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Nutrient Removal from Recirculating Aquaculture System Water

Eliza M. Costigan
University of Maine, eliza.costigan@maine.edu

NUTRIENT REMOVAL FROM RECIRCULATING

AQUACULTURE SYSTEM WATER

By

Eliza Costigan

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Advisory Committee:

Jean D. MacRae, Associate Professor of Civil & Environmental Engineering, Advisor

Onur G. Apul, Assistant Professor of Civil & Environmental Engineering

Deborah A. Bouchard, Director, Aquaculture Research Institute, University of Maine Cooperative Extension and Aquaculture Research Institute

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Thesis Advisor: Dr. Jean D. MacRae

An Abstract of the Thesis Presented
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Recirculating aquaculture systems (RAS), where only 10% of the total system water is exchanged per day, have grown in popularity in recent years due to their potential to provide a high-quality protein source in a contained environment. With increased production comes the need for RAS water treatment to mitigate recirculation and discharge of nutrients produced by fish; mainly phosphorus and nitrogen. When discharged, nutrients can contribute to eutrophication in surrounding water bodies, harming the fish and other aquatic life. Therefore, RAS effluent should be treated before discharge. One method of phosphorus removal is adsorption, a surface phenomenon that is often used to bind dissolved pollutants to a solid-phase medium and remove them from water. Nitrogen is present in RAS as ammonia, which is toxic to fish even at concentrations as low as 0.05 mg L^{-1}. Therefore, the ammonia is transformed to nitrogen's non-toxic form, nitrate, before recirculation or discharge, by a process called nitrification. Both adsorption and nitrification can be affected by RAS process parameters such as salinity. Many anadromous fish such as Atlantic salmon require a change in salinity over their lifetimes; therefore, both of these processes should be investigated for their response to salinity changes.

An adsorption study was performed on an aluminum oxide-based material, RhizoSorb®, to assess its response to different RAS variables. It was found that both film diffusion and intraparticle diffusion are

rate-controlling steps in the adsorption process, and the removal efficiencies in batch tests were affected by time, salinity, and phosphate concentration. The Freundlich isotherm fit the equilibrium data better than the Langmuir isotherm, showing that adsorption is a multi-layer process and that the adsorbent is highly heterogeneous. The Clark model was better suited than the Thomas model for predicting the performance of the RhizoSorb® in a flow-through system. The results of this study showed that RhizoSorb® and other alumina-based sorbents have high potential towards application to the RAS water treatment process to remove and recover phosphorus.

A second study was performed to assess the effects of salinity changes on nitrifying biofilters. Acclimation to a small amount of salinity before transition to a higher salinity may help biofilters recover from the larger shift; therefore, a series of experiments was performed on both freshwater and brackish (3 parts per thousand (ppt)) biofilters to assess their respective levels of recovery after an abrupt change in salinity (3, 20, and 33 ppt). Tests were run for a two-week period in which the nitrification rates were monitored. The freshwater biofilters quickly recovered from a shift to 3 ppt, but did not recover from a shift to 20 or 33 ppt. The brackish filters started to recover at the end of the two-week test, but did not recover from a shift to 33 ppt. DNA sequencing of the variable V4 region of the 16s rRNA gene showed that the heterotrophic communities in the biofilm were lysed at a greater proportion than the nitrifiers, though the nitrifiers were inactivated in higher salinities. A longer series of tests could fully characterize the effects of acclimation to salinity with the effects of ammonia concentration and organic matter, which could help to fully understand the microbial community dynamics.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1. Recirculating aquaculture systems

As the world's population grows, the need for sustainable food sources grows as well. Current food production systems around the world are unsustainable due to the amount of water and energy used during production, packaging, and transportation of the goods. Traditional agriculture, which is operated on land to produce plant- and animal-based products for consumption, is ubiquitously inefficient across the globe. Not only does agriculture take up over a third of the land space on the planet, but it also accounts for 70% of the world's freshwater use (FAO, 2020). Furthermore, much of this water is not conserved within the system. Water that passes through agriculture systems often becomes contaminated with nutrients, pesticides, and pathogens; thus, agricultural runoff can heavily pollute the surrounding environment. Many of these compounds and microorganisms are toxic to humans and animals, and may additionally pollute the ecosystems in which they are discharged. Therefore, we must find new methods of farming that both conserve water and prevent harmful substances from entering the environment.

One method of farming that has become increasingly popular in the last several decades is aquaculture, due to its potential to provide a high value protein source (FAO, 2020). In 2017, around 425 different species were raised in aquaculture, including finfish, shellfish, and seaweeds (Naylor *et al.,* 2020). Aquaculture can also be a good option for sustainably mitigating food scarcity. Fish are a major source of protein, micronutrients, and fatty acids (Pradeepkiran, 2019), which make good supplements to a malnourished diet. Not only does aquaculture provide a food source, but it also provides a source of income and creates jobs for fish farmers. Over 20.5 million people were employed in aquaculture ventures

in 2018 (FAO, 2020), a number that has steadily increased since the 1990s. Figure 1.1 shows the trends of capture fishing and aquaculture from the last 50 years.

