Solids Retention Governs Nitrification of Poultry Processing Wastewater using an Algal-bacterial Consortium

by

Dillon Sprague

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Approved by

Brendan Higgins, Chair, Assistant Professor, Dept. of Biosystems Engineering
David Blersch, Associate Professor, Dept. of Biosystems Engineering
Mark Dougherty, Associate Professor, Dept. of Biosystems Engineering

Abstract

As water demand increases worldwide, the utilization of alternative water sources, such as wastewater, is becoming necessary. The poultry processing industry generates nutrient-rich wastewater that could be treated and reutilized as irrigation water for hydroponic systems. Unfortunately, antimicrobials are often found in poultry processing wastewater, which have been found to have a negative effect on the bacteria within wastewater treatment systems. Antimicrobial chemicals such as peracetic acid (PAA) have particularly acute effects on nitrifying bacteria. The function of nitrifiers is critical to repurposing this water for irrigation. In our lab, research has shown that the addition of algae can aid bacteria, and particularly nitrifiers, in the treatment of poultry wastewater. The objective of this work was to determine if and under what conditions algae promote nitrification during treatment of poultry processing wastewater in continuously operated reactors over a long (200 day) time horizon. Also, we aimed to test the resilience of nitrification to shock events such as the addition of the antimicrobial, PAA. In this study, two reactors were used, one containing bacterium and the other containing an algal-bacteria consortium. From this study, it was revealed that the effluent composition of the algal-bacterium reactor can be manipulated by the amount of solids retained (p-value <0.05). In the algal reactor, low solids retention led to a photosynthetic dominant system that had high nitrogen and phosphorous removal. A high solids retention led to a bacteria dominated system, supported by algae, which helped oxidize nutrients and made the water suitable for hydroponic irrigation. Overall, both systems were negatively affected by stressor events, but the algal system exhibited greater resilience by continuing to remove phosphorous and transform ammonium to nitrate.

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AOB - Ammonium-oxidizing Bacteria

AS – Activated Sludge

DO – Dissolved Oxygen

HRT – Hydraulic Retention Time

NAS - Nitrifer-enriched-activated-sludge

OD – Optical Density

PAA – Peracetic Acid

qPCR – Quantitative PCR

SRT – Solids Retention Time

TSS – Total Suspended Solids

Introduction

With increasing water demands throughout the world, utilizing alternative and water resources, including wastewater, is becoming necessary. The food processing industry holds a unique position within the water landscape, generating nutrient-rich wastewater that is continually expelled and treated as a waste. In the past, scientists have focused on the meat processing industry, specifically on beef processing plants and the large wastewater streams generated in those procedures (Flugaur, 2003; Ziara et al., 2018). Similarly, the poultry processing industry generates approximately 62 billion gallons of wastewater annually (Julie K. Northcutt & Deana R. Jones, 2004) and (Carawan et al., 2014) at a cost of over $247 million for treatment. These numbers are based on an assumed 6.9 gallons (26 L) of water per bird (J.K. Northcutt & D.R. Jones, 2004) and a treatment cost of $4 per 1000 gallons (Carawan et al., 2014). In 2018, the broiler industry within the United States, produced approximately 9 billion chickens, with a total economic impact of $31.7 billion (NASS, 2019). The waste stream created from the poultry slaughter and meatpacking industries are rich in nitrogen, phosphorus, and potassium, all of which are essential for plant growth (Uchida, 2000). However, roughly two thirds of the poultry processing plants discharge this nutrient-rich wastewater to their city sewer systems, while the remainder utilize on-site treatment to be directly discharged to the environment (Julie K. Northcutt & Deana R. Jones, 2004). By continuing to dispose of wastewater in this manner, the industry is letting valuable nutrients go to waste.

In addition to high levels of nutrients, poultry processing wastewater also contains antimicrobial chemicals that are used in processing plants. Although these antimicrobial chemicals are used to kill pathogens on the meat products, they have an unfortunate side-effect of interfering with the beneficial bacteria used to treat wastewater. Little is known about these

antimicrobials and the effect they have on bacteria within the wastewater treatment system. However, peracetic acid (PAA), a common antimicrobial, has been shown to inhibit nitrate formation (Pavlostathis, 2019).

Our goal is to treat poultry processing wastewater so that it can be repurposed as irrigation water. However, there are three major challenges that need to be addressed to ensure this water is appropriate for irrigation: 1) the nutrients need to be transformed into forms suitable for stable plant growth, 2) the negative effects of antimicrobial chemicals need to be mitigated, and 3) pathogens need to be eliminated. This thesis specifically focuses on the first two issues. Most importantly, the nitrogen in the system should be in the form of nitrate. Nitrate is essential to plant growth as it provides nitrogen to create amino acids. Nitrate is the preferred form for hydroponic plant production as reduced forms of nitrogen, such as ammonium, lead to unstable production due to ammonium toxicity (Hachiya et al., 2012). The antimicrobial chemicals themselves could prove detrimental to plant production both through direct interaction with plants and through interference with nitrogen oxidation.

In past work, researchers in our lab have been found that algae can aid bacteria in the treatment of poultry wastewater. The algae, themselves, are effective at taking up nitrogen and phosphorus pollutants and reducing the load on the bacteria within the system. We have shown in batch cultures that adding the green algae species, *Chlorella sorokiniana,* can increase the abundance and function of nitrifying bacteria within the system. This work also suggested that algae can mitigate the detrimental effects of the two most common antimicrobial agents, specifically PAA and cetylpyridinium chloride (CPC), on nitrifying bacteria. However, batch cultures (run over the course of 120 hours) often are not representative of what would happen in continuously operated reactors over long time horizons. Therefore, the objective of this work

was to determine if and under what conditions algae promote nitrification during treatment of poultry processing wastewater in continuously operated reactors over a long (200 day) time horizon. We also aimed to test the resilience of nitrification to shock events such as the addition of the antimicrobial, PAA. We hypothesized that combining algae with bacteria in continuously-operated wastewater treatment systems will lead to improved nitrification similar to what was previously observed in batch cultures. We also hypothesize that the algal-bacterial system would exhibit greater resilience to antimicrobial chemicals when compared to a treatment reactor with only bacteria. These hypotheses were tested through long-term operation (200 days) of two reactors: one inoculated with algae and a bacterial consortium and the other reactor inoculated with only the bacterial consortium.

Literature Review

2.1 Wastewater Treatment

The treatment of wastewater dates back to the 1800s when the first municipal water treatment plant was built in Scotland (Mohsenpour et al., 2021). Since then, wastewater treatment has become essential for every city and municipality as a way of mitigating and reducing the negative effects of releasing contaminated waters into the environment. The goal of wastewater treatment is to reduce the quantity of carbonaceous materials, along with excessive concentrations of nitrogen and phosphorus compounds, prior to downstream discharge (Grady Jr et al., 2011; Gray, 2004). The release of large amount of decayed organic material and debris can use up the dissolve oxygen in water bodies, resulting in an environment where aquatic biota and fauna cannot survive (USGS, 2021). Additionally, the addition of nutrients, such as nitrogen and phosphorus, can cause eutrophication. Eutrophication is the enrichment of an aquatic environment by nutrients leading to excessive plant and algal growth (Chislock et al., 2013). The dense blooms of algal growth reduce water clarity and harm water quality. The blooms limit light penetration, reducing growth and causing die-offs of plants and lowering the success of predators requiring light to pursue their prey (Lehtiniemi et al., 2005).

To help prevent the negative effects of wastewater discharge, conventional wastewater treatment consists of a combination of physical, chemical, and biological processes and operations to remove solids, organic matter, and nutrients (Sonune & Ghate, 2004). Typically, wastewater treatment uses several steps which are preliminary treatment, primary treatment, and secondary treatment. Preliminary treatment removes large materials and coarse solids, such as wood, cloth, and paper from the wastewater stream. Next, primary treatment is designed to remove organic and inorganic solids by sedimentation and flotation. This step removes

approximately 25-50% of the incoming biological oxygen demand (BOD), 50-70% of the total suspended solids (TSS), and 65% of oil and grease (Sonune & Ghate, 2004). Finally, the goal of secondary treatment is to remove the residual organics and suspended solids remaining in the stream. To accomplish this, secondary treatment utilizes different microorganisms that can metabolize the organic material (Sonune & Ghate, 2004). Beyond the three standard processes, wastewater treatment facilities have begun adding steps to improve their treatment methods, called tertiary treatment. Tertiary treatment involves a series of additional steps after secondary treatment to further reduce organics, turbidity, nitrogen, phosphorous, metals and pathogens (Pepper et al., 2011). Both physicochemical treatment and combined biological-physical treatment are included in tertiary treatment. Physicochemical treatment is defined as a treatment in which biological and physical-chemical process are intermixed to achieve the desired effluent (Sonune & Ghate, 2004). Finally, combined biological-physical treatment combines biological and physicochemical treatments (Sonune & Ghate, 2004). New technologies related to wastewater treatment are continually being developed and improved. These new technologies consist of membrane treatment technology, desalination technology, reverse osmosis, electrodialysis, ion exchange, and freeze desalination.

Wastewater that is generated in the food processing industry has differing characteristics than municipal waste but still follows a similar set of treatment procedures. Wastewater generated in the food industry, typically, has higher concentrations of BOD and suspended solids (SS) (Tchobanoglous et al., 2003). Additionally, the influent stream is constantly changing due to the seasonal nature of food processing and post harvesting. Animal slaughtering, such as in the poultry industry, leads to very strong organic waste from bodily fluids (Tchobanoglous et al., 2003). In addition to this, the wastewater can be contaminated with concentrations of

antimicrobials, growth hormones, and pesticides (Tchobanoglous et al., 2003). In the poultry industry, preliminary treatment using dissolved air flotation is used to remove particulate matter like fat and animal tissue. Next, approximately two-thirds of plants discharge this effluent into the sewer where it is treated with other municipal water. At this stage, the wastewater is treated by conventional processes such as activated sludge. Activated sludge is a biochemical process that uses oxygen and microorganisms to biologically oxidize organics in the waste stream (Tchobanoglous et al., 2003). Typically, these systems use an aeration tank where the oxygen is injected into and mixed with the wastewater. Next, a clarifier allows the waste sludge to settle, where the sludge and water can be removed for further treatment. The remaining one-third of plants treat their wastewater onsite with aerated lagoons prior to environmental discharge.

2.1.1 Wastewater Treatment with Algae

Many current wastewater treatment processes have several drawbacks that researchers are looking to improve upon. Wastewater processes require substantial amounts of electricity, which in turn, carbon emissions are released. Approximately 0.6 to 3% of the total electricity generated in developed nations is expended on treating wastewater (Chae & Kang, 2013; Plappally & Lienhard V, 2012; Wang et al., 2016). Of the electricity consumed, approximately 50% or more is used during aeration processes (Pabi et al., 2013). Additionally, as more nitrogen and phosphorus need to be removed from the system, energy and electricity requirements and costs increase. Another drawback described by Mohsenpour et al. (2021) is the high production of sludge in activated sludge systems. In the United States, approximately 13.8 million tons of dry solids were generated annually from the 15,000 public-owned treatment facilities (Seiple et al., 2017). The disposal and handling of the sludge generated presents a challenge to those managing wastewater as the sludge cannot always be used in direct application to agricultural land

(Mohsenpour et al., 2021). Typically, the sludge generated must be treated for pathogens, toxic metals, and other chemicals, prior to land application (Singh & Agrawal, 2008). The use of microalgae, alongside current wastewater treatment processes, provides a unique opportunity to lower energy consumption by reducing aeration requirements and operation complexity without affecting performance and while meeting effluent standards (Mohsenpour et al., 2021).

The use of microalgae has been reported to be highly efficient in removing phosphorus and nitrogen, along with capturing CO_2, from wastewater (Cai et al., 2019). The microalgae can utilize both organic forms of nitrogen, such as urea, and inorganic nitrogen, such as ammonium, ammonia, nitrite, and nitrate (Ross et al., 2018). Because algae can utilize all these forms of nitrogen, the wastewater does not need to transition between different operating conditions to facilitate the nutrient transformation or removal. The wastewater treatment process can occur in a single-step treatment stage, reducing the complexity and energy required, compared to a typical wastewater treatment system (Gouveia et al., 2016; Sturm & Lamer, 2011). Additionally, as the algae species perform photosynthesis, dissolved oxygen is produced, reducing the cost of energy and electricity spent on aeration.

Many species of microalgae have been studied on varying types of wastewater, such as municipal, agricultural, brewery, refinery and industrial (Mohsenpour et al., 2021). The algae, *Scendesmus obliquus*, has been shown to successfully remove nutrients from piggery wastewater, whereas *Chlorella pyrenoidosa* successfully grew in dairy production effluent (Ji et al., 2013; Kothari et al., 2012; Prandini et al., 2016). The success and performance of microalgal treatment is affected by several different parameters of the wastewater stream. A significant influence on microalgae success is the ratio of carbon, nitrogen, and phosphorus in the wastewater (Mohsenpour et al., 2021). To ensure optimal nutrient removal efficiency, an optimal

ratio of nutrients that is reflective of the microalgae elemental stoichiometry is required (Mohsenpour et al., 2021). The utilization of these specific nutrients is closely tied to the growth of the organism so a limited supply of a primary nutrient can significantly reduce their growth rate (Xin et al., 2010). Hence, supplementation of a limiting nutrient to the wastewater is helpful.

Several systems for microalgae cultivation techniques have been designed and can be classified as either suspended or immobilized systems (Christenson & Sims, 2011). The different systems can be categorized further as either being open to the environment or enclosed. A few specific examples of algal systems are photobioreactor (PBR), high-rate algae ponds (HRAP), matrix-immobilized microalgae, and attached microalgae biofilm systems (Borowitzka & Moheimani, 2013). The system with the greatest potential for widespread adoptions is high rated algae ponds or HRAP. In immobilized cells, the immobilization of microalgae is achieved through the self-attachment to a bedding material either fully or partially submerged to support biofilm development, or throughout entrapment in gel matrices by flocculants or chemical agents (Mohsenpour et al., 2021).

2.1.2 Impact of Antimicrobials on Wastewater Treatment

Although the reuse of wastewater is not a new concept, there are specific barriers related to the poultry processing industry that prevent widespread adoption. A major issue is the presence of antimicrobial agents within the wastewater. These antimicrobial agents negatively affect microorganisms that are known to be involved in the nitrogen cycle (Pavlostathis, 2019; Semedo & Song, 2020; S.R. Woodruff, 2019). In addition to restricting the microorganisms within the system, many antimicrobial agents are known to persist in the environment (Semedo & Song, 2020) and harm plant production by negatively affecting essential microbial processes (S.R. Woodruff, 2019). Although these antimicrobials are known to affect microbial processes

such as nitrogen cycling, the direct effect on plants are not understood. The antimicrobials used to kill pathogens in poultry processing have led to cases of "nitrite locking" during wastewater treatment where nitrite is unable to be converted to nitrate by bacteria (S.R. Woodruff, 2019). Nitrite locking is detrimental as nitrite is toxic in the environment, whereas nitrate is more harmless and the preferred form for plant production (Hachiya et al., 2012). Several types of antimicrobial agents are used throughout the poultry wastewater process to meet specific food safety standards. A common agent used is PAA and is known to inhibit nitrate formation at concentrations as low as 9 mg/L (Pavlostathis, 2019). There is no known research on how algae interact with either PAA or other antimicrobials used within the industry, however, preliminary data conducted in our lab indicates that algae exhibit a similar response to that of several bacteria groups within wastewater.

The first experiment conducted by a previous researcher in our lab used primary municipal wastewater that was treated with activated sludge (AS) bacteria. Two treatments were used, one with the primary wastewater and the second with primary wastewater and cetylpyridinium chloride (CPC). CPC is a common antimicrobial used in the poultry processing industry. A concentration of 1.5 mg CPC/L was found to suppress ammonia oxidation and nitrite oxidation. The treatment without antimicrobials found a decrease in nitrite concentration as it was being transformed into nitrate. Throughout the batch culture, a period of 120 hours, nitrite concentrations slowly increased in the antimicrobial treatment. During this time, very little nitrite oxidation was recorded, suggesting that the antimicrobial agents suppressed the nitrifying capability of the AS bacteria.

2.1.3 Algal Impacts on Nitrifying Bacteria

Although algae have been widely studied for the removal of nutrients including nitrogen, there is considerably less study on how algae affect nitrogen transformation by bacteria. Given our interest in using the water for irrigation, our primary interest focuses on transformation rather than removal. Throughout literature, there are conflicting studies on how algal species impact nitrifying bacteria and whether the effect is positive or negative. Typically, examples of negative relationships involve algae and cyanobacteria competing with nitrifying bacteria, decreasing their populations and their capacity to transform ammonium (Nils, 2003). In this study, alga-colonized sediment had between 4 -51% of the coupled nitrification-denitrification activity as sediment without algae activity, depending on the nitrogen load. Additionally, this study indicated that the presence of active microalgae might reduce the population of nitrifying bacteria capable of having an active metabolism due to nitrogen limitation. Similarly, the same researchers found significant declines of ammonia-oxidizing bacteria when there is competition for ammonium between benthic microalgae and nitrifying bacteria (Risgaard-Petersen et al., 2004). In alga-colonized sediments, 20% less ammonia-oxidizing activity was recorded, again suggesting that the cells were starved for ammonium. The competition presented by the algal species affected the entire ammonium-oxidizing bacteria (AOB) community. In this study, data suggested a direct competitive interaction between the algae and AOB community and that benthic algae were superior competitors due to higher nitrogen uptake rates and faster growth rates. Although limited ammonium concentration is typically credited to the decrease in nitrification activity and increased competition, a study by Choi et al. (2010) suggested that algae suppress nitrification even at relatively high ammonium levels. In this system, the nitrifying reactor was fed with a solution containing 8.3 mM ammonium nitrate (~150 mg/L NH4). Even by providing ammonium to the system, the growth of cyanobacteria and algae inhibited the

maximum nitrification rate by a factor of 4 (Choi et al., 2010). The reason for this suppression was not investigated.

