 24 samples were centrifuged at 12,000 rpm for five minutes. A biomass pellet was saved from the second 2-mL

sample and frozen. The remaining supernatant was syringe filtered (0.2 um), and frozen until further use.

2.2 Experiment 2

A graphical outline of the experimental design for Experiment 2 can be found in Figure 11. This experiment contains four (4) treatments, defined below in Table 3. In this case, only the Low CEC Biochar was used because of the promotion effect it provided in Experiment 1.

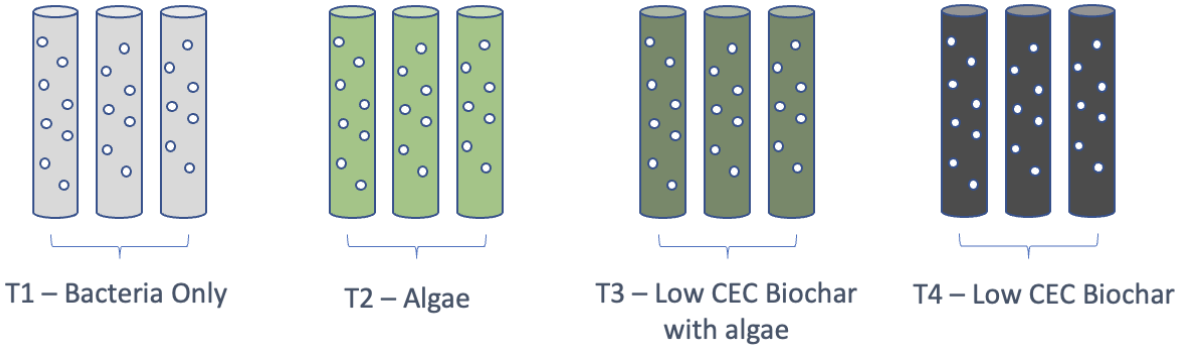

Figure 11: Graphical outline of Experiment 2 bioreactors

Table 3: Experiment 2 Treatment Outline. PPWW = Poultry Processing Wastewater, AS = Activated Sludge.

Treatment 1	PPWW + AS
Treatment 2	PPWW + AS + Algae
Treatment 3	PPWW +AS + Algae + Low CEC Biochar
Treatment 4	PPWW + AS + Low CEC Biochar

This was a batch culture experiment conducted for 5 days (120 hours). Each day, a single 2-mL sample was taken from the cultures and measured for Optical Density (OD) at wavelengths 550 and 680 using a spectrophotometer. These measurements were recorded, and all 12 samples were centrifuged at 12,000 rpm for five minutes. The supernatant from these samples was

syringe filtered (0.2 um), and frozen until later use. The pellet from these samples was discarded. Additionally, on days 0 and 5, a second 2-mL sample was taken from each of the twelve cultures. After reading Optical density, all 24 samples were centrifuged at 12,000 rpm for five minutes. A biomass pellet was saved from the second 2-mL sample and frozen. The remaining supernatant from both samples was syringe filtered (0.2 um), and frozen until further use.

3 Data Analysis

The daily samples collected throughout both experiments were analyzed via ion chromatography. Previously published standard methods were used to evaluate the cation and anion concentrations in (Wang et al., 2019). Nitrification potential was evaluated through time-course tracking of ammonium, nitrite, and nitrate concentrations. Additionally, growth curves of all treatments were calculated though OD and dry weight data collection as previously described (Wang et al., 2021a).

Experiments were all conducted in triplicate. Statistical analyses (ANOVA and Turkey's HSD multiple comparisons test) were conducted in R with the ‘car’ package and ‘agricolae’ package. Significance was evaluated at $\alpha = 0.05$. Standard deviations were calculated in Microsoft Excel

Results and Discussion – Experiment 1

As seen in Figure 12, significant ($p = 0.002$) promotion of nitrification was seen in the cultures with low CEC biochar. This conclusion can be made by the large nitrate accumulation in Treatment 3. This treatment, along with Treatment 2 also showed complete ammonium consumption. Because this was not accompanied by a large increase in nitrite or nitrate in Treatment 2, it is likely that the algae were responsible for this consumption. In contrast, the

combination of algae, AS, and biochar in Treatment 3 led to much greater oxidation of ammonium to nitrate.