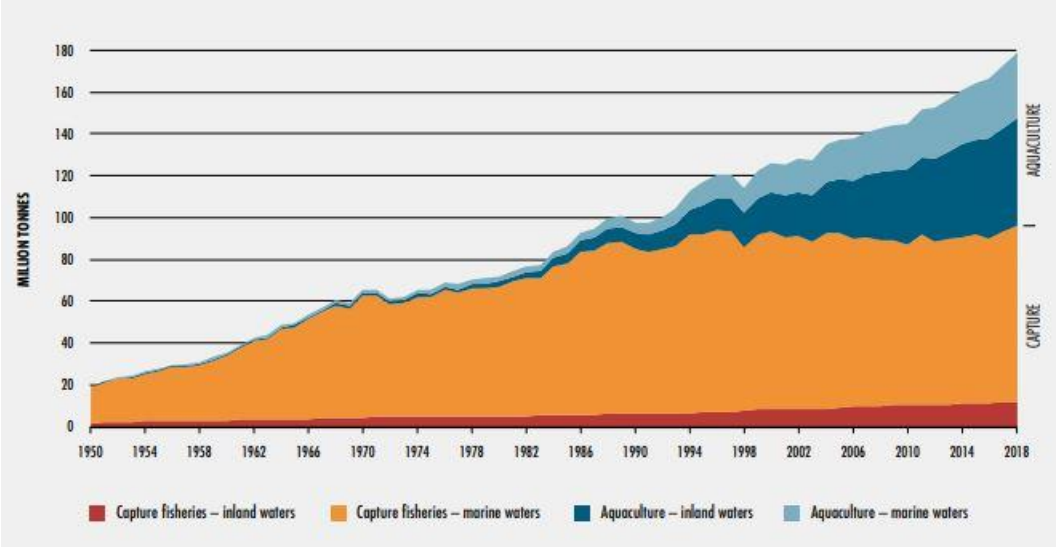


Figure 1.1. Fishing and aquaculture trends since 1950 (FAO, 2020).

Traditionally, land-based aquaculture systems have been operated as flow-through systems, where the water is run through a series of ponds and discharged. However, there are disadvantages of this method, several of which include: excessive water usage, water pollution from effluent wastewater, and the disturbance of natural ecosystems (Boyd, 2003). Recirculating aquaculture systems (RAS) mitigate some of these issues, as the water is treated and then circulated back into the system. This keeps the fish isolated from the surrounding environment, preventing fish from escaping and interfering with the surrounding ecosystem, and eliminates much of the fresh water needed to run the system. Furthermore, RAS allow for optimal fish rearing parameters regardless of location and outside environment. A RAS typically has a water exchange rate of 10% or less of the total system water volume per day (Davison, 2019). Because the majority of the water is recirculated rather than discharged, it must be treated to maintain a level of quality in which the fish will thrive (van Rijn, 2013). Due to the low water exchange

rate and high fish stocking densities, RASs can produce wastewater that is 10 to 100 times more concentrated than traditional aquaculture systems (Martins *et al.,* 2010). Therefore, the portion of the water discharged should be treated to remove constituents that can harm the surrounding environment. In particular, nutrients such as phosphorus and nitrogen should be removed before discharge or recirculation, as high nutrient concentrations can affect the health of the fish and contribute to eutrophication in surrounding water bodies.

Fish need feed with high levels of proteins and nutrients, which are then converted to energy, fish biomass, and waste products. Phosphorus is an essential nutrient; therefore, fish need supplemental phosphorus in the feed as it is not present at high levels in plant-based feed materials. Any indigestible phosphate will be excreted, which can harm the surrounding environment through eutrophication. Therefore, phosphorus should be adequately removed from the effluent before discharge. Nitrogen is excreted when fish consume proteins, typically via the gills in the form of ammonia (Lazzari and Baldisserotto, 2008). Ammonia can be toxic to fish at moderate levels, so it is imperative that the ammonia-nitrogen be converted to nitrogen's non-toxic form, nitrate, through a process called nitrification, before the RAS water is recirculated or discharged. This thesis will discuss both phosphorus removal as well as ammonia conversion from RAS water.

1.2. Nutrients and eutrophication

In the environment, certain elements, also called limiting substrates, hinder the growth of different organisms due to lack of abundance. Most of the time, the factor that limits algal growth is a nutrient such as phosphorus or nitrogen. Phosphorus often limits growth because it is present at fairly low quantities in natural ecosystems (Yang *et al.,* 2008). When the limiting nutrient is added, algae begin to grow exponentially, essentially taking over an ecosystem. These algae blooms can be harmful to other

organisms in the ecosystem, as blooms cover the surface of the water and block light from reaching deeper waters where other aquatic plants grow. Furthermore, as algae die and begin to decay, the decomposing biomass consumes the oxygen in the water, which can cause hypoxic zones (areas with little to no dissolved oxygen). Hypoxic zones kill fish and other plants, effectively destroying an ecosystem (BoQiang *et al.,* 2012). This phenomenon is called eutrophication.

Once a water body becomes eutrophic, it is very difficult to reverse (Carpenter, 2005). There are several different types of eutrophication: natural and cultural eutrophication. Natural eutrophication occurs when the area around a water body naturally contains high levels of nutrients, and this is the result of natural weathering, erosion, and sedimentation processes that occur over hundreds of years. As particles and associated nutrients are washed into water bodies over time, more and more algae are able to grow, and the particles build up and make the water body shallower. This results in a higher concentration of nutrients in the remaining water, which spurs further productivity in the water body. These natural ecological scenarios are very hard to alter without drastic land change; for example, the soil underneath a lake may be high in phosphorus concentration due to years of sedimentation from the surrounding area, and it may be impossible or impractical to alter the soil state to remove the phosphorus. Therefore, natural eutrophication will often persist.

Cultural eutrophication is slightly different in that it is typically a result of point- or non-point source pollution from human activities, such as wastewater treatment or agriculture, as discussed above. Wastewater treatment plant effluent can increase the discharge of nutrients directly into a water body, while agriculture increases the overall concentration of nutrients in a given area through runoff. Though humans can do little to mitigate natural eutrophication, cultural eutrophication can be mitigated by decreasing the concentrations of nutrients that we release into the environment. In particular, growing

industries such as RAS that produce nutrients should make nutrient removal an important factor in their system operation.