In contrast, many studies have found that when ammonium is not limited, there can be positive interactions between algae and nitrifiers. In E. Bankston et al. (2020) synergistic growth effects between *C. sorokininana* and certain activated sludge (AS) microorganisms were observed. Specifically, combining the AS microorganisms and the algal species led to greater nitrogen assimiliation by algae and increased nitrification rates by bacteria when compared to either organism group acting alone. The co-cultivation of *C. sorokiniana* and AS resulted in 2.7 times more nitrate production through nitrification when compared to only the AS community. Also, algae helped promote full oxidation of ammonium to nitrate by nitrifying bacteria, whereas incomplete nitrification was observed in the absense of algae. In a similar study performed by Su et al. (2012), an algal-bacterial culture, composed of wastewater-born algae and AS was cultivated to treat domestic wastewater and accumulate biomass. The study tested different inoculation ratios on phosphate and nitrogen removal. The researchers found that the interaction of *C. vulgaris* and bacteria can enhance the ammonium removal rate than systems composed of only algae or bacteria. In E. Bankston et al. (2020), the presence of algae helped promote a 57% increae in organics removal by heterotrophic bacteria. The researchers from this study suggest that the poultry litter digestate used acted as an inhibitor to the nitrite oxidzing bacteria within the system, depressing their numbers and function. By adding algae, the negative outcomes are mitigated and full nitrification was observed, along with a slower decline in the nitrifying bacteria population. The growth promoting effects that the AS microorganisms had on *C. sorokinina* consisted of greater cellular nitrogen uptake, along with faster phosphate removal when both the AS and algae were cultured together. The algae added into the system produced

excess dissolved oxygen, which helped benefit nitrification reactions and support the nitrifying populations.

The concept of adding microalgae to help provide oxygen to bacteria has been noted by other researchers, expecially in the marine and aquatic ecology field. Typically, in these marine environments, oxygen limits nitrification activity but the addition of benthic microalgae enhanced the rate of nitrification when ammonium was not limited (An & Joye, 2001). Similarly, periods of zero nitrification were found during times of darkness and periods of high nitrification were found during illumination (Lorenzen et al., 1998). This result indicates that the O_2 produced by algal photosynthesis can have a stimulating effect on nitrification. Microalgae have been used as an oxygen supplier for nitrification beyond marine environments. Six different microalgae species were used to see if they could supply sufficient oxygen for nitrification for municipal wastewater treatment (Kwon et al., 2019). It was found that four of the species, *C. vulgaris*, *S. quadricauda*, *D. communis*, and *C. emersonii* were able to produce sufficient oxygen for nitrification in all mixing strategies. In these results, complete nitrfication was observed due to the addition of algae and the oxygen they produce. Similarly, in Sepehri et al. (2020), the effects of innoculation ratios of a *C. vulgaris* and nitrifer-enriched-activated-sludge (NAS) consortium on nutrient removal was investigated. They found 100% ammonium removal within 7 days in a photo-bioreactor containing 10% *C. vulgaris* and 90% NAS (w/w). In this photo-bioreactor, the DO concentration reached 4 mg/L due to the photosynthetic activity of the algal species. Because of the photosynthetic nature of *C. vulgaris*, the NAS had proper conditions to perform nitrification. In E. Bankston et al. (2020), the organisms were grown in well-aerated bioreactors (0.5 vvm) with low biochemical oxygen demand so oxygen limitation was unlikely, but anoxic conditions could be present in the center of cell flocs when algae was not present.

Another positive of adding algae to bacterial systems provided by E. Bankston et al. (2020), is that algal biomass can degrade toxic molecules that negatively impact nitrifying bacteria. In this paper, digestates are mentioned to inhibit nitrifying bacteria, as they are rich with fatty acids and phenolic compounds (Franke-Whittle et al., 2014; Hecht & Griehl, 2009; Hernandez & Edyvean, 2008). Beyond this, algae has been used to relinquish the impact of antimicrobials found in some wastewater streams on the nitrifying bacteria community. Research done by Box and Higgins (2020) shows the effect of algae on nitrifying bacteria that are in the presence of the antimicrobial, PAA. In this study, the addition of algae mitigated the suppression experienced by the bacterial community in the presence of PAA. The ammonium concentrations dropped significanly in the algae cultures due to the improved algal uptake and bacterial oxidation of ammonium. qPCR analysis of this study indicated that the PAA suppressed the ammonia and nitrite oxidizers, however, the algae helped support nitrogen oxidizers in both the presence and absence of PAA.

2.2 Wastewater Reuse in Irrigation

Reuse of treated wastewater for irrigation has been practiced for many decades and is increasing in popularity. The reuse of wastewater is beneficial for a number of reasons: water shortages can be resolved or alleviated; large amounts of wastewater can be disposed of during the entire year; high-quality resources could be used for potable uses; economic benefits attributed to the nutrient content of the wastewater (Lubello et al., 2004). Specifically, the use of treated wastewater for irrigation decreases the pollution of surface waters and provides an opportunity for groundwater recharge (Maurer et al., 1995). Many studies have been conducted worldwide, at both laboratory and field scales, to better understand the impact of wastewater reuse on the soil, plants, and public health (Ofori et al., 2021).

One of the most common reasons for utilizing treated wastewater is to reduce fresh water consumption. Agriculture irrigation consumes approximately 70% of the world's water resources (Pedrero et al., 2010). Additionally, it is estimated that 80% of water extracted from surface water, groundwater, or storage reservoirs is discharged as wastewater and 70% of this amount can be recovered (Yi et al., 2011). By collecting and treating wastewater, water extraction can be reduced. In fact, a field study conducted in Saudi Arabia found that 60% of groundwater withdrawal was saved when treated wastewater was used for irrigation (Balkhair et al., 2013). Beyond reducing water consumption, the application of treated water helps protect and promote water quality. This is done by avoiding discharge of effluent thus reducing organic and inorganic substances from entering receiving water bodies (Ofori et al., 2021).

The reuse of treated wastewater would be ineffective if the application had a negative effect on plant growth and production. In many studies, improved plant yield and accelerated growth was found due to easy nutrient availability and uptake (Aziz & Farissi, 2014). In one study, a significant difference in weight was noticed between wastewater irrigated lettuce and potable water irrigated lettuce due to the nutrients supplied during the growing season (Urbano et al., 2017). Overall, the application of treated wastewater provides many macro- and micronutrients available for plant uptake. However, the nutrients provided by the treated wastewater could lead to plant toxicity when in excessive concentrations. A study conducted by McCauley et al. (2009) looked at specific nutrients essential to plant growth. Excessive nitrogen can cause delayed maturity and weak steams and excessive phosphorus will inhibit zinc uptake leading to a zinc deficiency. Finally, excessive potassium will reduce magnesium uptake, leading to a magnesium deficiency. However, excess nitrogen and phosphorous is not likely to be a

problem for poultry processing wastewater which has levels of 300 and 17 mg/L, respectively (Fatima et al., 2021).

Beyond plant health, it is important that the reuse of wastewater does not negatively impact soil health and the microorganism community. There are conflicting studies indicating whether wastewater irrigation increases or decreases microbial activities and biomass (Adrover et al., 2012; Kayikcioglu, 2012). However, the majority of research conducted suggests that the activities and functions of soil microorganisms increase both short-term and long-term when using treated wastewater (Ofori et al., 2021). The use of wastewater impacts more than the microbial community, including the structure and hydraulic properties of the soil. Typically, the use of treated wastewater negatively affects the soil structure by increasing salt composition (Ofori et al., 2021). A study conducted in Israel found that long-term application of wastewater reduced the hydraulic conductivity, sorptivity, and cumulative infiltration when applied long-term (Assouline & Narkis, 2011). Also, a study conducted by Sou/Dakouré et al. (2013), found a significant reduction in structural porosity when irrigated with wastewater compared to non-wastewater. These negative impacts from using wastewater as irrigation need to be mitigated as crop production could be adversely affected (Ofori et al., 2021). However, all of these studies were conducted using soil-based agriculture. Far less is known about using wastewater in soilless or hydroponic-type production with the exception of aquaponics.

2.3 Hydroponic and Aquaponic Systems

Many countries are or will be facing major challenges related to water scarcity, nutrient-depleted soils, and pollution. More than half of the world's population is expected to experience water scarcity challenges by 2030 (Alcamo et al., 2000). To help mitigate this issue, the development of technologies allowing for nutrient recovery from municipal wastewater and

integrating them with irrigation is becoming popular. By combining these two systems, a closed-loop system is developed so that nutrients, such as nitrogen and phosphorus, and water that are essential for plant growth can be recovered and re-used for crop production (Magwaza et al., 2020). However, due to agricultural land shortage, alternative approaches are required to utilize open spaces in urban environments. The solution has been the development of hydroponic systems. Although hydroponic systems are not being developed or researched in this thesis, it is important to understand how hydroponic systems function. By having this understanding and foundation of hydroponic systems, data collected through this experiment can be interpreted with the idea of using the algae-treated wastewater as a future irrigation source for hydroponic systems.

Hydroponic systems that utilize wastewater consist of typical wastewater treatment steps, both primary and secondary treatment, along with an additional recycling loop feeding treated wastewater to crops (Magwaza et al., 2020). Like standard municipal wastewater treatments, primary treatment uses a septic tank to remove settleable solids and provide partial anaerobic treatment. Next, secondary treatment utilizes sand-based filters, anaerobic digestion by activated sludge, and anaerobic baffler reactors to remove organic matter within the waste stream. The final treatment stage, referred to as tertiary treatment stage, utilizes plants as a biological treatment (Magwaza et al., 2020). Greenhouses are used to help plant growth, enhancing, and promoting the conditions needed for plant growth with irrigation and nutrients are provided by the wastewater stream.

There are several classifications of hydroponics system that are determined by: the fate of drainage of the nutrient solution; how the nutrient solution is delivered; the type of crop used for nutrient removal (Pardossi & Incrocci, 2011). The two types of drainage categories are open

(free drain) and closed (recirculating). In one study, recirculation of drainage water resulted in a 33% reduction in water consumption when growing cucumber plants (Grewal et al., 2011). In this study, the treated wastewater contained 59%, 25%, and 55% of nitrogen, phosphorous, and potassium, respectively, of the original influent stream. The next classification is based on the type of substrate or growth medium used in the hydroponic system. According to Haddad et al. (2012), there are two main types being solution culture or media filled systems. The next categorization, crop selection, serves an important role as the crops uptake nutrients and promote microbial activity (Magwaza et al., 2020). The majority of hydroponic systems use either ornamental crops or vegetable spices in their studies (Magwaza et al., 2020). Common vegetables consists of tomatoes, cucumber, and lettuce due to their short growth cycle allowing for better control and standardization (Magwaza et al., 2020).

For hydroponic systems to become adopted worldwide, the system must be effective at treating municipal wastewater, while supporting and growing profitable plants. According to Magwaza et al. (2020), the use of hydroponic systems has many benefits compared to field crop production such as 30-50% faster plant growth, reduction of land requirements, and decrease of soil contamination.

Related to hydroponics, aquaponics is the process of growing aquatic organisms and plants symbiotically in which the effluent of the aquaculture undergoes microbial transformations to be used as a nutrient source for plant growth, while nutrient absorption from plants remediates water for aquaculture (Yep & Zheng, 2019). Most of modern day aquaponic systems are recirculating aquaculture systems (RAS) where the waste produced by aquatic organisms is filtered through tanks full of microbes. These microbes help break down organic compounds, allowing them to be available for plant uptake (Yep & Zheng, 2019). The goal of

aquaponic systems is to utilize nutrients provided by the aquatic organism before expelling them into the environment. Because of this, aquaponic systems provide an insight into utilizing wastewater as an irrigation source. A study done by Alarcón-Silvas et al. (2021) reported a removal efficiency 56-74% for nitrogen and 42-47% for phosphorus. The removal efficiencies reported in this study are comparable to conventional wastewater treatment methods highlighting the advantages of the aquaponics system (Alarcón-Silvas et al., 2021). Although aquaponic systems are relatively new, the success of the systems show they are capable of utilizing wastewater streams as an irrigation source.

Understanding the necessary parameters required of the wastewater stream to promote hydroponic plant growth is essential to developing a viable treatment system. The ideal pH for lettuce production is between 5.6-6 (Melissa Brechner, 2013). A common issue that has risen with the use of wastewater is excessive amounts of reduced nitrogen, such as ammonium, that lead to unstable plant production due to ammonium toxicity (Hachiya et al., 2012; Ikeda & Tan, 1998). Another important parameter is dissolved oxygen (DO). A DO level of at least 4 ppm supports lettuce growth (M. Brechner, 2013). This means that most biodegradable organics should be removed from wastewater via treatment prior to application in hydroponic systems. Beyond the nutrients required, the wastewater must be suitable for plant irrigation by minimizing the presence of human foodborne pathogens within. However, the issue of pathogens will not be investigated in this work.

Methods

The objective of this study was to determine if and under what conditions algae help promote nitrification during wastewater treatment in a continuously operated reactor. Additionally, the resilience of the nitrifying community was to be tested through several stressor events such as the addition of PAA. It was hypothesized that the addition of algae to an activated sludge system would lead to improved nitrification. Also, it was hypothesized that the algal-bacterial consortium would exhibit greater resilience to the antimicrobial chemicals that were introduced to the system.

3.1 Preparation of Algae Stock

In this experiment, a strain of *C. sorokiniana* (UTEX 2805) was used. This strain was isolated from a wastewater treatment facility in Mexico (de-Bashan et al., 2008) and has been shown to be very effective in diverse wastewater treatment applications (E. M. Bankston et al., 2020; Higgins et al., 2018; Wang, Prasad, et al., 2019). A stock culture of *C. sorokiniana* was cultivated in N8-NH_4 media (Higgins & VanderGheynst, 2014) for one week. Inoculation of the algae stock occurred in a biosafety cabinet to minimize contamination of the stock. To inoculate the stock, individual colonies of plated algae were collected using loop and swirled in 2 sterile tubes containing 1 mL of sterile water. The contents of the tubes were poured into 2 1L bottles of sterile N8-NH_4 medium. The stock was placed on stir plates for one week under light and 400 mL/min aeration with 2% CO_2 in air. pH was monitored and adjusted daily to maintain a pH of 7.5. The two bottles were rotated daily to minimize impacts from differences in light, air, and stir plates. Optical density (OD) was read daily at 550 nm and 680 nm to monitor the growth of the stock culture (Wang, Peng, et al., 2019). When an OD550 reading of approximately 0.2 – 0.3 was achieved, the stock was placed on the bench and allowed to settle for 24 hours.

3.2 Collection of Activated Sludge and Poultry Scald Wastewater

Activated sludge was collected from a mesophilic digester at the South Columbus Water Resource Facility in Columbus, Georgia. This sludge was used to provide an inoculum of nitrifying bacteria into the treatment reactors. Batches of poultry scald tank wastewater were shipped to Auburn University from a processing plant in northern Georgia multiple times throughout this study. Upon delivery, the wastewater was stored in a cold room (4 C) until the specific bottle was opened and used as an input. Once opened, the bottle was stored in a refrigerator at 4 C.

3.3 Inoculation of Bioreactors

To test the nitrification potential and resiliency of the algal consortium, two 1L reactors were used. The first reactor, known as the bacterial reactor, contained only the activated sludge, whereas the second reactor contained the activated sludge and the algal consortium. Table 1 shows the experimental design for the two reactors.

To prepare the activated sludge, 50 mL of the sludge slurry settled on the bottom of the container was collected using a serological pipette. 40 mL was allocated to the bioreactors (20 mL in each reactor) and 10 mL was weighed for solids content analysis. To prepare the poultry scald wastewater for use, 800 mL of poultry scald water was centrifuged (5000 rpm, 5 min) to remove most suspended solids. Supernatant (650 mL) was transferred to a 2-L beaker and diluted with 1.35 L of DI water to achieve a ~3x dilution. Once diluted, the scald water was divided equally into two 1-L wide-mouth bottles. In the BSC, the supernatant from the cultured algae stock bottles was removed and centrifuged to concentrate the cells. Algae concentrate was added to the algae-bacteria treatment reactor. As the experiment progressed, additional activated sludge was added into the reactors to increase cell density. This was done because growth performance

in the bacteria-only reactor was generally poor and sustaining high biomass density was a challenge in this reactor. A timeline of additional inputs and the specific input dates can be observed in Figure 1.