Treatment 3 – the Low CEC Biochar – shows a promising ability to promote nitrification in poultry processing wastewater. The High CEC Biochar did not show such a promotion. There seems to be a suppression of nitrification by the High CEC Biochar. The hypothesis for these two treatments was that the High CEC Biochar not promote nitrification due to its increased interaction with positively charged ions. Ammonium, being a positively charged ion, was hypothesized to be immediately sorbed by the High CEC Biochar, leaving none for nitrification. Based on the ammonium consumption trends seen, this did not seem to be the case. The ammonium concentration was not significantly different across any of the treatments in the first 24 hours. It is likely that there is another mechanism or characteristic in the High CEC Biochar that is limiting promotion of nitrification.

While the Low CEC Biochar did promote nitrification significantly above the other three treatments, this does not mean that low cation exchange capacity is the driving characteristic behind this biochar's promotion effect. Both biochars have many different properties that have yet to be categorized. There are many possibilities as to why this specific biochar promoted nitrification, such as surface area, biochar composition, or even the chemicals used in the creation process. Further data needs to be collected in order to define these characteristics. Once this characteristic is isolated, the biochar can be tuned or engineered to further optimize the nitrification potential.

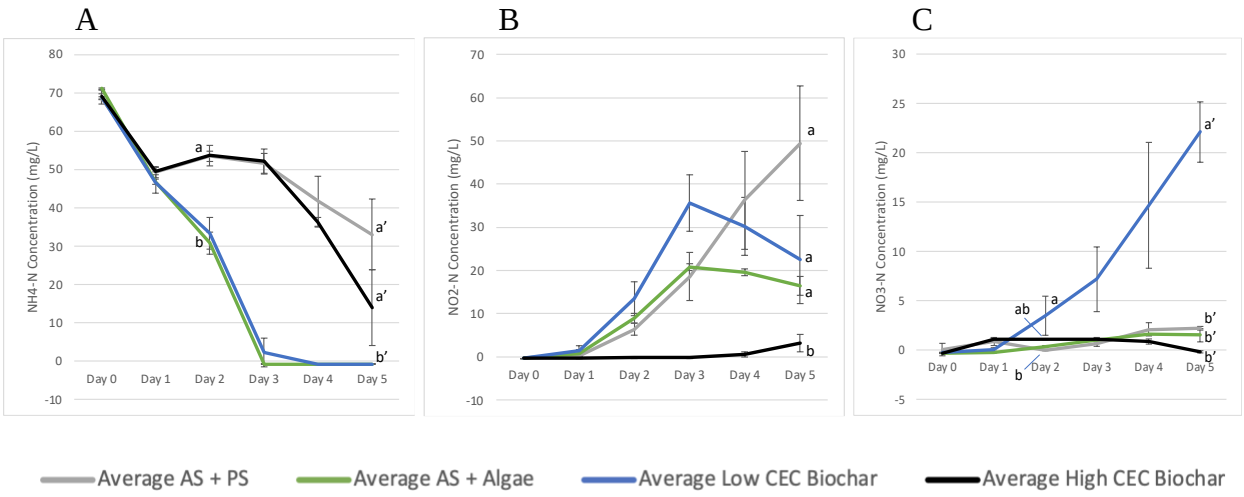

Figure 12: Nitrification parameter data for Experiment 1, Days 0-5. A = ammonium-nitrogen removal, B = nitrite-nitrogen accumulation, C = nitrate-nitrogen accumulation. AS: Activated Sludge, PS: Poultry Scald Wastewater. Note: within each panel, letters with ‘, and ‘’, are compared to each other. Letters without punctuation are also compared to each other.

The accumulation of nitrate in treatment 3 does not continue over time – as seen in Figure 13. Due to overgrowth of algae (seen in Figure 14), there is no nitrate accumulation in any of the treatments containing algae after Day 5. The control treatment (Treatment 1) continued to nitrify over time, suggesting that this lack in nitrification in other treatments was not due to fitness of nitrifiers or toxins in the wastewater. This phenomenon is hypothesized to be due to extremely dense algal cultures being grown in the bioreactors. Such cultures likely assimilated ammonium rapidly, depriving nitrifying bacteria of their electron source.