1.3. Phosphorus removal and recovery from RAS

In addition to the prevention of eutrophication by excess nutrient loading to the receiving environment, an advantage to removing phosphorus from RAS effluent is the potential for phosphorus recovery and reuse. Phosphorus is an essential nutrient for the fertilization of crops, though it is a non-renewable resource. Most of the phosphorus used in agriculture is mined and combined with other minerals to form fertilizer; however, this is not an efficient process, as only about 25% of the phosphorus applied to crops is taken up by the plants (Bhattacharya, 2019). Therefore, much of the world's mined phosphorus runs off agricultural fields, after which it is washed into surface water bodies or leached through the soil into the groundwater. When it ends up in lakes, estuaries, or other shallow water bodies, it can lead to eutrophication; furthermore, it can be washed into the deep ocean with soil particles, an effective loss of a valuable nutrient.

As a mineral, non-renewable resource, readily accessible phosphorus supplies will eventually be depleted. Early estimates of the total amount of phosphorus on the planet suggested that we would face a shortage within 50 to 100 years (Cordell *et al.,* 2009); however, others argue that the total amount of P in the world may sustain our society for up to 600 years (Alewell *et al.,* 2020). Practical limits on the use of different P sources will depend on their locations and the economic, social, and environmental effects that come with harvesting (Alewell *et al.,* 2020). To prolong its availability and reduce eutrophication, the phosphorus already in circulation in agriculture and other industries should be reused. Recovering the wasted phosphorus from RAS water can provide a supplement to agriculture and potentially provide a

source of income for the RAS facilities involved. A promising method of harvesting phosphorus from water for reuse is through adsorption.

1.3.1. Adsorption of phosphorus

Adsorption is a process where a chemical substance, called the adsorbate, adheres to the surface of a solid, called the adsorbent, or sorbent. Materials with small sizes (in the millimeter range or smaller) generally make good adsorbents, as small size is often correlated with high surface area, which provides more opportunities for pollutant binding. Porous materials may also make good adsorbents, as a well-developed pore structure greatly increases surface area (Yang *et al.,* 2017). For example, the high surface area of activated carbon is a result of an extensive pore distribution; this along with its low cost and broad availability make activated carbon the preferred adsorbent for many substances (Li *et al.,* 2018). However, many other cost-effective materials may be used as adsorbents. Both organic and metal compounds are often considered for their phosphate adsorption capacity. Organic compounds have advantages because they are often produced as solid waste; therefore, there is a large opportunity to reuse them to supplement the wastewater treatment process (Xu *et al.,* 2009). Metal compounds have advantages in ensuring selectivity of a pollutant as well as the potential to be combined with activated carbon or other metals. Once the pollutant is adsorbed to the adsorbent, the adsorbent can be removed from water. However, many metal adsorbents such as aluminum, iron oxides, dolomite, zeolite, or others are often powdered, which makes them difficult to remove from water. Thus, they can sink to the bottom of water bodies, causing secondary pollution (Liu *et al.,* 2013). Powdered adsorbents often require a binder to assist in the removal process. These binders form larger beads that can be much more easily removed from suspension (Jung *et al.,* 2017); however, an appropriate binder should be chosen to avoid significantly decreasing the available surface area on the adsorbent (Argalis *et al.,* 2021). For some metal compounds, magnetic removal is an effective separation option.

Many different adsorbents have been investigated for their phosphate adsorption capacity. As phosphate (PO_4^{3-}) is a negatively charged trivalent anion, the best metal compounds for pulling the phosphate from solution should be cations, cationic polymers, or ion exchange resins with an equal or greater positive charge. Some metals that are commonly used for adsorption (or are added to activated carbon to supplement the adsorption process) are iron (Fe^{3+}), zirconium (Zr^{4+}), lanthanum (La^{3+}), aluminum (Al^{3+}), zinc (Zn^{2+}), and others. Aluminum oxide, in particular, has been used for wastewater treatment for many years, and has shown promise for the recovery of phosphorus.

Adsorption efficiency and capacity are greatly affected by water chemistry, such as pH, alkalinity, and coexisting ions. The effect of coexisting ions on an adsorbent is particularly relevant to the aquaculture industry because many systems use seawater for some (if not all) of their fish rearing, depending on the species raised. Common seawater contains chloride (Cl^-), sodium (Na^{2+}), sulfate (SO_4^{2-}), magnesium (Mg^{2+}), calcium (Ca^{2+}), and potassium (K^+), and bicarbonate (HCO_3^-) (Millero, 2008). Many other ions are present in seawater, but these make up the majority. Therefore, when investigating an adsorbent for use in RAS, the effect of coexisting ions should be investigated. The second chapter of this thesis presents a study on an aluminum oxide-based adsorbent material, RhizoSorb®, for its applicability for phosphorus recovery from RAS water.

1.3.2. Application in agriculture

A major advantage of using adsorption for the recovery of phosphorus is the potential to use the nutrient-saturated adsorbent as a controlled-release (also called slow-release) fertilizer (CRF). When nutrients are added once and slowly released rather than added in repeated feedings, less overall nutrients will be lost to runoff. Traditional slow-release fertilizers are typically a bundle of nutrients, including nitrogen, phosphorus, and potassium, held together with a thin thermoplastic resin coating,

made from polyolefin, polyvinylidene chloride or other polymers (Lawrencia *et al.,* 2021). The coating breaks down over time, releasing the nutrients. Unfortunately, in some cases, the coatings break down into microplastics, which are becoming an increasingly problematic issue due to the potential for animal ingestion and adsorption of harmful pollutants (Costigan *et al.,* 2022). Therefore, using metal-based adsorbents for slow-release fertilizers is an attractive alternative. Adsorbents as slow-release fertilizers work through the desorption of nutrients over time. Desorption is the opposite of adsorption, and occurs when the adsorbate moves from the adsorbent surface back into the bulk liquid; in agriculture, this substrate is the soil porewater, which will be depleted in phosphorus as it is taken up by plants, prompting further desorption. Investigation of desorption parameters of the RhizoSorb® was not within the scope of this study, though such an investigation may be important for characterizing the material for use in agriculture.