Table 1. Experimental Design for Bioreactors

	Reactor 1 (Bacteria Reactor)	Reactor 2 (Algal Reactor)
3x diluted Poultry Wastewater (1 L)	+	+
Activated sludge (20 mL)	+	+
DI Water (36 mL)	+	-
UTEX 2805 (36 mL)	-	+

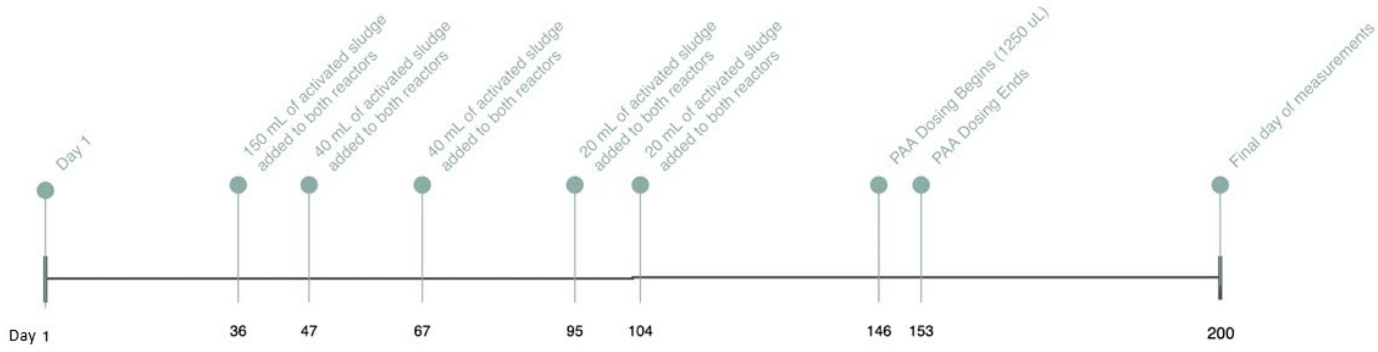


Figure 1. Timeline of Experimental Inputs

3.4 System Design

The two reactors were located on the benchtop and placed on stir plates. Both reactors received aeration at 400 mL/min of an air and 2% CO_2 mixture to support algal growth and autotrophic nitrification. Both the algal and bacterial reactor were open to the atmosphere. The two bioreactors were fitted with dissolved oxygen and temperature probes. Additionally, the algal reactor was fitted with a dissolved CO_2 and pH probe. The probes were used with a

Mettler-Toledo InPro system which allowed for continuous process monitoring. The system design can be observed in Figure 2.

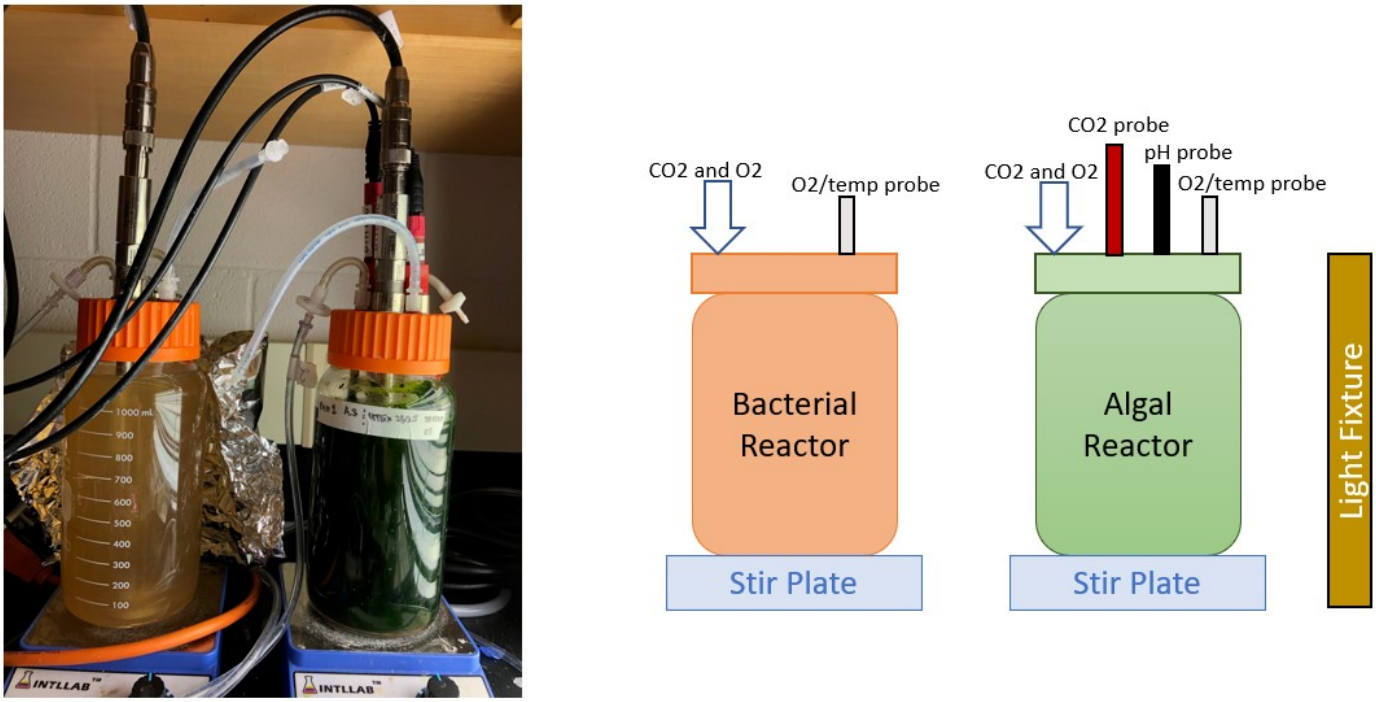


Figure 2. System Design for Bacterial and Algal Reactors

3.5 Operation and harvesting of Bioreactors

Every 2-3 days (Mon, Wed, Fri), 75% of the liquid (750 mL) was removed from each reactor and replaced with fresh, 3x diluted wastewater. The effect was a semicontinuous reactor with an HRT of 3.1 days. This was set based on the results of batch cultures which showed a 72 hour timeframe to result in peak nitrate formation (Box & Higgins, 2020). The collected liquid was centrifuged at 5000 rpm for 5 minutes and the supernatant was aspirated using a vacuum pump and serological pipette. Solids collected from the bacteria reactor were reintroduced back into the system with the new wastewater input resulting in a near infinite solids retention time (SRT). This was done in an effort to sustain the maximum possible cell density in this reactor which suffered from die-off in this wastewater.

In contrast, the algal reactor exhibited robust biomass growth and went through three different phases of solids removal in order to sustain three different target total suspended solids (TSS) levels. TSS was correlated to optical density at 550 nm. Initially, a harvest occurred every 5-6 days to allow adequate time for growth within the algal reactor resulting in an SRT of about 7 days. The goal of this phase was to create a stabilized community within the algal reactor. Additionally, this first phase helped provide data on nitrification and nutrient removal rates for decision-making with regard to how the algal reactor would be operated as the experiment continued. The algal reactor was operated at this SRT for 67 days and operation stabilized starting around day 50. This operating regime resulted in nearly complete nutrient removal. Since the long-term goal was to transform nutrients rather than remove nutrients, alternative operating regimes were considered. We hypothesized that this relatively short SRT was resulting in an algal-dominated system resulting in high nutrient removal that deprived nitrifying bacteria of nutrients. Moreover, the frequent biomass removal was likely depleting the reactor of its nitrifying populations. Therefore, subsequent stages of operation used progressively longer SRTs. Instead of setting a constant SRT, biomass harvests were instead timed to when target TSS levels were reached. During the second treatment phase (Day 67-104), biomass harvests occurred when the OD550 was recorded above 1.0. From day 104-200, the reactors were switched to the third treatment phase where biomass harvests only occurred when the OD 550 read above 1.5. However, HRT was maintained at 3.1 days throughout the entire study period. Higher TSS levels were expected to sustain larger nitrifying populations while limiting photosynthetic activity through greater light blockage. Figure 3 shows a timeline of the three SRT phases.

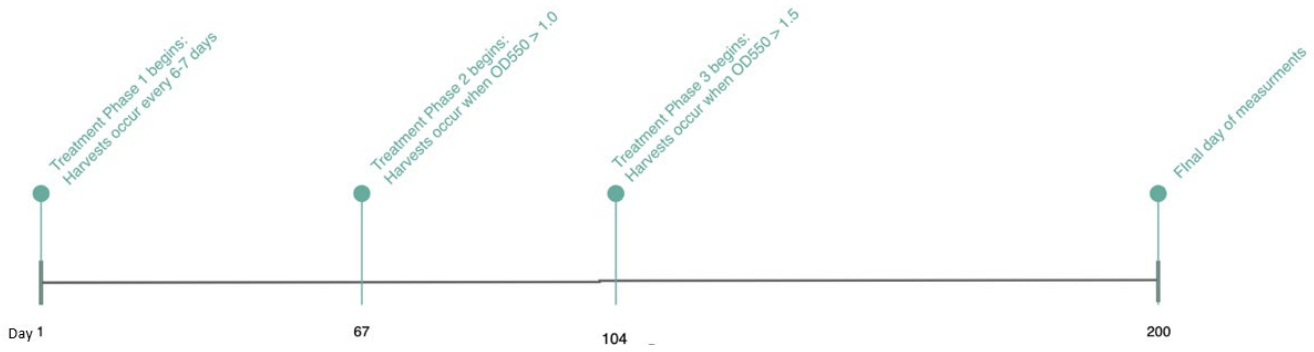


Figure 3. Timeline of SRT Phases

Samples were collected before and after the change of wastewater to measure nutrient ions. Prior to wastewater exchange, two 2 mL samples were taken from each reactor using a 5 mL serological pipette. From these samples, 200 µL of each was used to measure the OD 550 and OD 680. A wavelength of 550 nm was measured because it excludes the absorbance of chlorophyll, suppressing bias from changes in chlorophyll content (Wang, Peng, et al., 2019). A wavelength of 680 nm is associated with chlorophyll absorption so measuring this wavelength gives insight of the growth and health of the algal population The remainder of the samples were centrifuged (12000 rpm, 2 min) to pellet and the supernatant was syringe filtered (0.2 um) for IC and COD analysis. Immediately after the fresh, diluted wastewater was introduced into the system, an additional two, 2 mL samples were taken for each reactor following the same procedure described. The samples, taken prior to harvest, are referred to as the "treated" samples, while the samples taken after the addition of fresh wastewater are referred to as the "untreated" samples. The pH of the two reactors was monitored and adjusted after wastewater exchange by adding 3M NaOH or 3M HCl as needed.

3.7 Peracetic Acid Phase

The two reactors were challenged with doses of peracetic acid (PAA) starting on day 146 and dosing occurred three additional times on days 148, 151, and 153. During this phase, all

sampling and wastewater exchanging procedures continued as normal but with the addition of PAA. The dosing resulted in a PAA concentration of 7 mg/L in the two reactors. This concentration was determined using work by Box and Higgins (2020) as the EC50 for *C. sorokiniana*. After the PAA dosing, the harvest procedures continued as normal, during the third SRT phase.

3.8 Water Quality Analyses

Soluble inorganic macronutrients were analyzed by suppressed ion chromatography on a Prominence Liquid Chromatography (LC) system (Shimadzu). A Dionex IonPac CS16 column (4x 250 mm, Thermo Scientific) and Dionex CERS 500e 4 mm regenerative suppressor were used to measure cations (Na^+, K^+, NH_4^+, Ca^{2+}, and Mg^{2+},). A Dionex IonPac AS22 column (4x 250 mm, Thermo Scientific) plus regenerative suppressor (Dionex AERS 500 carbonate 4 mm) was used for the analysis of anions (Cl^-, NO_2^-, NO_3^-, PO_4^{3-}, and SO_4^{2-}). Soluble chemical oxygen demand (COD) was measured using the HACH high range assay kit.

3.9 Data Analyses

Basic statistics such as mean and standard deviation was conducted in Microsoft Excel. R Studio was used to conduct backward stepwise multiple regression analysis for the collected data. The predictor variables that were investigated were pH, dissolved oxygen, temperature, nutrient (phosphate, nitrate, nitrite, and ammonium) concentration in the specific reactor, OD550, OD680, wastewater input nutrient (phosphate, nitrate, nitrite, and ammonium) concentrations and PAA dosing. The response variables investigated were nutrient (phosphate, nitrate, nitrite, and ammonium) concentration in the specific reactor. Both the algal and bacteria reactor had backward stepwise multiple regression analysis conducted.

3.10 Production and Removal Rate Equations

To determine the removal and production rate of various nutrients during the different treatment phases, equation 1 and 2 were used.

Equation 1 – Removal Rate

$$Removal\ Rate = \frac{c_1 - c_2}{t_2 - t_1}$$

Where c_1: the concentration of nutrient in the reactor after wastewater input (mg/L)

c_2: the concentration of nutrient in the reactor after treatment (mg/L)

t_1: the day of wastewater input

t_2: the day of sampling after wastewater treatment

Equation 2 – Production Rate

$$Removal\ Rate = \frac{-(c_1 - c_2)}{t_2 - t_1}$$

Where c_1: the concentration of nutrient in the reactor after wastewater input (mg/L)

c_2: the concentration of nutrient in the reactor after treatment (mg/L)

t_1: the day of wastewater input

t_2: the day of sampling after wastewater treatment

Results and Discussion

4.1 Biomass Density

The optical density (OD) was recorded when samples were taken from the two reactors. OD reading taken prior to the wastewater harvest are labeled as “treated” and readings taken after the wastewater change are labeled “untreated”. Figure 4 shows the optical density

throughout the experiment beginning at day 50. During the first phase of the experiment, harvests were occurring every 6-7 days and not based on OD readings. The first phase had an average OD550 of 0.62 ± 0.3 in the algal reactor. On day 67, it was decided to return algal biomass back into the algae reactor if the OD550 <1. After this point, it can be observed that the OD of the system steadily rises until a harvest occurs. In the algae reactor, the average OD550 during the second phase was 0.88 ±0.27. On day 104, it was decided to return the algal biomass back into the algal reactor if the OD550<1.5. During the third phase of the treatment, the OD550 had an average of 1.19 ± 0.26.

During two of the treatment phases, the OD was used to determine when algal biomass should be removed from the reactor. Unfortunately, due to biofilm development within the reactor, the OD was not a good measurement of solids. Because of biofilm development, solids remained unaccounted as they were stuck to the sides of the reactor instead of in suspension. With this, the OD gives an idea of the total suspended solids at the time of sampling as opposed to total solids in the system. Because of the biofilm development, there was more solids within the algal reactor than measured during each phase. Developing a better way of removing biofilm from the reactor walls would help improve OD readings and help generate a better idea of the solids within the reactors. The reactors used were 1-L bottles, which have rounded sides making any attempt to remove biofilm difficult. To improve upon this, the reactor should have flat walls allowing for better biofilm removal.

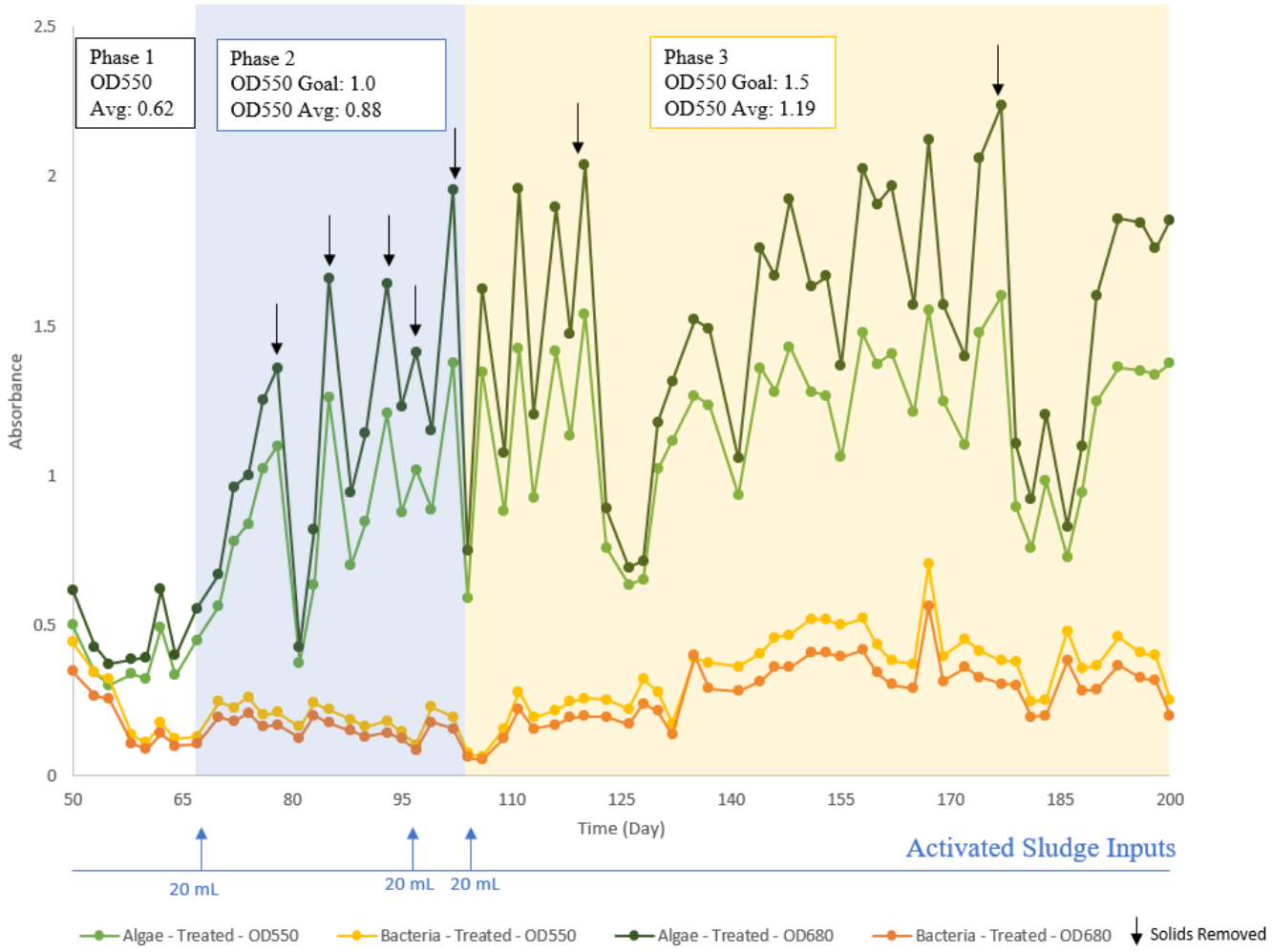


Figure 4. Optical Density Throughout Treatment Phases

Throughout the experiment, both wavelengths of 550 nm and 680 nm were used to monitor the growth of the bacteria and algal communities in the reactors. Changes in the ratio of OD550/OD680 provides insight into changes in relative abundance of algal versus bacterial populations. An increase in this ratio would suggest an increase in the bacteria to algae ratio.