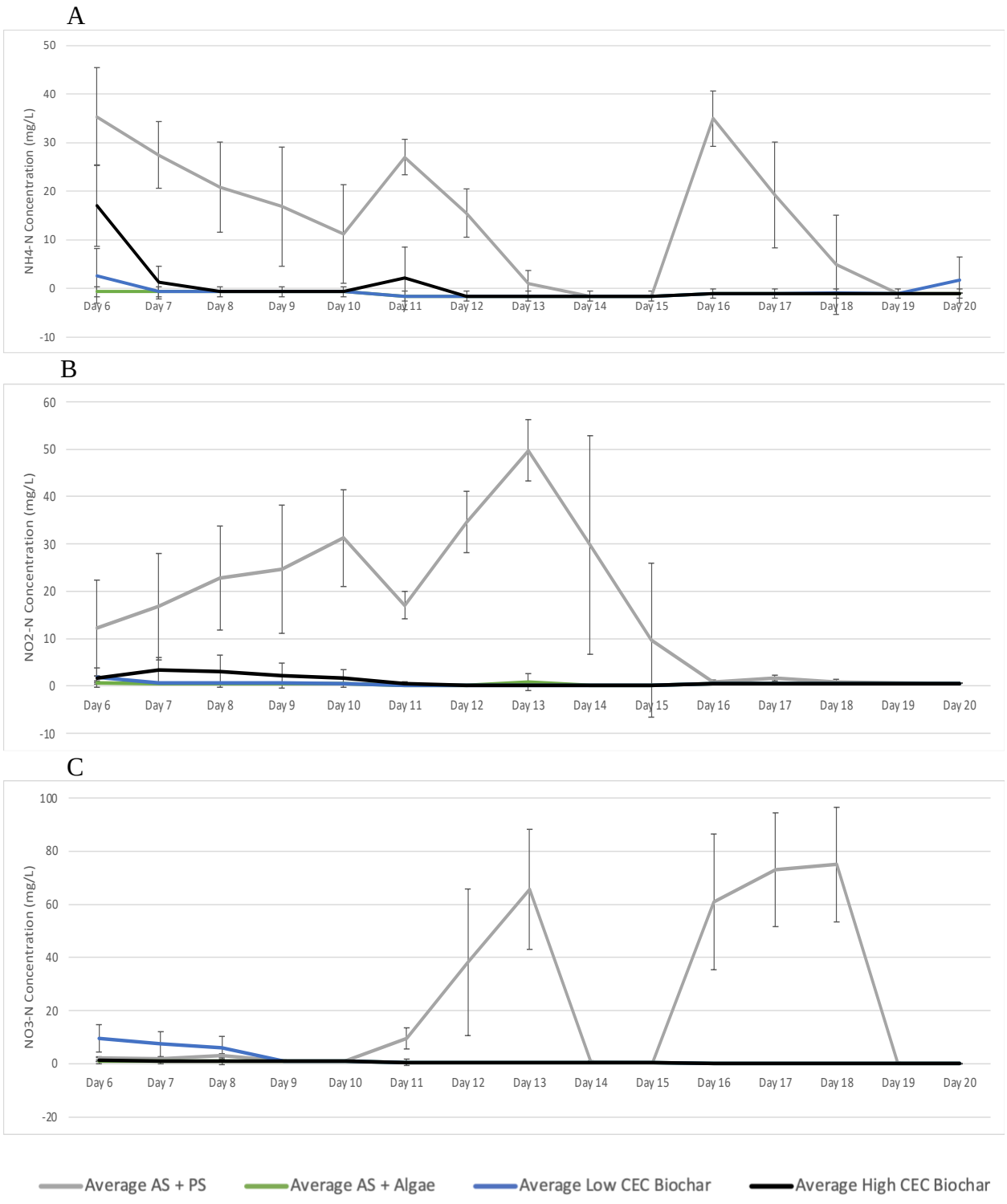

Figure 13: Nitrification parameter data for Experiment 1, Days 6-20. A = ammonium-nitrogen removal, B = nitrite-nitrogen accumulation, C = nitrate-nitrogen accumulation. AS: Activated Sludge, PS: Poultry Scald Wastewater.