1.4. Ammonia conversion to nitrate

1.4.1. Nitrogen cycle

Nitrogen is another important nutrient for life, and is the most abundant component of most fertilizers. Farming methods are notoriously inefficient with nitrogen usage, as only about 30 to 50% of the nitrogen added to crops is actually taken up by the plants. This can be due to improper amounts of fertilizer, low plant stocking, or poor application methods (Anas *et al.,* 2020). The remaining 50 to 70% of the nitrogen is wasted and can be washed into the surrounding environment in runoff, contributing to eutrophication.

Nitrogen exists in several stable inorganic forms, the most abundant of which are nitrogen gas ($N_{2(g)}$), ammonium (NH_4^+), and nitrate (NO_3^-). Atmospheric nitrogen, N_2, makes up 78% of the atmosphere, but is not a form that is useful to most organisms. Ammonium and nitrate, as well as organic nitrogen are

the forms taken up and used by most organisms (Takai, 2019). Forms of organic nitrogen include amino acids, proteins, urea, nucleic acids and other compounds that are produced by living organisms. The nitrogen cycle (Figure 1.2) involves the conversion between different forms of nitrogen. When air comes into contact with water, a portion of the nitrogen gas in the air dissolves into the water. Aqueous nitrogen can be "fixed" by a relatively small group of nitrogen-fixing microbes, which can convert N_2 to ammonia at great metabolic cost. The ammonia produced can then be incorporated into amino acids and other forms of organic nitrogen. As these compounds break down, in a process called ammonification, ammonium is released. Once ammonium becomes available, certain microbes may perform another step in the cycle, nitrification, which is a two-step process that first oxidizes ammonium to nitrite ($NH_4^+ \rightarrow NO_2$), before oxidizing nitrite to nitrate ($NO_2^- \rightarrow NO_3^-$). This conversion is performed in an aerobic environment.

The microbes that perform this process are typically categorized into two groups, ammonia oxidizers and nitrite oxidizers. There are three common distinct groups of autotrophic ammonia oxidizers, ammonia oxidizing bacteria (AOB), ammonia oxidizing archaea (AOA), and comammox bacteria (complete oxidation of ammonia to nitrate) (Lehtovirta-Morley, 2018). AOB were thought to be the sole source of ammonia oxidation until AOA were discovered, and even more recently, when comammox were discovered. There are several genera of ammonia oxidizing bacteria that are commonly found in wastewater treatment, including *Nitrosomonas, Nitrosospira, and Nitrosococcus*, while some less commonly found genera include *Nitrosolobus* and *Nitrosovibrio* (Samocha and Prangnell, 2019). *Nitrosomonas* are often found in traditional wastewater treatment, while *Nitrosospira* typically have higher abundance in soils (Lehtovirta-Morley, 2018). *Nitrosococcus* can be found in wastewater treatment, but it has shown to be better suited for saltwater than freshwater (Fumasoli *et al.,* 2017). *Nitrosolobus* and *Nitrosovibrio* are very similar to *Nitrosococcus*; therefore, some studies have described

them as indistinct from *Nitrosococcus* (Robertson and Groffman, 2015). Ammonia oxidizing archaea contain the genera *Nitrososphaera, Nitrosocosmicus, Nitrosocaldus, Nitrosotalea,* and *Nitrosopumilus*. Many of these AOA thrive in low-ammonia concentrations (Lehtovirta-Morley, 2018), and they are commonly found in soils, hot springs, soils, and wastewater treatment plants. Comammox bacteria is made up of certain types of the genus *Nitrospira,* a microbe traditionally believed to only be capable of nitrite oxidation. However, commamox *Nitrospira* were discovered in 2015 (van Kessel *et al.,* 2015). These bacteria have been found in water and wastewater treatment plants, sand filters, aquaculture ponds, and nitrifying biofilters, though they may be the most abundant in water treatment plants (Maddela *et al.,* 2021). Unfortunately, it can be difficult to distinguish comammox *Nitrospira* from nitrite-oxidizing *Nitrospira* with traditional sequencing methods.

Nitrite oxidizing bacteria (NOB), along with anammox bacteria (anaerobic ammonium oxidation), make up the two categories of nitrite oxidizers. There are no known nitrite oxidizing archaea. The major genera of NOB are *Nitrospira, Nitrobacter, Nitrococcus,* and *Nitrospina* (Samocha and Prangnell, 2019). Up until the 2000s, *Nitrobacter* were thought to be the most abundant NOB in most wastewater treatment systems, though studies have indicated that NOB *Nitrospira* may be the most abundant (Mehrani *et al.,* 2020). *Nitrococcus* can be found in saltwater (Fussel *et al.,* 2017), while *Nitrospina* has been found in both domestic wastewater and saltwater (Huang *et al.,* 2021; Lucker *et al.,* 2013). Anammox bacteria, which oxidize nitrite in the absence of oxygen, can oxidize high concentrations of nitrite and may have potential to decrease costs of the wastewater treatment process, as they do not require aeration (Weralupitiya *et al.,*2021). However, these bacteria are a fairly new discovery; thus, more research should be performed to fully understand their behavior.

The end product of nitrification is nitrate. The next step that returns the nitrogen to N_2 is denitrification, a process performed by microbes in an anaerobic environment. The nitrate is converted

back to nitrite ($NO_3^- \rightarrow NO_2^-$), nitrite is converted to nitric oxide ($NO_2^- \rightarrow NO$), nitric oxide is converted to nitrous oxide ($NO \rightarrow N_2O$), which is then converted to nitrogen gas ($N_2O \rightarrow N_2$). Here, the cycle can begin anew. Denitrification is not within the scope of this thesis, so the specific microbes that perform this process will not be discussed.