Figure 5 shows the OD ratio for both the algal and bacterial reactor. The algal reactor OD ratio had an average of 0.80 ± 0.09. The bacteria reactor had an average OD ratio of 1.24 ± 0.07. The ratio of optical density was expected to be lower for the algal reactor as the OD680 value should be much larger than that in the bacteria reactor. Additionally, the optical density ratio was

used as insight to ensure algal contamination did not occur in the bacteria reactor. If contamination did occur, a significant drop would be observed in the OD ratio. A decrease, as previously described, was observed on day 135, where the OD ratio had a value of approximately 1.0. The decrease in the OD ratio raised concerns about contamination but was dismissed because a normal OD ratio was observed the next sampling period.

It was expected that the second SRT phase of the algal reactor would have a higher OD ratio value than the third SRT phase as less algal biomass was kept in the system. However, both phases of solids retention have similar OD ratios throughout the treatment phases. During these phases, a more bacteria-dominated system was expected in the algal-bacteria reactor, leading to an increase in the OD ratio, however, the ratio of the two phases remains similar. The average OD ratio for the second SRT and third SRT was 0.78 ± 0.05 and 0.79 ± 0.06, respectively. Although there was more biomass in the system during these two phases, the ratio of algae to bacteria remained relatively unaffected. Another explanation as to why the two SRT phases may have had similar OD ratios could be due to the build-up of algae on the sides of the reactor. As the SRT shifted phases, much more algae grew and "stuck" to the sides of the reactor as opposed to being in suspension. Because of this, the algal growth that occurred on the wall was not recorded on the OD readings. If the algal-bacterial biofilm was able to be captured by the OD readings, the OD ratio would have decreased due to the increase of algae concentration and associated chlorophyll concentration. Although this effect was mitigated by scraping the sides of the reactor prior to each sampling, complete removal was unsuccessful and could be leading to these observations.

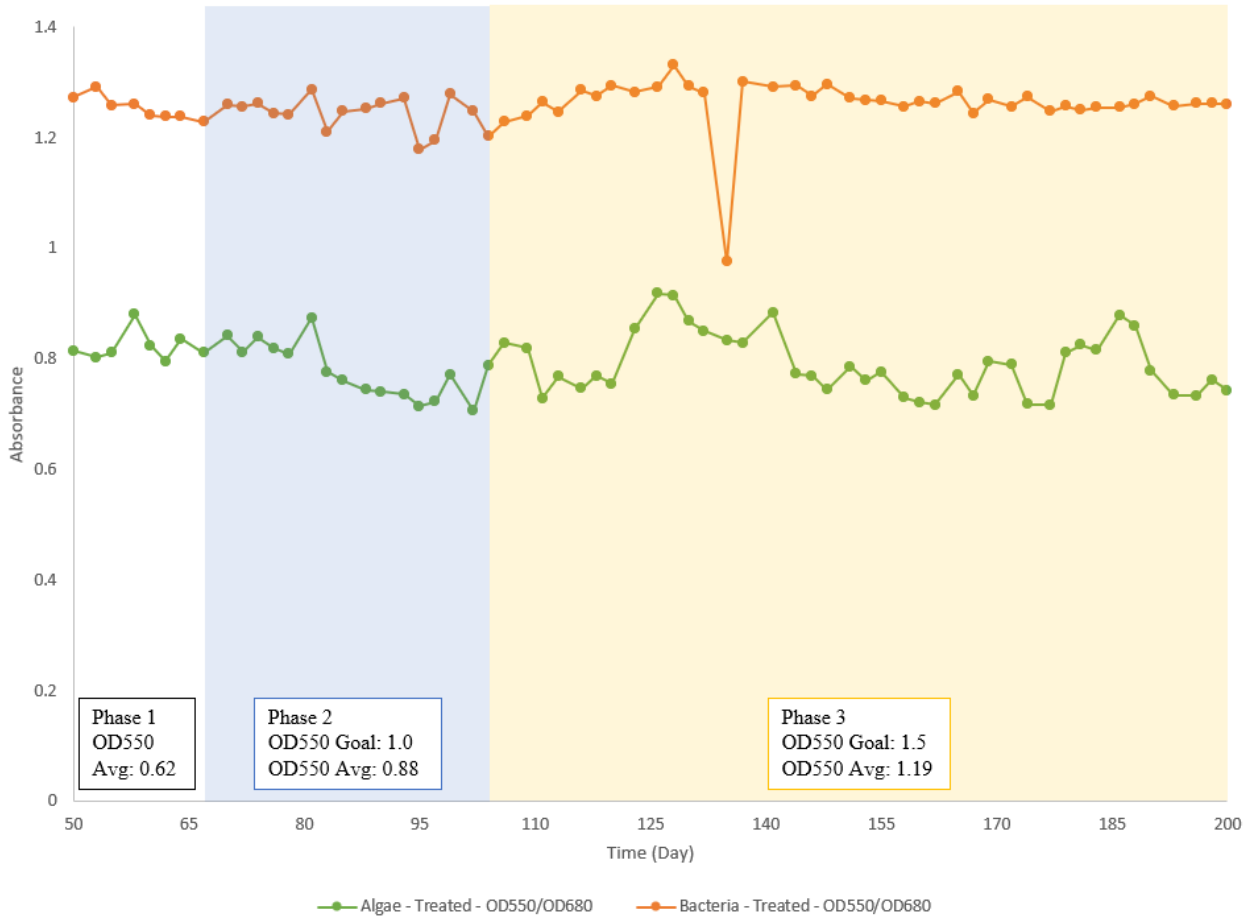


Figure 5. Optical Density Ratio during Treatment Phase

4.2 pH

The pH of the algal reactor was continuously monitored throughout the treatment phase. Figure 6 shows the pH measurements throughout the experiment. It can be observed that the pH cycles from approximately 5.0 to 8.0. The pH increased to 7.5 or greater when new wastewater was introduced to the system and decreased over time as ammonium oxidation occurred in the reactor. As the reactor continued operation, the buffer capacity decreased allowing for greater variability within the reactor. The composition of the wastewater batches differed throughout the experiment accounting for the change in buffer capacity. *C. sorokiniana* has been studied and grown in many different pH conditions. The maximum biomass productivity was observed at a

pH of 6.0 and the least productive was observed at a pH of 9.0 (Qiu et al., 2017). The pH of the algal reactor remained between these values throughout the experiment. The pH in the bacteria reactor was not regularly recorded but was checked manually with pH strips after wastewater input.

The pH of the reactors is an important parameter as many processes occurring within are affected by it. Additionally, depending on the use of the "treated" water, pH needs to be regulated and balanced between any plant or microbe species (Zou et al., 2016). The pH affects the nitrification activity within the reactors directly. It was found that a pH of 8.5 had a significantly faster ammonia conversion to nitrate than at pH 7.5 or 6.5 (Tyson et al., 2004). The pH within the algal reactor fluctuated mostly between a pH of 6 and 8, meaning nitrification activity was not being maximized. For the water to be used as irrigation water within a hydroponic system, the water should have a pH of 5.5 to 6.5 (Tyson et al., 2004). Extreme pH conditions should be avoided, within the reactor, as plants and bacteria are affected negatively by high alkaline conditions (Tyson et al., 2004).

In addition to nitrification, pH affects the availability of phosphorus. As the pH increases, the phosphorus concentration decreases due to phosphorus binding to several cations (Cerozi & Fitzsimmons, 2016). As the pH increases, phosphorus binding increases, meaning less free phosphate ions are available for uptake for either algae or plant species. Because of this, hydroponic systems should have a pH range of 5.5-7.2 for optimal phosphorus availability and uptake by plants (Cerozi & Fitzsimmons, 2016). Understanding what "treated" water will be used for will affect how the reactors are monitored and how the pH should be maintained. Acids and bases can be used to adjust and manipulate the pH to the specified ranges depending on

intended use of the generated water. However, no pH adjustments were made on the generated water over the course of this experiment.

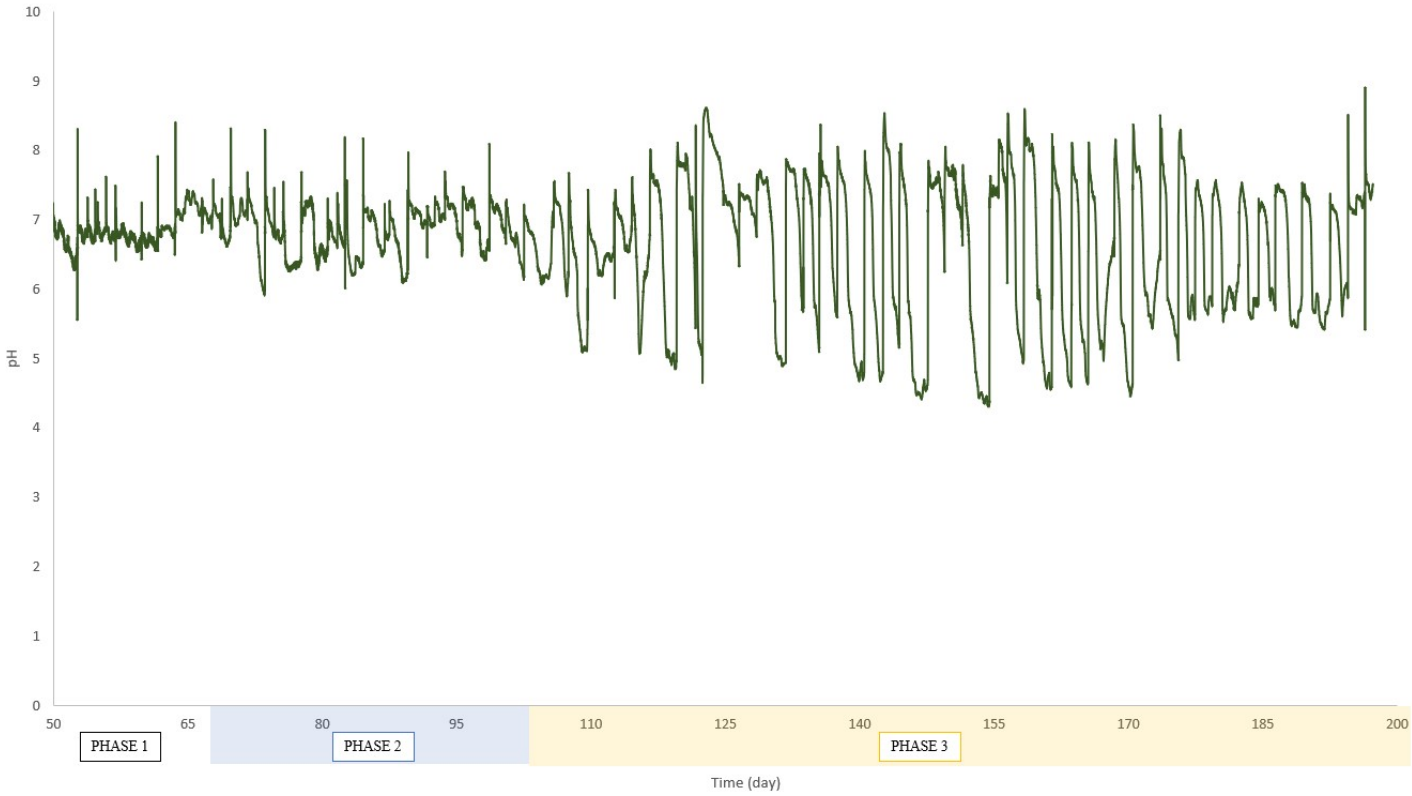


Figure 6. pH Throughout Treatment Phase in Algal Reactor

The O_2 concentration of the bacteria and algae reactor is shown in Figure 7. The O_2 concentration in the algae reactor cycled throughout the day, peaking in the afternoon. A 48-hour period can be observed below in Figure 8, where a light/dark cycle can be observed. The cycle seen can be contributed to the algae performing photosynthesis within the reactor. The sawtooth nature of the figure can be explained by particulate in the reactor blocking and sticking to the probe momentarily, leading to small shifts in measurements.

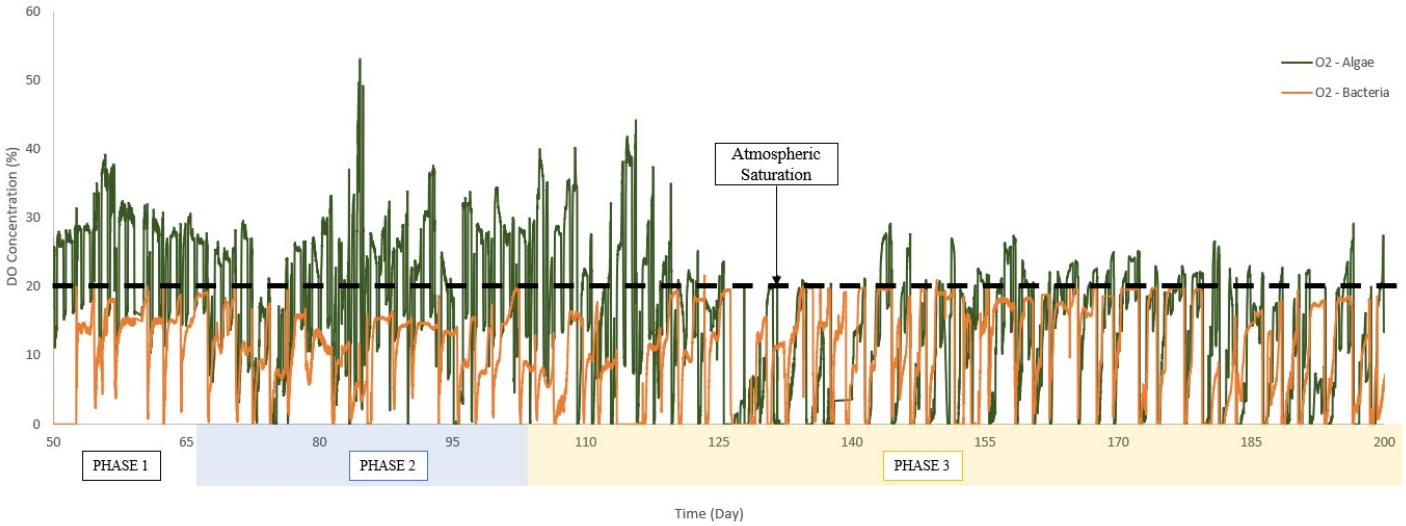


Figure 7. Dissolved Oxygen Concentration Throughout Treatment Phase

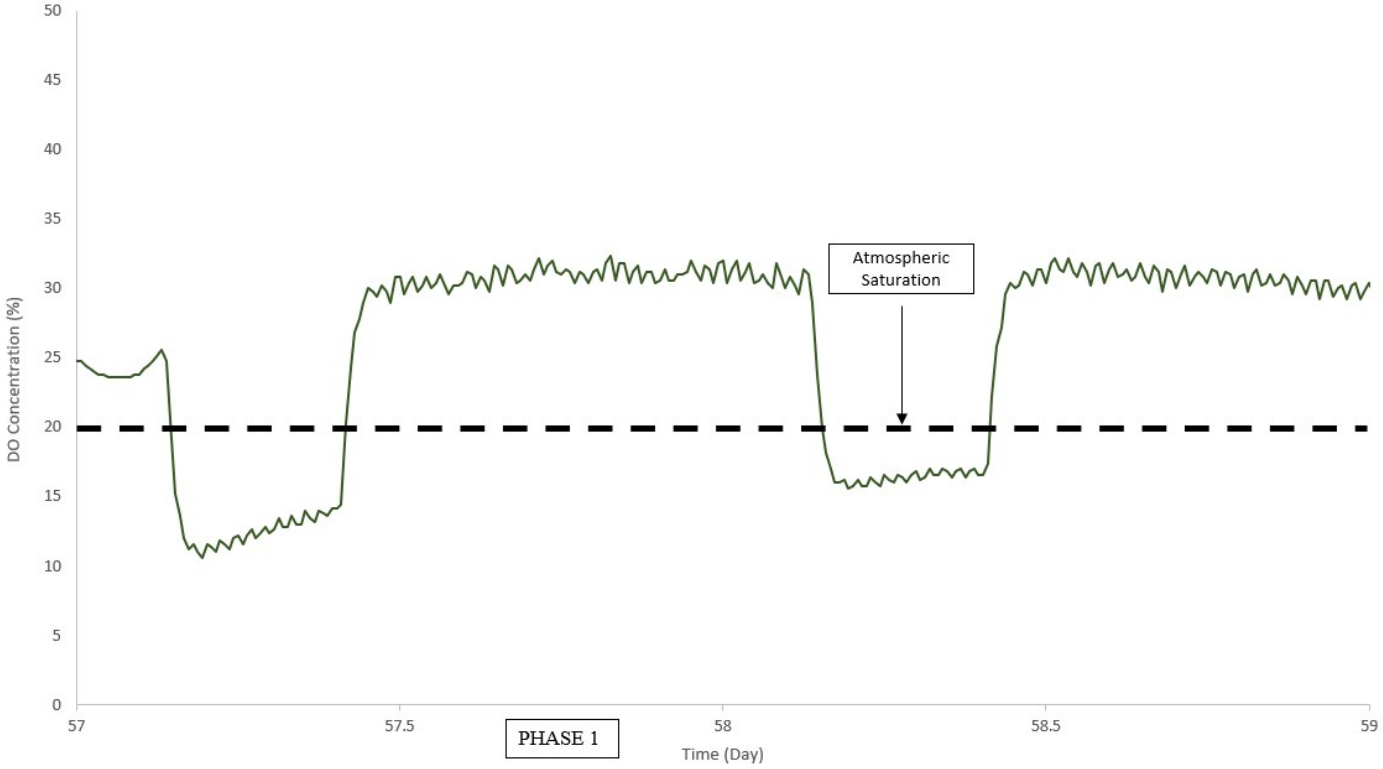
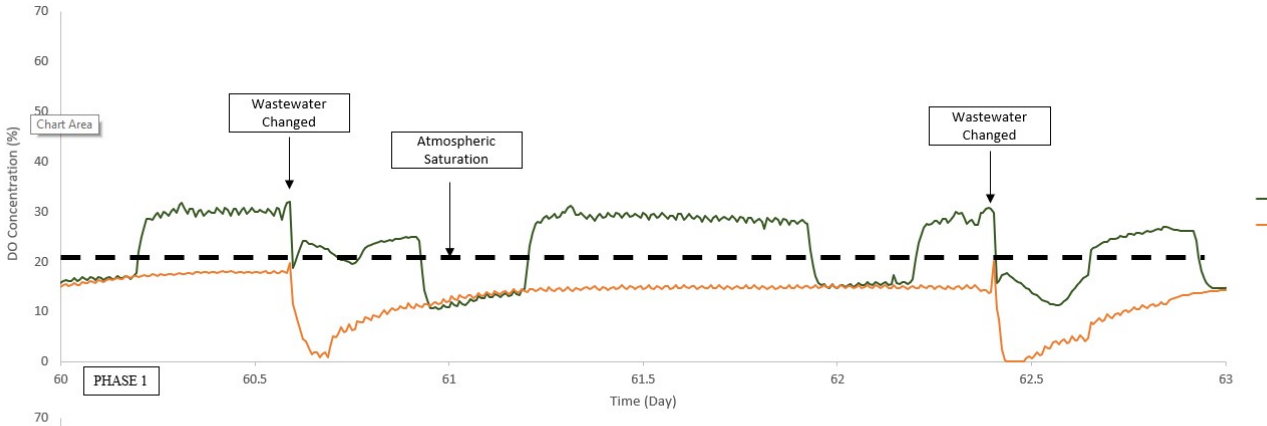