The ammonium concentrations in these cultures decreased to limiting values after the first wastewater replacement. Typically, when ammonium is limiting, promotion of nitrification in algal-bacterial communities ceases. This is due to the competition between the two microorganisms (Choi et al., 2010; Nils, 2003). Algae require nitrogen to survive and are often more robust organisms than nitrifying bacteria. They therefore out-compete the bacteria for the available ammonium nitrogen, leaving none left in the culture to be nitrified by the bacteria. During the experiment, it was observed that the algae looked "yellow-ish-green," which usually happens when the algae are grown heterotrophically and starved of nitrogen (Miao and Wu, 2006). Poultry processing wastewater contains large quantities of soluble COD including volatile fatty acids (VFAs) and protein. *A. protothecoides* is a known consumer of VFAs and amino acids (Higgins et al., 2015; Kind et al., 2012) and was likely growing mixotrophically in the poultry processing wastewater. This would explain the very high culture densities on the order of 3 g/L (seen in figure 14) which have been observed previously in mixotrophic culture of this organism (Higgins and VanderGheynst, 2014). The algae will produce more lipids than when they are healthy, which gives this lighter, yellow-er visual. If the algae were nitrogen-starved, then each time the culture was replaced with fresh wastewater, the algae would immediately uptake all available nutrient into their biomass, which would leave nothing left for the nitrifiers to oxidize. There was not enough ammonium being replaced into the system to accommodate the needs of both the dense algae culture and the nitrifying bacteria.

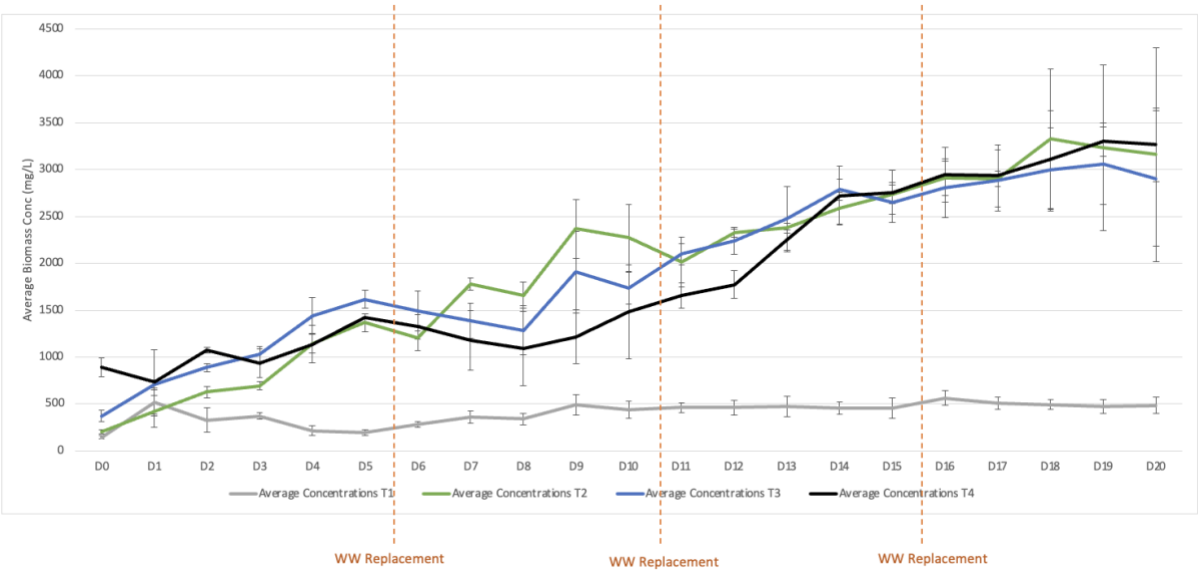

Figure 14: Experiment 1 Growth Curve/Biomass Accumulation. T1 = Control Treatment, T2 = Algae Treatment, T3 = Low CEC Biochar Treatment. T4 = High CEC Biochar Treatment.

Results and Discussion – Experiment 2

The data presented in Figure 15 is the nitrification parameter data for Experiment 2. The purpose of this experiment was the evaluate the nitrification potential of the Low CEC Biochar further. After seeing the benefit of the Low CEC Biochar in Experiment 1, an additional experiment was devised to see if the promotion of nitrification was reliant on algae. There is significant promotion of nitrification seen by both treatments containing biochar, regardless of the presence of algae through day 4 ($p = 0.0002$). This suggests that there is some characteristic or property that the biochar has that aids in nitrification despite the presence of algae.