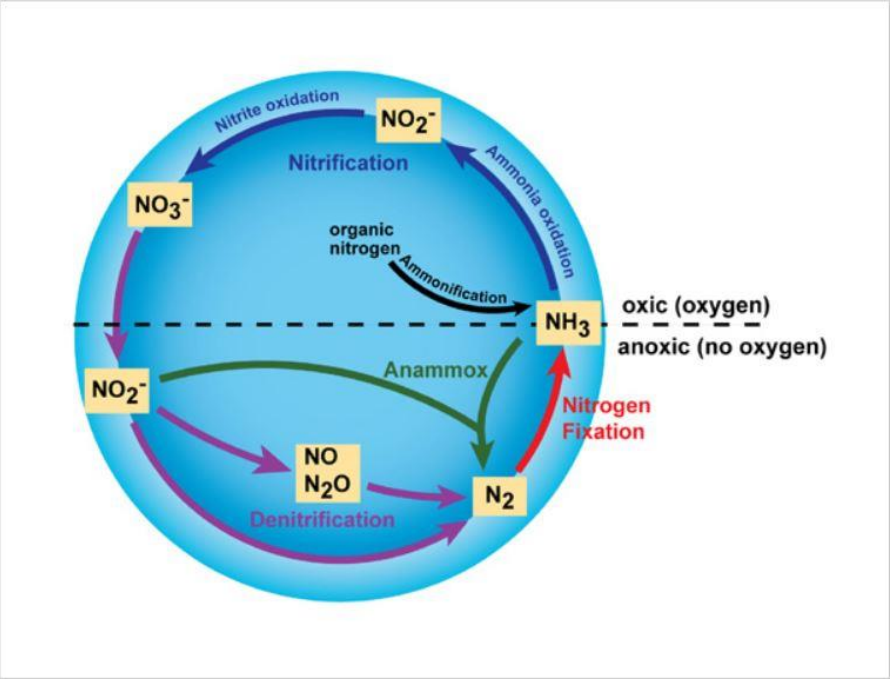


Figure 1.2. Simplified nitrogen cycle (Bernhard, 2010).

1.4.2. Nitrogen in RAS

Nitrogen enters RAS systems in fish food, which is then converted to tissue (Pucher and Focken, 2017), or is excreted in feces and via the gills in the form of ammonia. Ammonia must then be converted to nitrate, the less-toxic form of nitrogen, to avoid causing harm to the fish in RAS. The nitrification process is usually performed by nitrifying biofilms suspended on a media through which the water can pass. This media can be made from plastic, fiberglass, ceramic, quartz, or other porous materials that have high

surface areas to facilitate biofilm growth (Losordo and Delong, 2015). The water is then typically recirculated or discharged.

Denitrification is often not performed in RAS due to the cost of implementing specific denitrification reactors as well as the lack of incentive to remove nitrate, a non-toxic chemical (Suhr *et al.,* 2013). Therefore, if denitrification is not implemented, there may be other opportunities for nitrogen removal, such as recovery. Nitrogen recovery is not typically performed because it is a very abundant nutrient and the benefits of recovery may not outweigh the costs. Ammonia, in particular, is readily abundant (Minnesota Department of Agriculture, n.d.), but it is difficult to remove from water as it is a soluble, monovalent ion. However, recovery of nitrogen from RAS would help to prevent environmental issues associated with discharge. Recovering N-rich aquaculture wastewater could have sustainable applications in aquaponics, as aquatic plants also require a source of nutrients (Gebauer *et al.,* 2022). This could be a practical method of nitrogen recovery that would reduce waste from the RAS while also benefitting the hydroponics system.

There are other interesting options for nitrate recovery in RAS that may not have been extensively investigated. One particular option is the application of microalgae, which can supplement the feed of other organisms such as oysters (Lu *et al.,* 2020). Microalgae are not commonly used in RAS water treatment because algae require high levels of nitrogen, lots of sunlight (requiring more energy), and are very fast growing, and many RAS effluents are not high enough in nitrogen concentration to adequately feed the microalgae (Lu *et al.,* 2020). Additionally, microalgae often adjust the pH of the effluent water, making it too alkaline for recirculation. However, there are options that may help to make algae a more viable option for nitrogen recovery, such as photobioreactors that evaporate the water and increase the N concentration. These and other such options require more research to become viable and to increase their benefit to the RAS industry.

1.4.3. Nitrification and contributing factors

All five categories of nitrifying bacteria, AOB, AOA, NOB, anammox, and comammox bacteria, have been found in RAS biofilters (Hupeden *et al.,* 2020; Skoyles *et al.,* 2020). Nitrifiers need a specific set of conditions to thrive, and these conditions will influence the microbial communities in different types of biofilters.

The first major factor that affects the growth and efficiency of biofilter media is the total ammonia-nitrogen concentrations (TAN). The dominant form of ammonia/ammonium in the water is highly affected by pH, as shown in Figure 1.3. For the most part, biofilters are better equipped to oxidize ammonia than ammonium, so the pH of the RAS water should be kept at a minimum of 7.0 for biofilter efficiency (Bregenballe, 2015). This is also the ideal pH level for most fish raised in RAS (Pattillo, 2014).

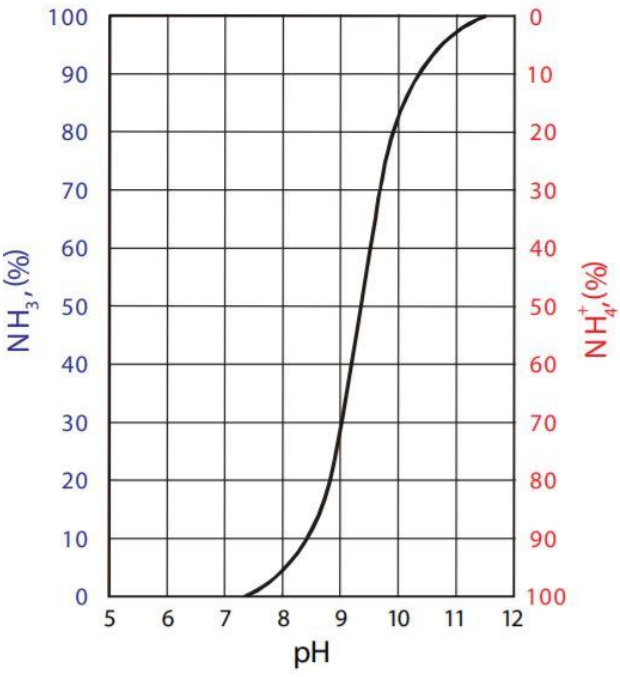


Figure 1.3. Equilibrium of ammonia and ammonium as a function of pH (Bregenballe, 2015).