Figure 8. Dissolved Oxygen over 48-Hour Period in Algal Reactor

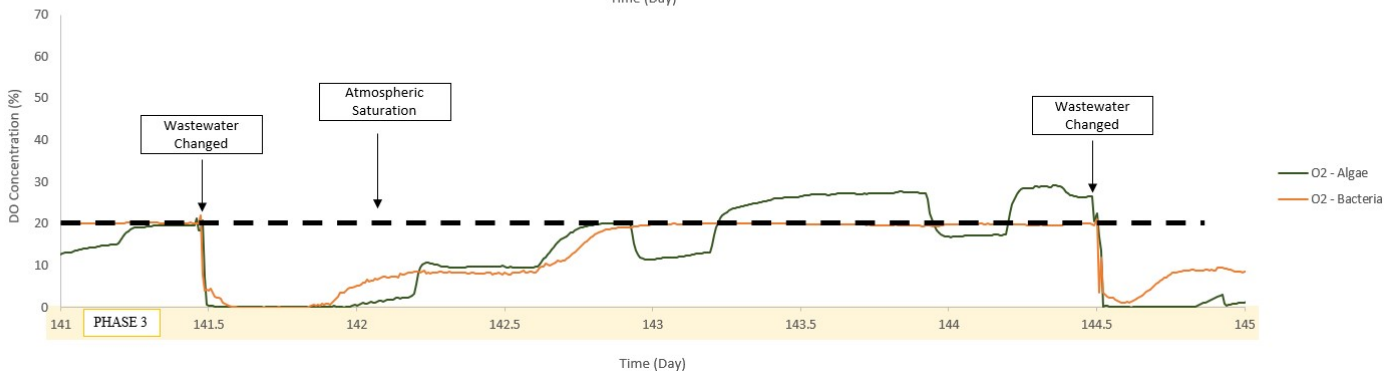
decrease and stabilize around atmospheric saturation for the rest of the day. This can be observed in Figure 9a. As the reactor continued operation, the O_2 concentration would decrease to 0% when new wastewater was introduced (Figure 9b). In these later treatment phases (higher solids retention), the algal reactor became bacteria-dominated, the effects of which can be observed in Figure 9b. Less photosynthesis was occurring in the reactor, resulting in lower DO concentrations. A day/night cycle can still be observed during this phase, suggesting photosynthesis was still occurring, however, the algae species was not as productive during the later phases. In the bacteria reactor, the O_2 concentration would drop to 5% or less when new wastewater was added.

As mentioned above, there was a shift in O_2 conditions within the algal system, which occurred around day 126 (6/17/2021). Prior to this day, the algal reactor was dominated by algal growth leading to high levels of photosynthesis. The highest concentrations of O_2 occurred during this period, with levels of 35% or greater being recorded. When the SRT phase shifted, from a second SRT to third SRT, the algal biomass was being left in the reactor for longer periods. The oxygen concentration within the reactor started experiencing effects from this shift around day 126, when the system shifted to a bacteria-dominated system within the algal-bacterial reactor. With high algal biomass remaining in the system, limited light penetration likely led to a decrease in algal growth. Consequentially, low algal growth led to low nutrient uptake and more oxidation of nitrogen. Due to this, the oxygen concentration cycled around 20% and peaked around 30%.

a)

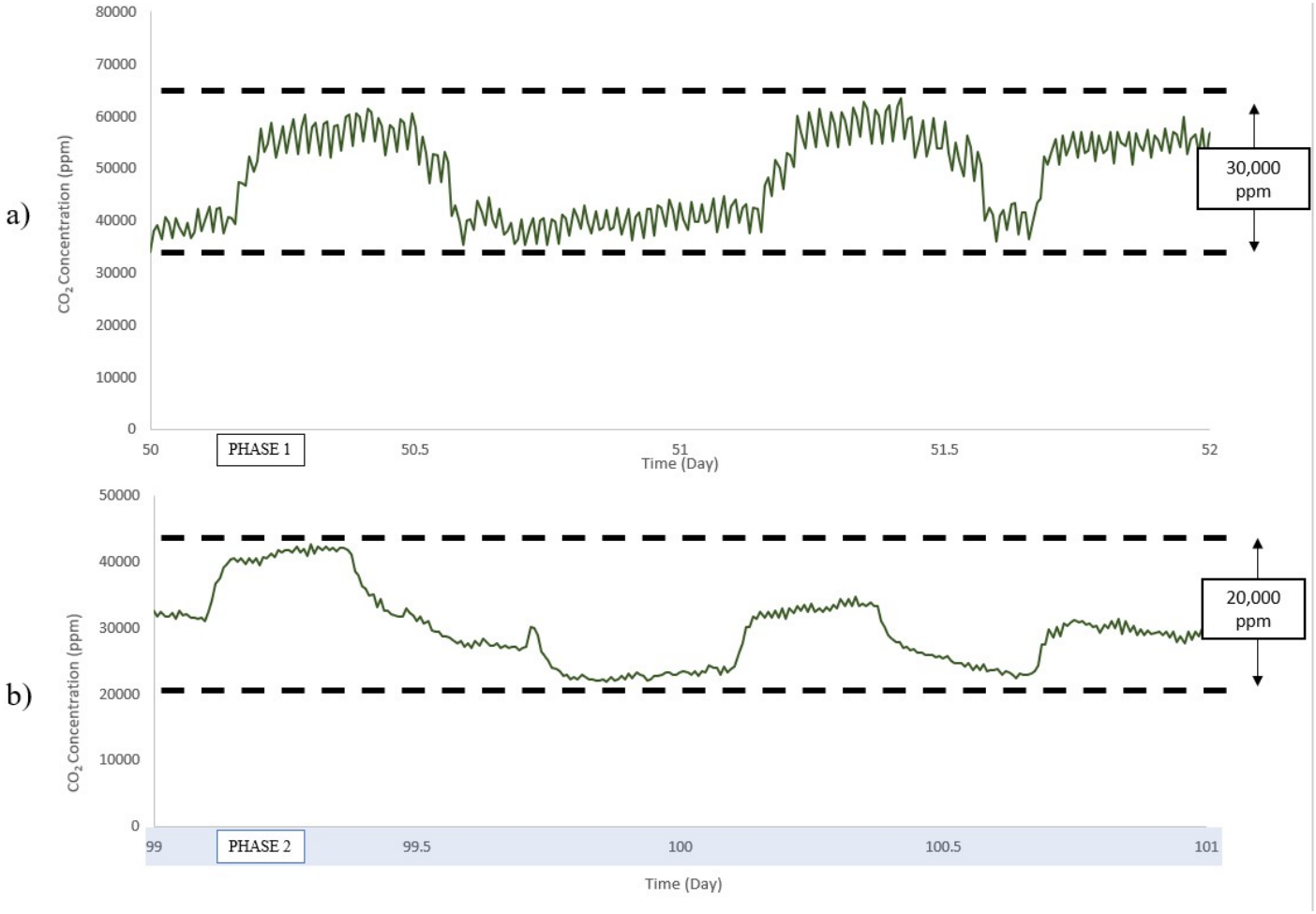


b)



4.4 CO_2 Concentration

The CO_2 cycled throughout the day, peaking in the early morning around 3 AM. Like the oxygen concentration, this is directly related to the photosynthesis being performed by that algal biomass. Figure 7 shows a comparison between two 2-day periods in the first SRT phase (a) and second SRT phase (b). In both graphics, a distinct day/night cycle can be observed with CO_2 decreasing during the daytime as photosynthesis occurs. During the first SRT phase, the CO2 concentrations were higher and showed greater variability, whereas during second SRT phase, the CO2 concentrations were lower and with less variability. There is less oscillation in CO2 concentration during the second SRT phase due to less photosynthesis being performed by the algal species.



4.5 Temperature

Figure 11 shows the temperature in both reactors throughout the treatment phase. The temperatures of both reactors cycle throughout the day, peaking in the afternoon. The temperature cycle closely follows the lighting pattern (16:8) due to the heat provided by the lighting fixture. The average temperature in the algal and bacteria reactors were 25.8 ± 2.4 C and 23.7 ± 1.6 C, respectively. The temperature difference between the two reactors was due to the algae reactor being closer to the lighting fixture. Additionally, the bacteria reactor was covered with aluminum foil to prevent light from entering the system, essentially reducing the amount of

radiation from the light fixture from entering the reactor. Because of this, the bacteria reactor had a lower temperature and less temperature variability.

During the treatment phase, three temperature spikes can be observed, occurring on 4/27/2021, 5/3/2021, and 6/27/2021 (Day 75, 81, 136). These events occurred due to air conditioning failure in the laboratory. These dates were treated as stressor events, similar to the PAA dosing, which could happen in real-life treatment systems. During the A/C failure, temperatures reached approximately 33 C and 31 C in the algal and bacteria reactor, respectively. *C. sorokininana* has been known to grow in temperature conditions of greater than 40 C and is known to be capable of growing in high temperatures at very intense levels of insolation (de-Bashan et al., 2008). The conditions within the algal reactor, even during the temperature spikes, were within *C. sorokiniana's* ideal temperature range.

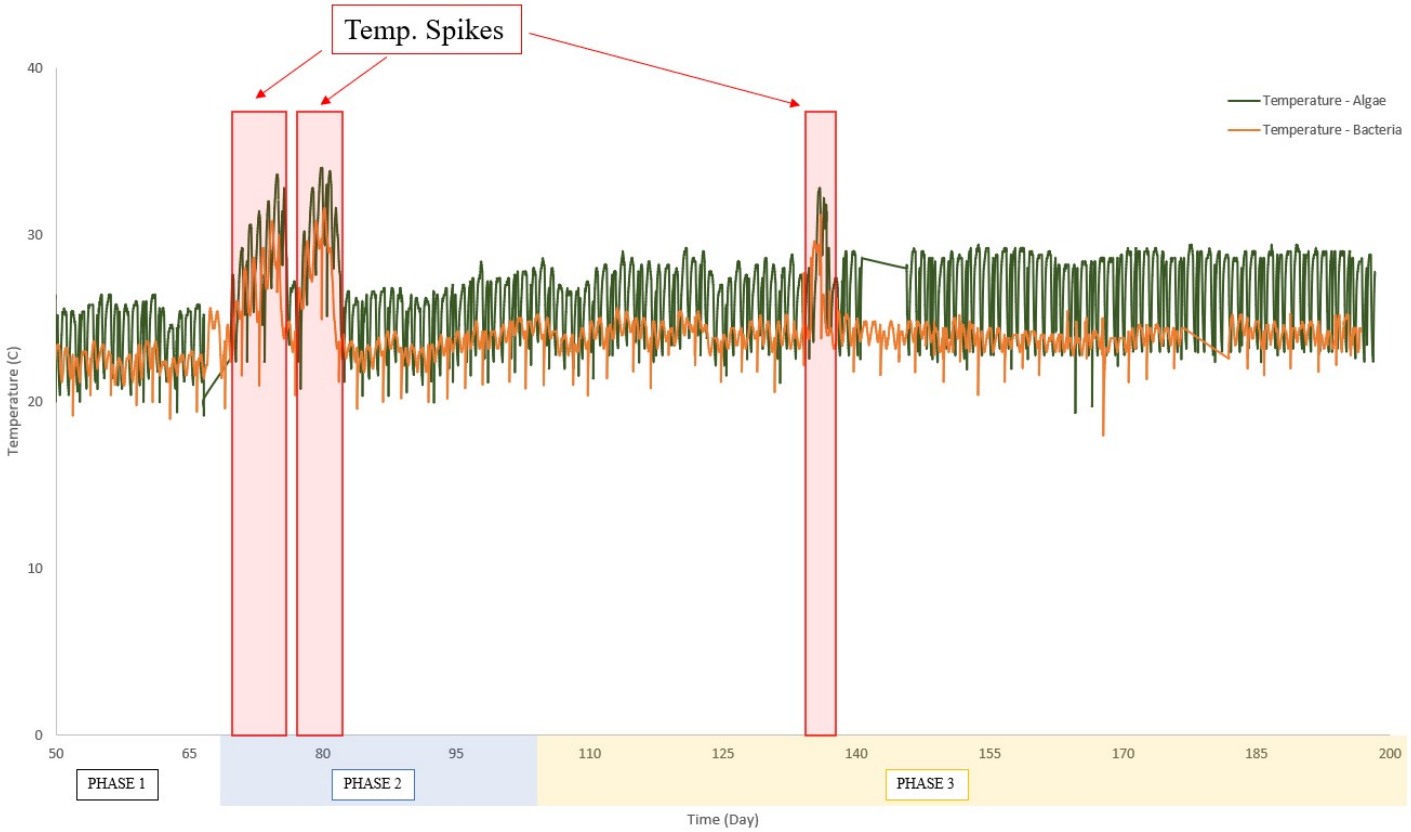


Figure 11. Temperature in Reactors throughout Treatment Phase

4.3 Forms of Nitrogen

Figure 12 shows the ammonium concentration throughout the treatment phase. Looking at each heat shock period, the algal-bacterial reactor did not seem affected by temperature increase as ammonium was still being oxidized and ammonium concentrations decreased, however, the ammonium oxidation rate may have been affected by the PAA dosage. Figure 13 shows the multiple regression analysis results, which shows temperature was not statistically significant for the ammonium concentration within the algal reactor. The ammonium concentration within the algal-bacterial reactor decreased during the PAA dosage but spiked on the last day. It cannot be concluded that the ammonium spike is solely due to PAA, but the ammonium concentration spike should be noted. The dosing of PAA was not significant to ammonium concentration in the algal reactor (Figure 13). During all stressor events, the bacteria reactor's ammonium concentration increased. During the PAA dosage, the ammonium concentration within the bacteria reactor increased from 20 mg NH_4^+- N/L to 60.8 mg NH_4^+- N/L. This suggests that bacteria by themselves were less resilient than the algae-bacteria consortium to handle these stressor events. Although the increases in ammonium concentration was observed during both stressor events, only temperature, not the PAA dosing, was statistically significant (p-value <0.05). The average ammonium concentration of the wastewater input was 100.0 ± 51.1 mg NH_4^+- N/L, but the input varied greatly depending on the wastewater batch.

During the second SRT phase, the average ammonium concentration was 14.6 ± 11.0 and 25.1 ± 26.2 mg NH_4^+- N/L in the algal and bacteria reactors, respectively. When the reactors were switched to the third SRT phase, the average ammonium concentration decreased to 14.1 ± 15.5 mg NH_4^+- N/L in the algal reactor and increased to 37.7 ± 23.1 mg NH_4^+- N/L in the bacteria reactor. This occurred despite no change in how the bacteria reactor was operated

throughout the entire study period (infinite SRT). In the bacteria reactor, during the second SRT phase, an ammonium concentration of 0 mg NH_4^+- N/L was recorded several times, whereas, during the third SRT phase, the ammonium concentration rarely dropped below 20 mg NH_4^+-N/L. An explanation for this could be that the bacteria community never recovered from the early stressor events, such as A/C failure 1 and 2. Additionally, the third SRT phase had a much greater ammonium concentration input from the diluted wastewater, which may suggest that the ammonium oxidizers in the bacteria reactor were not as well equipped to handle these concentrations. The input concentration of ammonium was statistically significant ($p < 0.05$) in the algal reactor but not the bacteria reactor (Figure 13).