There are many possible characteristics that could be responsible for the promotion seen in the present work. The prevailing hypothesis from other researchers who have seen similar behavior is that the addition of more surface area to the system provides a substrate for the nitrifying bacteria to form biofilms. In a paper by Su, et al. from 2020, it was found that nitrate accumulation was increased with the increase in biochar concentration in their pilot-scale

aquaponics system. They concluded that the increased concentration of nitrate can be attributed to the increased colonization of NOB (Nitrite oxidizing bacteria) on pyrolysis biochar as indicated by the increased mass gain of biofilm formed on the biochar (Su et al., 2020). This further protects them from any inhibitors in the wastewaters and allows them to nitrify more effectively. To define decisively what property is responsible for the observed nitrification promotion, additional experiments would need to be done.

After day 4, many of the replicates (notably, one replicate of Treatment 2, as well as some others to a lesser extent) began to show visible signs of culture death. The cultures looked more brown or red in tint than the others in the same treatment. As for the algae treatment in this experiment, it can be observed that the treatment containing only algae did not provide further nitrification promotion. This is contrary to the previous data conducted in Objective 1 where *A. Protothecoides* was seen to promote nitrification in poultry processing wastewater. There are many reasons why this algae treatment may have not showed visible promotion of nitrification in this experiment, and it may also be related to the lysing/culture death that was observed later in the experiment. The large error bars and inconsistent significance levels on day 5 (seen in Figure 15) are attributed to this lysing phenomenon.

Since the algae treatment did not show nitrification promotion, it cannot be concluded whether the presence of algae would further promote nitrification along with the biochar if the algae are healthy and if there is no competition for nutrients. Further experiments would need to be conducted in order to make any conclusion about this. However, if the promotion is provided by biochar acting as a physical substrate and providing surface area for the bacteria to colonize on, it can be hypothesized that the algae would also benefit from this characteristic.

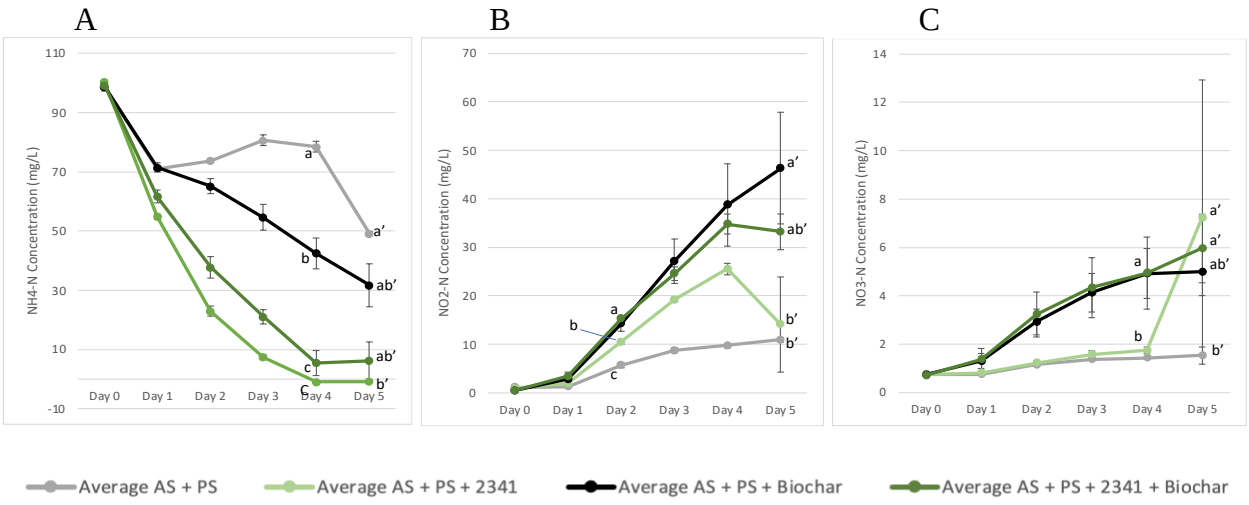

Figure 15: Nitrification Parameter data for Experiment 2. A = ammonium-nitrogen removal, B = nitrite-nitrogen accumulation, C = nitrate-nitrogen accumulation. AS: Activated Sludge, PS: Poultry Scald Wastewater.

Conclusions

The data presented in this study provides evidence that the Low CEC Biochar can promote nitrification in poultry processing wastewater with and without the presence of algae. This research prompts many following studies including but not limited to evaluation of different characteristics of biochar for nitrification promotion, isolation of the biochar characteristic that provides a benefit to the nitrifying bacteria, and a follow-up study to see if the presence of healthy algae would further promote the nitrification in a biochar-supplemented system. This research also provides insight into the relationship between biochar and nitrifying bacteria for use in waste-to-resource systems such as aquaponics and "poultry-ponics."