Determining the typical input concentrations of TAN to an average biofilter is difficult, as the levels of nitrogen in the system depend on the size of the RAS as well as the number of fish raised in the system. Often, TAN levels in the water widely vary over time, tending to spike when the fish in the system are fed. However, studies have mostly reported levels of 1 to 5 mg L^{-1} d^{-1}, though the concentrations of some spanned from 0.10 to 10 mg L^{-1} d^{-1} (Gao *et al.*, 2020; Navada *et al.*, 2019; Kinyage *et al.*, 2019).

Carbon is another important element for successful nitrification. Both inorganic carbon and organic carbon are important factors to consider. At typical pH levels, inorganic carbon in RAS systems is in the form of HCO_3^- with some CO_2/H_2CO_3 based on carbonate speciation (Al-Rawajfeh and Al-Amaireh, 2009). This carbon provides the system with alkalinity, which is the acid-buffering capacity of a system (or its ability to resist changes in pH). The flux of ammonia entering the system as well as the consumption of ammonia and nitrite (both acids) means that there are constantly changing levels of acidic compounds in the water; therefore, the system needs to be able to resist the changes in pH that come along with these fluxes. For every gram of ammonia-nitrogen that is converted to nitrate-nitrogen, 7.05 g of alkalinity as $CaCO_3$ is required to maintain the pH in the system (Timmons *et al.,* 2018).

Organic carbon enters the RAS system in the fish feed; some is taken up by the fish to produce biomass while some may be excreted. Both the fish feed and feces that come into contact with the water result in concentrations of dissolved organic matter (DOM). DOM supplies heterotrophs in the biofilms with an energy source, and these heterotrophs coexist with the nitrifying bacteria and help with biofilm structure by producing exopolysaccharide (EPS). However, a C:N ratio that is too high can cause the heterotrophs to dominate and inhibit nitrification (Navada *et al*., 2020). Therefore, it is important that the water going to the nitrification step be low in feed, feces and other sources of organic matter. It is also important to mention that throughout the nitrification process, the microbes need an adequate supply of dissolved oxygen (DO), so the biofilters must be aerated. Typical levels of DO in RAS are about 6.0 to 8.0

mg L^{-1} to ensure the health of the fish (Gao *et al.*, 2020). As the microbes oxidize the ammonia and convert it to nitrite and then nitrate, biomass is produced and eventually builds up and sloughs off the biofilters. This biomass can either be composted or landfilled with the rest of the solid waste in the system.

Nitrifying bacteria are particular about their environment; therefore, they are sensitive to most environmental changes, including salinity. Nitrifying biofilters can grow in any salinity up to full strength seawater (Hupeden *et al.,* 2020), but changing salinities are often difficult for the microbes to handle (Kinyage *et al.,* 2019), an issue exacerbated by the slow growth rate of the bacteria. The nitrifying microbial community is often made up of a combination of halotolerant, halophilic, and stenohaline species. Halophilic organisms require salinity to survive, while halotolerant do not, though they do have the ability to grow under saline conditions. Stenohaline organisms, the group that most freshwater organisms belong to, can only tolerate a narrow range of low salinities (Anton, 2011). In some stenohaline microbes, external osmotic pressure can cause water influx or efflux, leading to cell swelling, dehydration, or lysis (Wood, 2015). It is likely that some of the species present in the biofilters will experience these adverse effects when stressed with changing salinity, depleting the population and leaving the more halotolerant types of bacteria. Additionally, it has been shown that osmotic stress preparation can help to adapt freshwater biofilters to salinity (Navada *et al.,* 2020). The third chapter of this thesis presents a study on two different types of biofilters: freshwater and brackish biofilters, to assess their response to abrupt salinity shifts.

1.5. Overview of the thesis

This thesis presents two separate projects in the field of recirculating aquaculture, one that investigates phosphorus removal and recovery, and one that investigates ammonia conversion to nitrate. Both of these projects can help to further the field of RAS. In particular, both studies delve into the effects

of salinity on their respective nutrient mitigation techniques, as many anadromous fish, such as Atlantic salmon, experience a change in salinity over the course of their lifetimes. Therefore, RAS should be fully prepared for any changes in system operation that may come with shifts in salinity. The purpose of this work is twofold: first, the work will help to optimize several RAS processes that handle nutrients so that the industry can make informed decisions about process design. Second, this work helps to give fundamental scientific insights into these RAS processes, and can help to determine future work that may be required in this area.

The first study, outlined in Chapter 2, investigates a phosphorus adsorbent, RhizoSorb®, for its applicability as a method of phosphorus recovery from RAS. The study uses batch kinetic, batch equilibrium, and up-flow column tests along with corresponding models to determine the effects of salinity and other factors on the adsorption efficiency of the sorbent. The second study, outlined in Chapter 3, characterizes the microbial community and nitrogen concentrations in two types of nitrifying biofilters, freshwater and brackish. The tests are used to determine whether brackish biofilters are better equipped to handle shifts in salinity than freshwater biofilters. Finally, Chapter 4 reiterates and discusses the major conclusions made from this work, and outlines areas for potential future research.