High ammonium concentrations are known to be toxic to plants and suppress growth in a phenomenon known as ammonium toxicity (Hachiya et al., 2012). Because of this, if the intended use of the water is to be a source of irrigation water, it is important to transform the ammonium to more useable forms of nitrogen, such as nitrate. In a study performed by Ikeda and Tan (1998), plants were grown where the only nitrogen source was ammonium. In this experiment, the plants experienced partial yellowing, mottled chlorosis, and curling and wilting of leaves. These observations were made when the ammonium concentration was 84 mg/L or greater (Ikeda & Tan, 1998). Throughout the treatment phase, the ammonium concentration in the algal-bacterial reactor remained below the 84 mg/L level suggesting the "treated" water from the algal reactor would be suitable for irrigation. Alternatively, the bacteria reactor experienced periods of high ammonium concentrations, where the ammonium concentration spiked upwards of 90 mg NH_4^+-N/L. Concentrations of these levels could greatly affect the product generated in a hydroponics system.

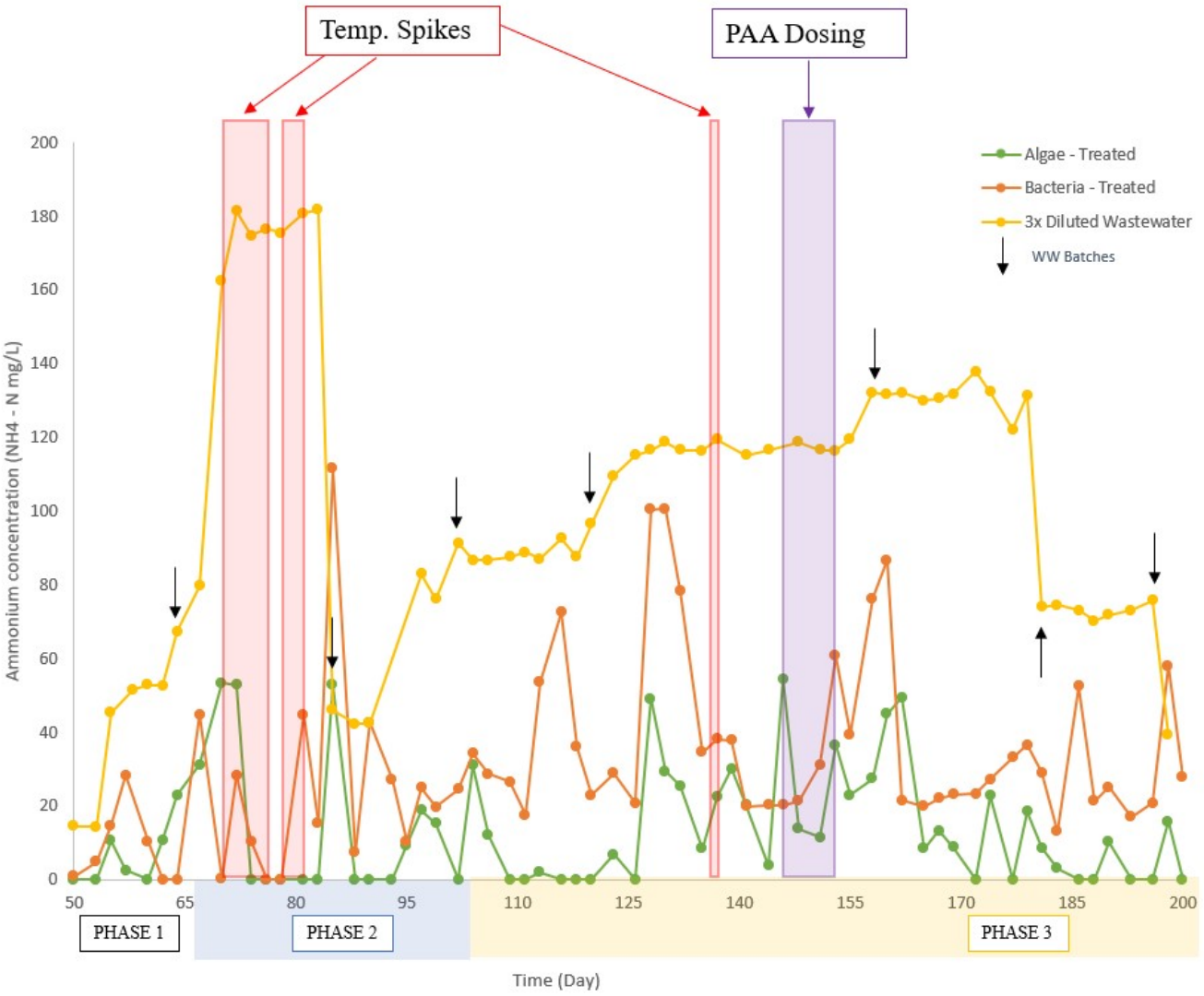


Figure 12. Ammonium concentration throughout treatment phase

a)

Algal Reactor

```
Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)                8.98236    9.86417   0.911  0.36615
Compiled_Data$`WW - NH4`   0.14718    0.05366   2.743  0.00802 **
Compiled_Data$`WW-NO2`    -5.94566    3.69003  -1.611  0.11237
Compiled_Data$`WW - PO4`  -0.34941    0.33172  -1.053  0.29643
Compiled_Data$PAAYes      16.52362   10.50314   1.573  0.12093
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 20.06 on 60 degrees of freedom
Multiple R-squared:  0.168,     Adjusted R-squared:  0.1126
F-statistic: 3.029 on 4 and 60 DF,  p-value: 0.02424
```

b)

Bacteria Reactor

```
Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)             -80.08250   62.41261  -1.283 0.204737
Compiled_Data$`O2 - B`   -1.59874    0.67278  -2.376 0.020931 *
Compiled_Data$`Temp - B`  5.73540    2.46663   2.325 0.023710 *
Compiled_Data$PO4        -0.63633    0.61275  -1.038 0.303512
Compiled_Data$NO2        -0.83456    0.32994  -2.529 0.014267 *
Compiled_Data$NO3        -0.73052    0.18458  -3.958 0.000216 ***
Compiled_Data$`WW - NH4`  0.08133    0.05775   1.408 0.164613
Compiled_Data$`WW-NO2`   -4.75876    3.93995  -1.208 0.232192
Compiled_Data$`WW - PO4`  0.98436    0.43358   2.270 0.027058 *
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 19.26 on 56 degrees of freedom
Multiple R-squared:  0.4755,    Adjusted R-squared:  0.4005
F-statistic: 6.345 on 8 and 56 DF,  p-value: 7.729e-06
```

Figure 13. Multiple regression analysis for ammonium concentration in algal reactor (a) and bacteria reactor (b)

Figure 14 shows the ammonium removal rate in the algal and bacterial reactors throughout the treatment phase. The removal rate differed in the algal reactor between the three treatment phases, with averages of 9.5 ± 5.6, 37.9 ± 18.3, and 32.5 ± 8.9 mg NH_4^+-N/L/day for treatment phase 1, 2, and 3, respectively. From this, it appears that the higher OD threshold, allowing more TSS in the reactor, provided greater ammonium removal, however optical density was not statistically significant (p-value < 0.05) when conducting multiple regression for the

ammonium concentration within the algal reactor. Within the bacterial reactor, the average ammonium removal rate for the three phases were 23.4 ± 24.5 , 35.0 ± 19.7, and 24.3 ± 12.8 mg NH_4^+-N/L/day showing a consistent removal rate throughout the experiment. During the first two temperature spikes, the ammonium removal rate decreased in both reactors suggesting that the ammonium oxidizers in both systems were affected negatively by the temperature increase. Temperature was found to be statistically significant (p-value <0.05) for the ammonium concentration within the bacteria reactor (Figure 13). For the bacteria reactor, the ammonium removal rate decreased through the PAA dosing, suggesting that the ammonium oxidizers were negatively affected by the antimicrobial, but it was not found to be statistically significant.

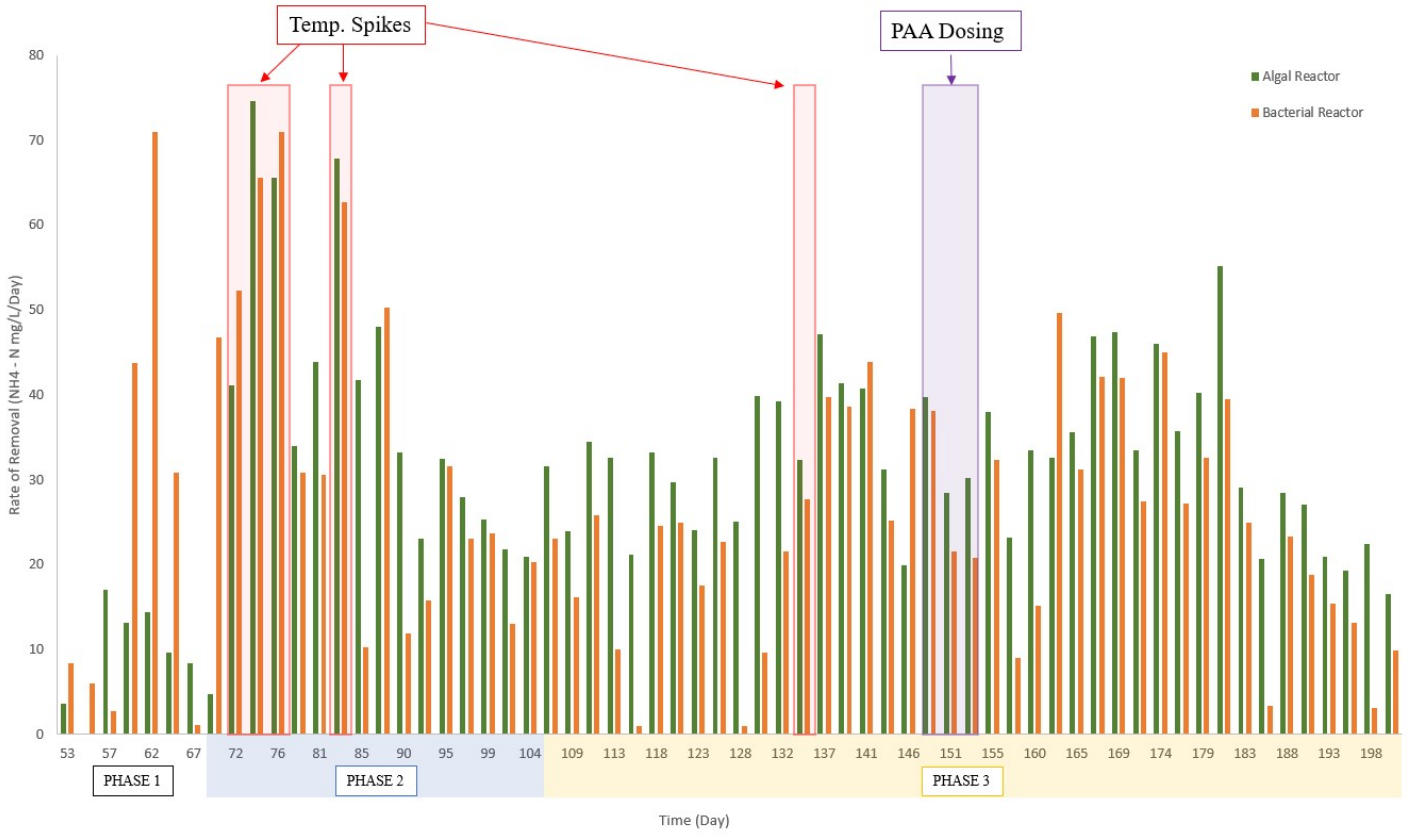


Figure 14. Ammonium rate of removal throughout treatment phase

The nitrite data, collected through ion chromatography, is shown below in Figure 15a. During A/C failure 1, 2, and 3, along with the PAA dosage, nitrite concentration increased in the algal reactor. The nitrite concentration spiked from 0 to 2.5 mg NO_2^--N/L on the last day of dosing, spiking to 5.1 Mg NO_2^--N/L two days afterwards. After each stressor event, it took approximately 1-3 days for the reactor to recover. Although these trends are observed, neither temperature nor the PAA dosing were found to be statistically significant for the nitrite concentration in the algal reactor (Figure 16). During all temperature shocks and the PAA dosing, nitrite increased in the bacteria reactor, however, both temperature and PAA dosing were not found to be statistically significant (Figure 16) .Prior to A/C failure 1, the bacteria reactor had a concentration of approximately 0 mg NO_2^--N/L of nitrite. However, afterwards, the nitrite concentration rarely dropped below 7 mg NO_2^--N/L. During the PAA dosing, the nitrite concentration increased from 13.3 mg NO_2^--N/L to 23.4 mg NO_2^--N/L. This is similar to the nitrite-locking cases that have occurred in real treatment systems for poultry processing wastewater as a result of PAA (Steven R. Woodruff, 2019), however, PAA was not found to be statistically significant for nitrite in the bacterial reactor. There was no nitrite in the input wastewater (Figure 15a) meaning all observed nitrite in the system was being transformed through ammonium oxidation. In the early stages in which lower levels of solids were retained, this was likely due to low levels of nitrification in the algal-bacterial reactor. In later (higher solids) stages of operation, the low nitrite levels were likely the result of efficient nitrification to nitrate. From the multiple regression analysis, a negative correlation was found between the OD680 and nitrite concentration suggesting that, as the chlorophyll concentration increased, the nitrite concentration decreased (Figure 16). This supports the idea that algae help reduce the

effects of stressor events and continue transforming the nutrients into suitable forms for irrigation.

Looking at the two SRT phases, the second SRT phase had higher nitrite concentrations than the third SRT phase in both reactors. During the second SRT phase, the algal reactor had a concentration of 2.71 ± 4.1 mg NO_2^--N/L. In contrast, the bacteria reactor had an average nitrite concentration of 18.35 ± 9.4 mg NO_2^--N/L. Similarly, during the third SRT phase, the algal reactor had a concentration of 2.29 ± 3.7 mg NO_2^--N/L, while the bacteria reactor had a concentration of 13.27 ± 6.9 mg NO_2^--N/L. At the end of the experiment, around day 181, the nitrite in the algae reactor began to increase, even beyond the concentrations within the bacteria reactor at times. This may be due to a harvest that occurred on day 177, where biomass was removed from the algae reactor and disturbed its nitrifying community. The harvest that occurred on day 177 was the first since day 139. The DO data in Figure 7 showed evidence of a return to an algal-dominant system. Additionally, the ammonia oxidizers may have been more productive than the nitrite oxidizers in the system, resulting in a surplus of nitrite in the system. This suggests that sudden biomass removal should be avoided with only gradual harvesting used over time, which is more realistic in a real continuously operating system. Even with the spike of nitrite at the end of the third SRT phase, the algal reactor transformed more nitrite than the bacterial reactor.

Nitrite is a toxic form of nitrogen to plants and fish species, so it is important for it to be transformed to nitrate in the reactor if the water is to be used in aquaponic or hydroponic systems. Nitrite concentrations of 0.4 -1.1 mg NO_2^--N/L have been observed in well-operated aquaponic systems using tilapia and basil, and were deemed adequate (Rakocy et al., 2004). Within both reactors, higher nitrite concentrations were observed, especially in the bacteria

reactor. To successfully use the water treated by the two reactors, nitrite will need to be better transformed. The algal reactor experienced periods of nitrite concentrations equal to 0 mg NO_2^--N/L, but the inconsistency of the nitrite concentration could cause issues if the water were to be used.

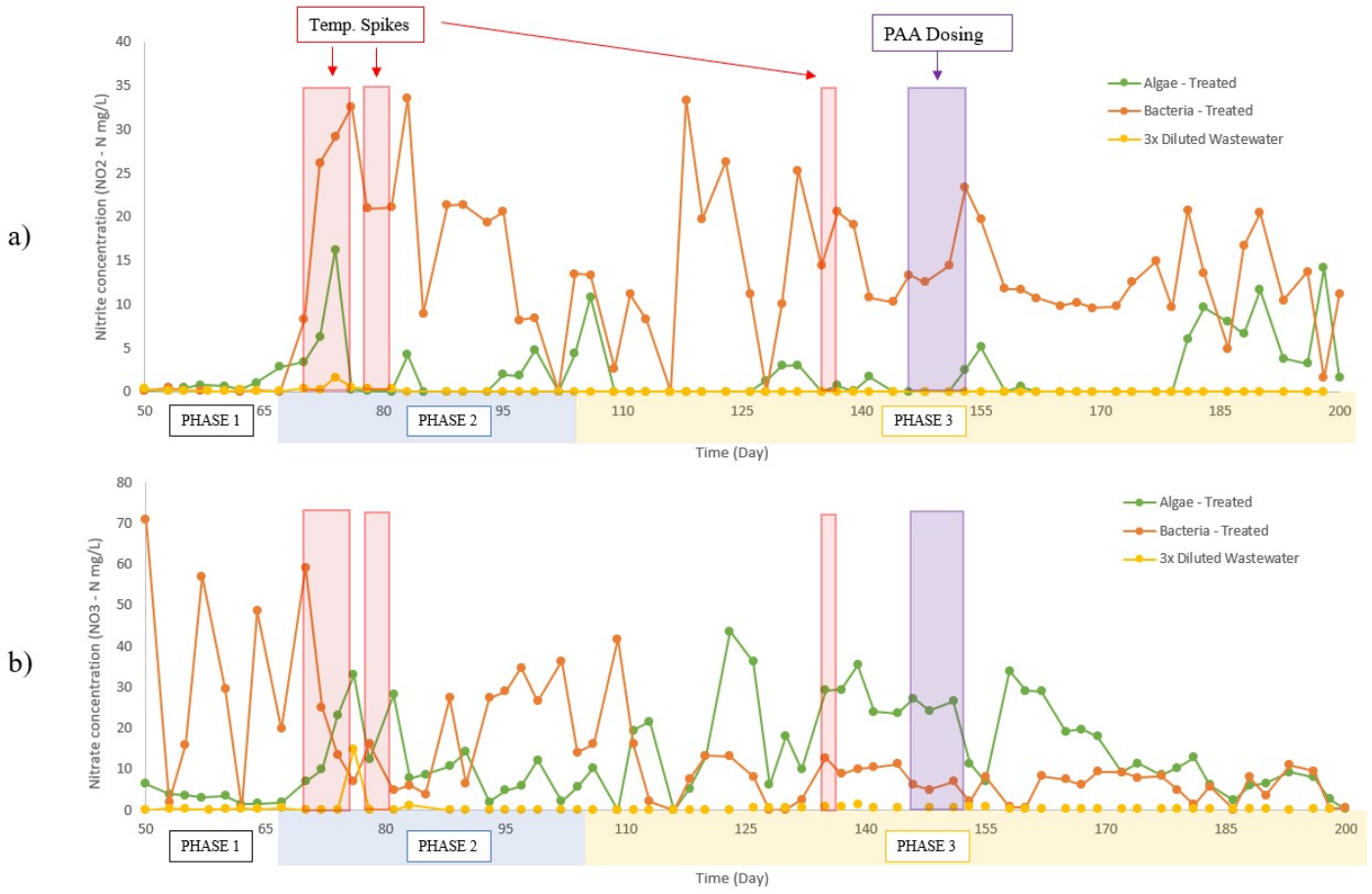


Figure 15. Nitrite (a) and Nitrate (b) Concentration Throughout Treatment Phase

a)