Conclusions and Future Plans

Takeaways

Although this research creates more questions than it answers, there are still multiple conclusions to be taken from the data collected. First, algae (both *Auxenochlorella protothecoides* and *Chlorella sorokiniana*) have the ability to promote nitrification in poultry processing wastewater provided that ammonium is not limiting. This finding further promotes the application of algae-aided nitrification in nutrient recycling systems (specifically poultry-ponics). Regarding the mechanism by which this occurs, the results are not conclusive. However, there does seem to be sufficient evidence herein to suggest that photosynthetic oxygenation alone does not provide enough benefit to account for this promotion. Additionally, "spent medium" and algal extracellular secretions seem to be partially responsible for nitrification promotion. Algal secretions were seen to play a role in nitrification enhancement in both objectives and should be further explored to identify its benefits more precisely.

Finally, it was also observed that some biochars do have the ability to promote nitrification in wastewater treatment with and without the additional presence of algae. Biochars are also known to help mitigate toxins, so this finding could have an even more beneficial application in poultry processing wastewater due to the presence of antimicrobials.

Future Plans

For this research to be entirely conclusive, there are a few important avenues of research to be explored. First, a run of the experiment from Objective 1 in which all of the components are successful would be necessary for this data to be publishable. Unfortunately, an ideal run of this experiment was not completed during the time allotted for a master's degree. This would need to be done to present this data conclusively in a publishable paper. Secondly, the other two hypotheses for algal promotion of nitrification need to be explored. QPCR and sequencing data

from these experiments would need to be evaluated to make conclusions about any community change the algae may have on the nitrifiers. Also, another experiment would need to be conducted to assess any physical sorption that the algae cells may be doing that could be affecting the performance of the nitrifiers. For the "spent medium" hypothesis, more specific experiments should be designed in order to truly observe the effect of algal secretions. An experiment where algal cells were purposefully lysed could provide insight into both the spent medium hypothesis from Objective 1, and the unexplained spike in nitrification seen in Objective 2.

The future scope of Objective 2 really pertains to the application of the "engineerability" of biochar for use in nutrient recycling systems. More experiments need to be conducted to narrow down the characteristics responsible for the promotion provided by this biochar. Once these are determined, the biochar can be tuned to provide the most benefit to the nitrifiers. The characteristics that are beneficial to nitrification and toxin mitigation should be enhanced in the biochar to create an ideal substrate for the poultry-ponics system.

Overall, the conclusions gathered from this research will inform future work in nutrient recycling and waste-to-resource wastewater treatment systems. Ideally, this work will be directly applied to the optimization of a poultry-ponics system. However, more information needs to be gathered in order to fully explore the questions posed in this research.

Appendix 1 : Objective 1 Wastewater Constituents

	Pilot Experiment 1	Pilot Experiment 2	Follow-Up Experiment 3	Follow-Up Experiment 4
Sodium	35.19951017	20.97246861	238.0383048	230.8193493
Ammonium	130.527	86.15881316	59.79620407	52.22226437
Potassium	77.59668202	32.03384442	57.58608714	56.44989891
Magnesium	36.94821067	28.30113607	19.16633897	14.79616384
Calcium	93.02524823	71.0011225	80.82441026	65.24139816
Chloride	104.4999842	27.80801823	151.4116161	144.3508545
Nitrite	0.842658971	0.889845575	0.74371669	0.74371669
Nitrate	2.52449843	2.062703118	5.180971493	5.95059489
Phosphate	21.30033723	24.10434883	32.54663988	28.74349995
Sulfate	43.99060008	21.60022254	N/A	N/A

*All data presented in mg/L

Appendix 2 : Objective 2 Wastewater Constituents

	Experiment 1	**Experiment 2**
Sodium	219.3149661	275.6611353
Ammonium	88.06839426	126.9933623
Potassium	67.05753683	89.36737776
Magnesium	10.89018221	42.06190212
Calcium	82.905289	153.1728514
Chloride	63.12480051	136.907313
Nitrite	0	1.146610539
Nitrate	0.158443318	0.757245732
Phosphate	11.86735	39.76950047
Sulfate	24.4534684	14.54152817

*All data presented in mg/L

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