CHAPTER 2

PHOSPHORUS RECOVERY FROM RECIRCULATING AQUACULTURE SYSTEMS: ADSORPTION KINETICS AND MECHANISM

2.1. Abstract

Recirculating aquaculture systems (RAS) have grown in popularity in recent years due to their potential to provide a high-quality protein source in a contained environment. With increased production comes the need for RAS wastewater treatment to remove waste products such as phosphorus, which can harm other aquatic life in the area by causing algae blooms. Additionally, there is potential to harvest the wasted phosphorus for use as a fertilizer and to combat nutrient scarcity. This study investigates a phosphorus adsorbent, RhizoSorb® (base material of aluminum oxide), for its applicability in RAS use under fresh water, simulated seawater, and real RAS water conditions. Film diffusion and intraparticle diffusion were both rate-controlling steps in the adsorption process, and the good fit of the Elovich model indicated that chemisorption is likely the dominating adsorption mechanism. Phosphate removal decreased with increasing salinity in all tests, which can be attributed to both anion competition and increasing pH. The Freundlich isotherm fit the equilibrium data slightly better than the Langmuir isotherm, possibly indicating that adsorption is a multi-layer process (the ratio of adsorbate to binding space is higher than 1:1) and that the adsorbent has a high number of binding sites available. The Clark model was better suited than the Thomas model for predicting the performance of the adsorbent in a flow-through system. The adsorbent showed high removal efficiencies and good selectivity for phosphate in all tests.

2.2. Introduction

As the world's population grows, the need for sustainable food sources increases as well. Aquaculture systems have grown in popularity in recent years due to their potential to provide a

sustainable protein source (FAO, 2020). Recirculating aquaculture systems (RAS) mitigate many issues posed by traditional systems, such as excessive water usage, pollution, and disturbance of natural ecosystems, as they are operated in tanks on land. This keeps the fish isolated from the surrounding environment and eliminates much of the fresh water needed to run the system; a RAS typically has a water exchange rate of 10% or less of the total system water volume per day (Davison, 2019). Due to the low water exchange rate and high fish stocking densities, RAS can produce wastewater that is 10 to 100 times more concentrated than traditional aquaculture systems (Martins *et al.,* 2010). Therefore, the portion of the water discharged should be treated to remove constituents that can cause harm to the surrounding environment. In particular, the phosphorus excreted by fish should be removed to prevent eutrophication in nearby water bodies. This phosphorus may be harvested from the water to supplement the world's current supply, which comes from mining and is mostly used for agricultural purposes (Aketo *et al.,* 2021; Daneshgar *et al.,* 2018). Mining is a nonrenewable method of obtaining phosphorus which will eventually deplete the Earth's available stores (Cordell *et al.,* 2009). As phosphorus is an important component of all living organisms, it is imperative to find ways to recover and reuse phosphorus to make the P cycle more sustainable. The recovery of phosphorus can not only benefit agriculture, but also reduce waste emissions from RAS at a potentially lower cost, due to the market for the spent adsorbent.

Phosphorus can be harvested from wastewater using several different methods; the most commonly used are biological uptake, chemical precipitation, and adsorption methods. Biological methods often struggle to remove an adequate amount of phosphate due to microbial uptake limitations (Jung *et al.,* 2017). Chemical methods work well to remove phosphorus from water, but some restrict actual recovery of the phosphorus due to the high costs and environmental impacts associated with the added chemicals (Pratt *et al.,* 2012). Adsorption is an attractive solution for phosphorus removal due to its simplicity and potential for beneficial use of the spent adsorbent. Many studies have demonstrated

that phosphorus can be cost-effectively removed from domestic wastewater by adsorption (Zhang *et al.,* 2021a; Dong *et al.,* 2020; Nguyen *et al.,* 2015; Li *et al.,* 2018; Liu *et al.,* 2015; Sun *et al.,* 2014; Khamidun *et al.,* 2017; Fritzen and Benetti, 2021). Therefore, adsorption methods have high potential to serve the aquaculture industry, resulting in environmental as well as financial benefits for the businesses involved.

The company Phospholutions has developed an adsorbent material, RhizoSorb®, which they pre-load with phosphorus for use as a controlled-release fertilizer. The active ingredient is aluminum oxide, variations of which have been used in water and wastewater treatment for many years. There is an opportunity to load this material with waste phosphorus from RAS where the effluent P concentration may be high. Thus, the purpose of this study is to assess whether RhizoSorb® can be used as a phosphorus recovery method for RAS wastewater. This can help contribute to sustainability and reduce potential harm associated with other commercial controlled-release fertilizers which are often coated in plastic polymers (Lawrencia *et al.,* 2021). Additionally, there are relatively few studies that investigate adsorbents for their potential for phosphorus recovery from RAS (Martins *et al.,* 2017), which have different constituents than domestic wastewater. Therefore, this work is necessary to further develop the sustainability of RAS water treatment.

Atlantic salmon are one of the most commonly grown species in RAS due to their low cost and nutritional benefits (Colombo and Mazal, 2020). These and other anadromous fish require different levels of salinity over their lifetimes, as they start their life cycle in freshwater and transition to saltwater as they reach adulthood (Global Salmon Initiative, 2017). Differing levels of salinity may affect the adsorption process; additionally, RAS wastewater will contain feed, excreta and other waste constituents, which may also affect the adsorption of phosphorus. Tests were performed to determine how phosphate adsorption was influenced by salinity as well as fish wastewater constituents.