Algal Reactor

```
Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)              -3.41771    5.35525  -0.638   0.5259
Compiled_Data$NO3        -0.13380    0.03191  -4.193 9.70e-05 ***
Compiled_Data$PO4         0.51890    0.22549   2.301   0.0251 *
Compiled_Data$OD550      58.10745   29.67360   1.958   0.0551 .
Compiled_Data$OD680     -40.55374   19.95364  -2.032   0.0468 *
Compiled_Data$`WW - NH4` -0.03488    0.02874  -1.214   0.2299
Compiled_Data$`WW-NO2`   10.16536    2.06295   4.928 7.52e-06 ***
Compiled_Data$`WW - PO4`  0.34312    0.17072   2.010   0.0492 *
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 9.522 on 57 degrees of freedom
Multiple R-squared:  0.4379,    Adjusted R-squared:  0.3689
F-statistic: 6.344 on 7 and 57 DF,  p-value: 1.587e-05
```

b)

Bacteria Reactor

```
Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)              -30.00459   22.08304  -1.359 0.179589
Compiled_Data$`Temp - B`   1.22719    0.91213   1.345 0.183819
Compiled_Data$NH4         -0.10446    0.04269  -2.447 0.017503 *
Compiled_Data$NO3         -0.18789    0.06786  -2.769 0.007579 **
Compiled_Data$`WW - NH4`   0.04856    0.01999   2.429 0.018294 *
Compiled_Data$`WW-NO2`     2.29313    1.32852   1.726 0.089751 .
Compiled_Data$`WW-NO3`     0.29971    0.10913   2.746 0.008051 **
Compiled_Data$`WW - PO4`   0.45095    0.11970   3.767 0.000393 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 6.921 on 57 degrees of freedom
Multiple R-squared:  0.4663,    Adjusted R-squared:  0.4008
F-statistic: 7.115 on 7 and 57 DF,  p-value: 4.187e-06
```

Figure 16. Multiple regression analysis for nitrite concentration in algal reactor (a) and bacteria reactor (b)

During all stressor events, nitrate decreased in the bacteria reactor. In comparison, during the A/C failures, the nitrate concentration increased or remained the same in the algae reactor. Temperature was found to be statistically significant for nitrate in the algal reactor. Multiple regression data for nitrate can be observed in Figure 17. In addition to elevated temperatures, two

major temperature events also coincided with a new batch of wastewater that had higher than typical ammonium concentrations. As shown in Figure 17a, the input concentration of nitrate, while small, significantly contributed to the nitrate concentration. The high levels of ammonium may have lessened competition for ammonium between algae and ammonia oxidizing bacteria during this period. During the PAA dosing, the nitrate concentration in the algae reactor remained constant and decreased on the last day, however, PAA was not found to be statistically significant to the nitrate concentration in the algal reactor. In the algal reactor, both OD550 and OD680, were statistically significant (p value <0.05) to the nitrate concentration. This supports the thought that the solids composition and solids retention can affect the effluent concentration.

Prior to the first A/C failure, the bacteria reactor had a higher concentration of nitrate than the algal reactor. This coincided with a period of lower suspended solids, rapid algal growth, and high levels of dissolved oxygen. It is likely that algae were consuming nitrogen for growth and depriving AOB of ammonium. *C. sorokiniana* is also able to consume nitrite and nitrate based on unpublished studies conducted in our lab. Thus, rapid algal growth during the first (low solids) stage likely obscured nitrification and suppressed the level of nitrate production. Increasing solids content in the algal-bacteria reactor is likely to not only prevent less wash-out of nitrifying bacteria, it also slows algal photosynthesis through light blockage. Increasing the solids level resulted in a transition period that lasted until day 120 when the nitrate concentration in algae reactor started to exceed that in the bacteria-only reactor. The average nitrate concentration in the algal reactor during the second SRT and third SRT periods were 11.7 ± 8.7 and 16.2 ± 11.2 mg NO_3^--N/L, respectively. However, no change in solids retention was made in the bacteria reactor (maximum possible retention continued throughout the study) and yet the concentration of nitrate decreased in the bacteria reactor. The decrease in nitrate concentration

coincides with the increase in nitrite concentration that was discussed prior, indicating a breakdown in nitrite oxidation that persisted to the end of the study. The nitrate data collected further supports a decrease in nitrite oxidation within the bacteria reactor around day 116. The mean nitrate concentrations in the bacteria reactor, during the second phase and third phase, were 21.1 ± 14.6 mg/L and 7.5 ± 7.1 mg/L, respectively. The nitrate concentrations observed in both reactors were not greatly contributed to directly by the wastewater input, as the wastewater input had an average nitrate concentration of 0.35 ± 0.33 mg NO_3^--N/L.

In aquaponics systems, nitrate concentrations have been recorded at high concentrations, such as over 200 mg N/L (Wongkiew et al, 2017). Concentrations of nitrate at this level, are adequate because nitrate is not toxic to fish, even at 150 – 300 mg N/L (Graber and Junge, 2009, Hu et al., 2014). Throughout the treatment phase, the nitrate concentration remained below this threshold, peaking to 45 mg NO_3^--N/L once. In contrast, the bacteria reactor had spikes of nitrate at concentrations of 50 mg NO_3^--N/L and greater several instances before day 110. From an irrigation standpoint, neither reactor would cause stress to tilapia or plants, as systems have reported nitrate concentrations between 10 mg N/L to over 200 mg N/L without issue (Lam et al., 2015, Seawright et al. 1998, Sikawa and Yakupitiyage, 2010). In fact, efforts to further increase N oxidation without N removal by algal uptake or other means would likely be beneficial.

a)

Algal Reactor

```
Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)               124.4165    71.4314   1.742  0.08704 .
Compiled_Data$`Temp - A`   -6.5650     2.8005  -2.344  0.02264 *
Compiled_Data$NO2          -1.3176     0.4658  -2.828  0.00648 **
Compiled_Data$OD550       240.9140   107.9819   2.231  0.02970 *
Compiled_Data$OD680      -155.1767    74.3488  -2.087  0.04144 *
Compiled_Data$`WW - NH4`    0.2991     0.1081   2.768  0.00764 **
Compiled_Data$`WW-NO3`      1.5361     0.6507   2.361  0.02175 *
Compiled_Data$`WW-NO2`     13.6491     8.0836   1.688  0.09688 .
Compiled_Data$`WW - PO4`    1.0250     0.6664   1.538  0.12962
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 37.05 on 56 degrees of freedom
Multiple R-squared:  0.4834,    Adjusted R-squared:  0.4096
F-statistic:  6.55 on 8 and 56 DF,  p-value: 5.282e-06
```

b)

Bacteria Reactor

```
Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)              -39.59321   36.49060  -1.085  0.28256
Compiled_Data$`O2 - B`     0.64176    0.45086   1.423  0.16017
Compiled_Data$`Temp - B`   2.80440    1.46538   1.914  0.06077 .
Compiled_Data$PO4         -0.51637    0.39069  -1.322  0.19164
Compiled_Data$NO2         -0.63546    0.18846  -3.372  0.00136 **
Compiled_Data$NH4         -0.25810    0.07469  -3.456  0.00105 **
Compiled_Data$OD550      -26.36312   13.17744  -2.001  0.05029 .
Compiled_Data$`WW-NO2`     2.43451    2.35376   1.034  0.30544
Compiled_Data$`WW - PO4`   0.48432    0.28340   1.709  0.09299 .
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 11.92 on 56 degrees of freedom
Multiple R-squared:  0.4663,    Adjusted R-squared:  0.3901
F-statistic: 6.117 on 8 and 56 DF,  p-value: 1.188e-05
```

Figure 17. Multiple regression analysis for nitrate concentration in algal reactor (a) and bacteria reactor (b)

Figures 18 and 19 show the nitrite and nitrate rate of removal throughout the treatment process. The bacteria reactor shows consistent nitrite production during all treatment phases, whereas the algal reactor has periods of removal and production. The nitrite production within the bacteria reactor spiked to over 10 mg NO_2^--N/L/day several instances, especially during the first two temperature spikes. Again, this suggests that the nitrite oxidizers within the bacteria system where not suited for the higher temperature conditions, even though temperature was not

statistically significant. Over the three treatment phases, the algal reactor had an average nitrite production rate of 0.26 ± 0.28, 0.96 ± 2.1, and 0.65 ± 1.6 mg NO_2^-N/L/day. In contrast, the bacteria reactor had an average nitrite removal rate of 0.08 ± 0.11 mg NO_2^-N/L/day in the first treatment phase and a nitrite production rate of 5.4 ± 4.8 and 3.4 ± 3.7 mg NO_2^-N/L/day in the second and third treatment phase. The shift to higher levels of production may be attributed to the temperature increase experienced at the beginning of the second treatment phase. The multiple regression data for nitrite, shown in Figure 16, suggests a positive relationship between temperature and nitrite concentration within the bacteria reactor. This relationship suggests that as the temperature increased in the bacteria system, the nitrite concentration increased. This can be explained by the nitrite oxidizers being negatively affected by temperature increases which allows for nitrite to accumulate.

Figure 18. Nitrite rate of production throughout treatment phase

The figure for nitrate formation can be seen below in Figure 19. Throughout the treatment phases, both reactors showed consistent nitrate formation, except for the algal reactor during the first phase. Initially, during the first two treatment phases, the bacteria reactor had greater rates of nitrate production than the algal reactor with the two temperature spikes being the exception. During these two phases, the bacteria reactor had an average nitrate production rate of 5.9 ± 8.9 and 6.5 ± 4.1 mg NO_3^--N/L/day, whereas the algal reactor had a production rate of only 0.75 ± 0.35 mg and 4.1 ± 4.4 NO_3^--N/L. Nitrate production increased in the algal reactor, beyond that of the bacteria reactor, during the final treatment phase when the TSS threshold was increased. Both the OD550 and OD680 was found to be statistically significant for nitrate in the algal reactor. This supports the idea that the solids content within the algal reactor can be manipulated to affect the nitrate concentration in the effluent. In this phase, the average production rate decreased to 2.5 ± 2.4 mg NO_3^--N/L in the bacteria reactor while increasing to 5.6 ± 4.6 mg NO_3^--N/L in the algal reactor. During the PAA dosing, the nitrate production decreased in the algal reactor, implying that the nitrite oxidizers were negatively affected when antimicrobials were present, however, the PAA dosing was not found to be statistically significant. Similarly, in the dates following the PAA dosing, the bacteria reactor experienced nitrate production rates of zero indicating the nitrite oxidizers were negatively affected, however, the PAA dosing was not statistically significant. The algal reactor had a greater rate of production than the bacterial reactor in terms of nitrate production during the PAA dosing and the week afterwards. The average rate of nitrate production was 7.1 ± 3.8 and 1.5 ± 2.4 mg NO_3^--N/L supporting the idea that algae can increase or sustain nitrifying activity with antimicrobials present.

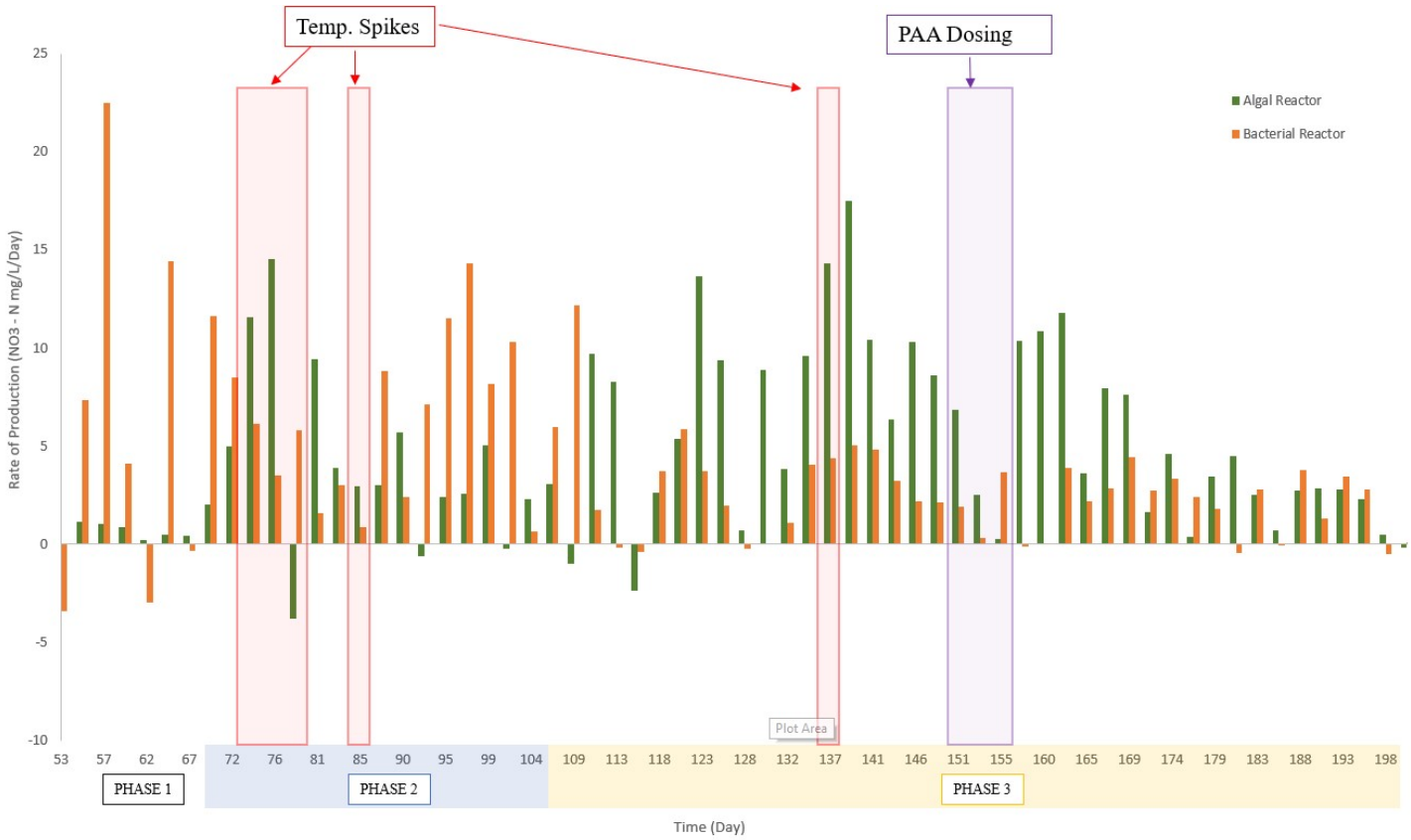


Figure 19. Nitrate rate of production throughout treatment phase

4.4 Other Nutrients of Interest

The phosphate concentration during the experiment can be seen in Figure 20. During all three A/C failures the phosphate concentration increased in the algal reactor, although it appears the phosphate concentrations were already increasing prior to these events. In contrary, during the PAA phase, the phosphate concentration decreased slightly.

On Day 67, the second SRT phase began, and biomass was returned into system after harvest of OD550 > 1. At this point, a small increase was observed in phosphate concentration, in the algal reactor, to 2.1 mg/L. After day 104, when the third SRT phase began, the phosphate concentration began to rise to 15.1 mg/L. The average phosphate concentration in the algal reactor was 2.2 ± 4.1 and 15.2 ± 6.2 mg/L in the second and third SRT phase. The bacteria reactor had an average phosphate concentration of 21.6 ± 5.1 and 26.2 ± 3.8 mg/L in the second

SRT and third SRT phases. The input concentration of phosphate remained consistent throughout the treatment process with an average of 27.1 ± 8.9 mg/L. The phosphate data collected during the treatment phases can be observed below in Figure 20. These data further confirm that the algal reactor moved from a period of high photosynthesis with high nutrient uptake to a system in which less growth and nutrient uptake occurred.

As discussed above, the phosphate concentration, in the algal reactor, remained low (around 2.1 mg/L) during the second SRT phase of the experiment. Because of the rapid removal of algal biomass, the algae continued growing throughout this phase meaning phosphate was being consumed. As the reactor was shifted to the longer SRT, algal biomass was no longer being removed as rapidly. This allowed for the algae to grow until the stationary phase where it was consuming less phosphate from the reactor. With this, a key difference is observed between the two SRT phases. As seen in Figure 21a, the OD550 and OD680 value was statistically significant to the phosphate concentration in the algal reactor. The significance of these two values suggests that the phosphate concentration in the reactor, and eventually the effluent, can be manipulated by the solids retained.

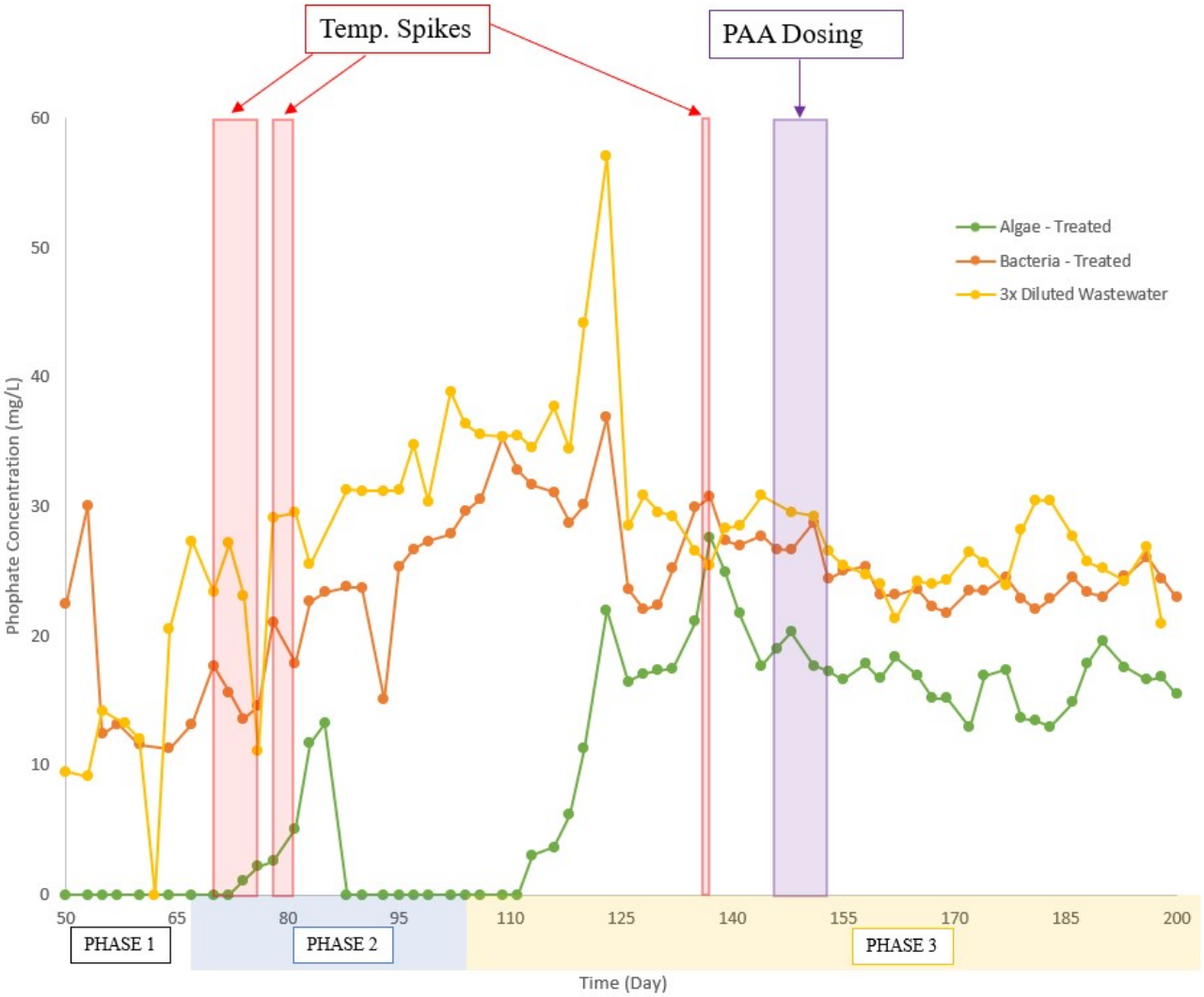


Figure 20. Phosphate Concentration Throughout Treatment Phases

The phosphate removal rate is shown in Figure 22 for both the algal and bacterial systems. A positive value suggests that phosphate is being removed from the system whereas a negative value suggests that phosphate is accumulating. The algal reactor removed more phosphate than the bacterial system throughout all treatment phases. The average removal rates for the algal reactor, during each treatment phase, was 3.6 ± 1.2, 8.4 ± 2.8 , and 4.6 ± 3.0 mg PO_4^{3-}/L/day. In contrast, the average removal rates for the bacterial reactor, during each treatment phase, was 0.57 ± 2.6, 2.7 ± 1.6, and 1.2 ± 1.5 mg PO_4^{3-}/L/day. Both systems had peak removal during the second treatment phase. During the first two temperature spikes, the

phosphate removal rate decreased in both reactors. It appears that the phosphate removal rate within both systems is affected negatively by both types of stressor events, however, only temperature in the algal reactor is statistically significant to phosphate concentration. Figure 21 shows the multiple regression data for phosphate and a negative correlation between temperature and phosphate can be seen. The negative correlation observed supports the decrease in phosphate removal during the temperature spikes.

As seen in Figure 21a, the nitrite and nitrate concentrations in the reactor are statistically significant to the phosphate concentration in the algal. In a study conducted by Arenberg and Arai (2021), it was found that higher nitrogen availability promoted phosphate mineralization. This studies objective was to evaluate P mineralization rates in temperate floodplain soils as a function of ammonium and nitrate. The researchers found that the P mineralization rate increased in the presence of ammonium and nitrate, which may explain the relationship between phosphorus and nitrate in the algal reactor.

a)

Algal Reactor

```
Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)               27.48914    9.56697   2.873 0.005762 **
Compiled_Data$O2          -0.20547    0.09052  -2.270 0.027157 *
Compiled_Data$`Temp - A`  -0.97621    0.35757  -2.730 0.008488 **
Compiled_Data$NO2          0.16009    0.06573   2.435 0.018141 *
Compiled_Data$NO3          0.06168    0.01700   3.628 0.000626 ***
Compiled_Data$OD550       43.41251   15.19802   2.856 0.006034 **
Compiled_Data$OD680      -24.84440   10.43391  -2.381 0.020749 *
Compiled_Data$`WW - NH4`   0.03203    0.01492   2.146 0.036264 *
Compiled_Data$`WW-NO2`    -4.95455    1.09553  -4.523  3.3e-05 ***
Compiled_Data$`WW - PO4`  -0.25449    0.08570  -2.970 0.004413 **
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 4.926 on 55 degrees of freedom
Multiple R-squared:  0.7195,    Adjusted R-squared:  0.6736
F-statistic: 15.68 on 9 and 55 DF,  p-value: 2.69e-12
```

b)

Bacteria Reactor

```
Coefficients:
                          Estimate Std. Error t value Pr(>|t|)
(Intercept)               16.80544    5.45679   3.080  0.00319 **
Compiled_Data$pH          -0.82694    0.66918  -1.236  0.22162
Compiled_Data$NO3         -0.04030    0.03793  -1.063  0.29248
Compiled_Data$NH4         -0.03015    0.02298  -1.312  0.19477
Compiled_Data$OD680       13.05548    5.37796   2.428  0.01838 *
Compiled_Data$`WW - NH4` -0.02119    0.01123  -1.887  0.06430 .
Compiled_Data$`WW-NO2`    -1.08714    0.76856  -1.415  0.16265
Compiled_Data$`WW - PO4`   0.50511    0.06994   7.222 1.36e-09 ***
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

Residual standard error: 3.883 on 57 degrees of freedom
Multiple R-squared:  0.5993,    Adjusted R-squared:  0.5501
F-statistic: 12.18 on 7 and 57 DF,  p-value: 2.138e-09
```

Figure 21. Multiple regression analysis for phosphate concentration in algal reactor (a) and bacteria reactor (b)

Figure 22. Phosphate removal rate throughout treatment phases

Sulfate is another important macronutrient required for algal growth. Sulfate data is shown in Figure 23. During the A/C failures, the sulfate concentration increased within the algal reactor. However, the sulfate concentration trended upwards until day 136, so the temperature increases may not have caused this increase. In contrary, during the PAA dosage, the sulfate concentration decreased in the algae reactor. However, during this period, the system's sulfate concentration was trending down. Again, the stressor event may not have caused the decrease. Within the bacteria reactor, the temperature spikes led to similar or an increase in sulfate concentrations. Additionally, the PAA dosage led to a decrease in sulfate concentration. Similarly to the algal reactor, the sulfate concentrations cannot be attributed to the various stressor events as the system was trending in their respective directions.

During the second SRT phase, the average sulfate concentration was 31.6 ± 5.4 and 48.4 ± 3.4 mg/L for the algae and bacteria reactors, respectively. When the system shifted to the third

SRT phase, the average sulfate concentration increased to 47.9 ± 6.2 and 52.9 ± 6.2 mg/L in the algae and bacteria reactor. Throughout the treatment phases, the average sulfate concentration of the 3x diluted wastewater input was 44.1 ± 10.6 mg/L. Similar to what was observed with phosphate, the sulfate concentration was lowest during the the first phase in the algal reactor. This can be attributed to the consistent algal growth occurring during this phase due to biomass being removed more frequently. As the system shifted to the longer SRT phases, the sulfate concentration rose in the algae reactor as the algae was not growing as rapidly. In this phase, sulfate was generated through transformation more rapidly than it was removed by algae (sulfate in the effluent exceeded that in the influent).

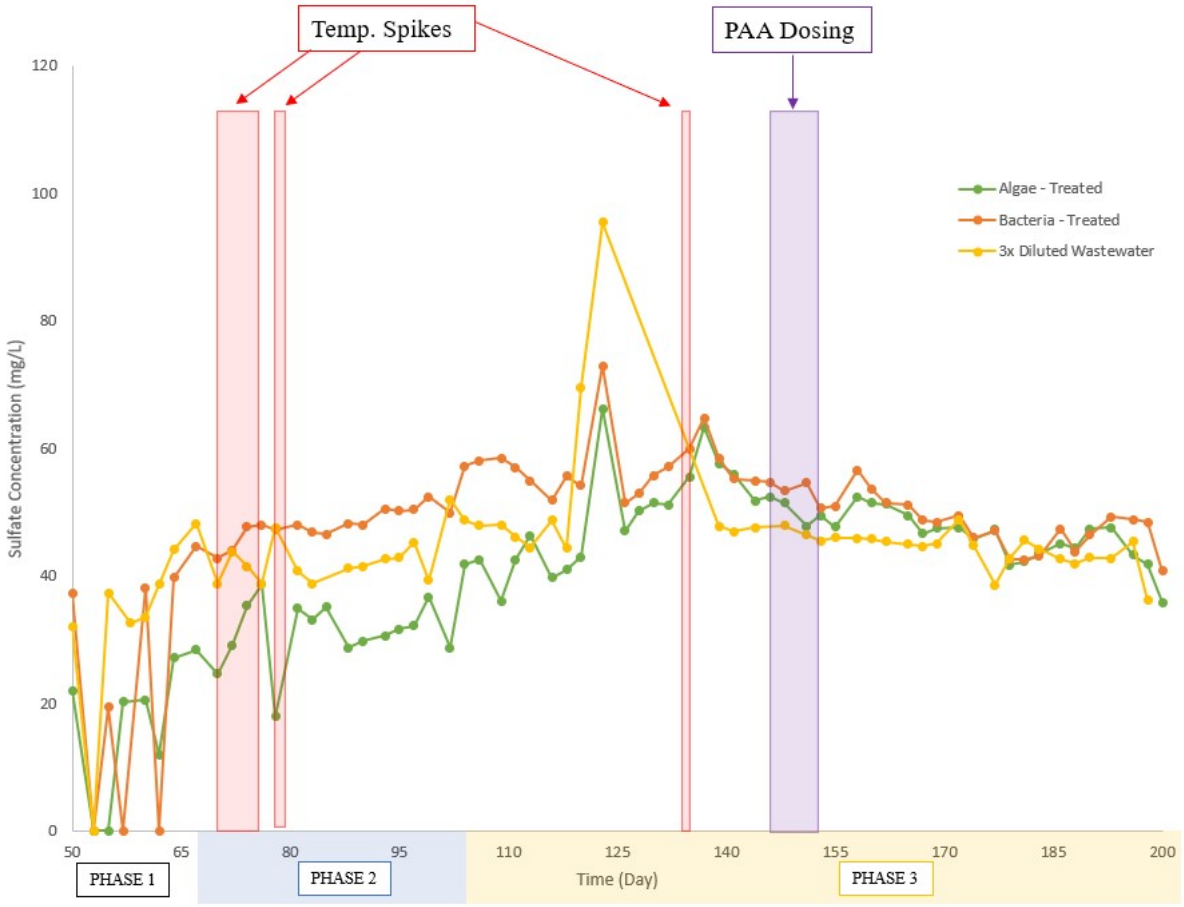


Figure 23. Sulfate Concentration Throughout Treatment Phase

4.5 COD Analysis

The COD of the diluted wastewater input varied throughout the experimental phases. Six samples were measured as representatives, to understand the COD concentrations in different batches of wastewater. The wastewater batches used were day 85 – 118, day 120 – 144, and day 148 – 196. The date ranges mark the beginning and end of the 3x diluted wastewater batch. As the experiment continued, the COD within the wastewater input increased, with the last batch having the highest COD. The table below shows the COD concentrations in the wastewater inputs. It appears that the COD decreased the longer the wastewater bottle was stored in the refrigerator. However, the batch that lasted from day 120 – day 144 was an exception to this.

Table 2. COD Analysis of 3x Diluted Wastewater Input

Day	Description	COD (mg/L)
85	Beginning of Bottle	666
118	End of Bottle	550
120	Beginning of Bottle	814
144	End of Bottle	1146
148	Beginning of Bottle	1194
196	End of Bottle	864

In addition to measuring the COD of the input wastewater, COD measurements were taken at specific time points during both SRT phases and can be observed in the table below. The input wastewater COD was measured prior to the addition of wastewater to the two reactors. Similarly, the treated effluent COD was measured prior to the addition of wastewater to the two reactors. During the second SRT phase, the algal reactor had a greater COD removal rate than the bacteria reactor. This is consistent with past studies in which algae have been shown to promote removal of organics by bacteria (Higgins et al., 2018). This was driven largely by the high levels of photosynthetic oxygen production (Holmes et al., 2020). COD data collected

through treatment phases 2 and 3 can be observed in Table 3. In Holmes et al. (2020), it was found that the presence of algae led to an 18-66% faster removal of COD by bacteria. This was attributed to the photosynthetic aeration provided by the algal species. Another possible explanation for the improved organics removal in the algal reactor is mixotrophy. *C. sorokiniana* has been found to be mixotrophic, suggesting that the algae can be cultivated on organic carbon substrates (Li et al., 2014). A study performed by Devi et al. (2012) reported a COD reduction of 18.3% when sterile municipal wastewater was treated with only a microalgae consortium under strict heterotrophic conditions.

Table 3. COD Analysis Through Treatment Phases 2 and 3

	Day	Input Wastewater COD (mg/L)	Bacteria System		Algal System	
			Treated Effluent (mg/L)	COD Removal Rate (mg/L/day)	Treated Effluent (mg/L)	COD Removal Rate (mg/L/day)
SRT Phase 2	85	666	-	-	-	-
	88	506	170	165.3	134	177.3
	90	558	136	185	96	205
SRT Phase 3	162	776	-	-	-	-
	165	1010	94	227.3	132	224
	167	500	112	449	110	450

The COD removal increased for both reactors during the third treatment phase; however, no benefit due to the addition of algae can be observed. Both reactors were effective at removal of COD in the range of 66 - 90%. The bacteria reactor had a removal percentage ranging from 66.4 % to 90.7% and the algal reactor had a removal percentage ranging from 73% to 86.9%. This is important when considering use of the water for hydroponic irrigation where high oxygen demand would lead to anoxic conditions that are harmful to plants.

Conclusions

It was found that the effluent of an algal-bacterium reactor can be manipulated by the amount of solids retained. Low solids retention led to a system dominated by photosynthesis, algal growth, and nutrient uptake. The result was removal of nitrogen and phosphorous from the wastewater. During this period, nitrate and phosphate concentrations remained low due to high algal growth and uptake. A high solids retention led to a bacteria dominated system, supported by algae, which helped oxidize nutrients without completely removing them. The resulting treated water is more suitable for hydroponic irrigation. With solids being retained, less phosphorous was removed from the system, leaving this essential nutrient in solution. Similarly, nitrate concentrations, in the algal system, rose during this period. By operating the treatment system at a high SRT, essential nutrients needed for plant growth remained in the effluent making it a good candidate for irrigation water. By controlling algal growth within the system, the nutrient output can be managed and manipulated depending on the intended goals. If the goal of the reactor is water treatment, a low SRT would be recommended as nutrients would be removed from the effluent. However, if the water is to be used in a hydroponics system as an irrigation source, using a high SRT may be recommended as nutrients are retained in the effluent and would benefit plant growth. The two reactors were presented with several stressor events, such as temperature spikes and PAA dosing. Overall, both systems were negatively affected by stressor events, but the algal system exhibited greater resilience by continuing to remove phosphorous and transform ammonium to nitrate.

Future Work

This study was able to find evidence that solids retention can govern effluent concentrations, however, further research should be conducted to improve upon this experiment. Further research should be done to optimize solids retention and to run the reactor as a truly continuous system. An issue that occurred during this study was the build-up of biofilm on the sides of the algal reactor. In the future, it would be recommended to conduct an experiment like this in a reactor with flat sides allowing for easy biofilm removal. By having the ability to remove biofilm from the sides, more accurate measures of total solids would be possible. Additionally, the process should be scaled up to test the effluents effectiveness for hydroponic irrigation. Finally, further antimicrobial dosings should be conducted for longer time periods. Approximately 70 sampling days occurred during this experiment and only four of which contained PAA. Any potential effect of the PAA dosing may have been obscured because of the amount of data that was collected outside of the dosing period.

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