2.3. Materials and Methods

RhizoSorb® was obtained from Phospholutions. Before use, the adsorbent was sieved with a Fisher Scientific 125 µm sieve to obtain a lower bound of particle size and remove fines. All labware was acid-washed in a 10% HCl bath and rinsed in a deionized (DI) water bath before use. Sea salt from Instant Ocean® was used to add salinity where necessary, the composition of which is as follows: 47.5% Cl^-, 26.3% Na^+, 6.6% SO_4^{2-}, and 3.2% Mg^{2+}, while the other >17% is composed of other ions and water (Christy and Dickman, 2002). Bicarbonate (HCO_3^-) is also present at 0.49% of the total weight. The 500 mg PO_4 L^{-1} stock solution of phosphate was prepared by dissolving KH_2PO_4 (Fisher Scientific) in deionized water, from which standards were prepared. RAS wastewater was obtained from the National Cold Water Marine Aquaculture Center (NCWMAC) located in Franklin, Maine, from three tanks of 0, 7, and 33 ppt salinity. All samples except the RAS wastewater samples were made up with DI water.

Samples of phosphate were prepared by adding stock solution of PO_4 to a flask and making up the total volume to 15 mL with deionized water or salt water to a specified concentration and salinity level. Phosphate concentrations of 5, 10, and 20 mg L^{-1} were used for the kinetic tests, while concentrations of 20 mg L^{-1} were used for the equilibrium tests. Masses of adsorbent from 5 mg to 20 mg were added to the tubes, and the samples were placed on a tube rotator and vigorously mixed at 20 rpm for a specified amount of time. For the kinetic tests, samples were removed and tested at various times up to two weeks until an equilibrium concentration was observed. The equilibrium samples were left on the tube roller for the observed equilibrium time, determined during the kinetic tests to be approximately 14 days.

For the column tests, tubing was attached to either end of a 5 mL column fitted with cotton above and below the desired mass of adsorbent (1.5, 2.0, or 2.5 g). The inlet tubing was placed in the test phosphate solution and pumped through the sorption column in an up-flow mode using a Masterflex ® #7518-60 peristaltic pump at rates of 0.4, 1.0, or 1.5 mL min^{-1}. The flow rates were chosen to have

comparable contact times to other phosphate adsorption column experiments in the literature (values range from ~2.5 min to 30 min) (Fulazzaky *et al.,* 2014; Liang *et al.,* 2022; Hussein and Mayer, 2022). The outlet tubing emptied into a beaker. The columns were prepared by pumping 50 mL of deionized water or solution with appropriate salt concentration through the system prior to use to ensure that the adsorbent was properly wetted before beginning the breakthrough experiments with added phosphate.

All solutions were adjusted to the appropriate salinity level (0, 10 and 20 ppt) before testing. These salinities were chosen because many RAS attempt to raise salmon in brackish water rather than saline water due to the higher operating costs and additional challenges that come with fully saline RAS (Moran, 2010). Furthermore, salmon have been shown to grow well in lower salinities such as 12 and 22 ppt (Ytrestøyl *et al.,* 2015). In all tests, the phosphate concentration was tested using the colorimetric total orthophosphate method (EPA method 365.3) with a spectrophotometer set to 880 nm.

Additional kinetic tests were performed with real Atlantic salmon wastewater from NCWMAC from three tanks of 0, 7, and 33 ppt salinity, corresponding to different stages of growth for the Atlantic salmon. Tests were also performed with DI and added sea salt at each of these salinities to compare the effect of other constituents in the salmon wastewater. Each wastewater stock solution was tested for its initial phosphate concentration, then both the DI and the wastewater solutions were adjusted up to 10 mg PO_4 L^{-1} for testing.

2.4. Data Analysis and Modeling

Adsorption capacity is the amount of target compound that can be bound by the adsorbent material, usually reported as mg of adsorbate per g of adsorbent. The adsorption process is a series of four major steps: bulk diffusion (mixing of adsorbate throughout solution), film diffusion (movement of adsorbate through a boundary layer), intraparticle diffusion (movement of adsorbate through pores), and interaction with surface sites (adsorption) (Sahoo and Prelot, 2020). The first and last step are generally

not considered time-dependent steps during the design of kinetic systems because they are fast processes compared to the second and third steps (Kajjumba *et al.,* 2018). Therefore, the rate controlling step is often either film diffusion, intraparticle diffusion, or a combination of the two. The relative influences of each can be approximated by fitting the data to diffusion models. Whether the final step, interaction between the adsorbent and adsorbate, is due to physical adsorption or chemical adsorption depends on the structure of molecules of the adsorbent and sorbate (Lavrenko *et al.,* 2018). The dominating phenomenon can be investigated by fitting various models to the adsorption data.

The measure of the amount of adsorbate that has been removed from solution per amount of adsorbent (q_t) is given below in Equation 1.

$$q_t = \frac{C_0 - C_t}{m_s} V \qquad (1)$$

Where q_t is the amount of adsorbed phosphate per gram of adsorbent at time t (mg g^{-1}), C_0 is the initial concentration of adsorbate in solution (mg L^{-1}), C_t is the concentration of adsorbate in solution at time t (mg L^{-1}), m_s is mass of adsorbent (g), and V is the volume of solution of the sample (L). The adsorption capacity, q_{max}, is reached when all binding sites on the adsorbent are saturated with adsorbate.

The removal percentage for the batch kinetic and equilibrium tests can be quantified below.

$$\textit{Removal efficiency}\ (\%) = 100\frac{C_0 - C_f}{C_0} \qquad (2)$$

Where C_0 is the initial concentration of PO_4^{3-} (mg L^{-1}), and C_f is the concentration of PO_4^{3-} in solution at the end of the test (mg L^{-1}).

2.4.1. Adsorption kinetic models

The time required to reach equilibrium varies with different adsorbents and adsorbates, as the adsorption process can be controlled by several different factors. Many models have been derived to

explain kinetic adsorption behavior (Wang and Guo, 2020), but two were applied here: The Weber-Morris model and the Elovich model. The Weber-Morris model was chosen to show the relative contributions of film and intraparticle diffusion, while the Elovich was chosen to investigate whether the bond between the adsorbent and adsorbate is a physical or chemical bond.

2.4.1.1. Diffusion model

The Weber-Morris model describes the diffusion process, and is one of the most widely used models for investigating the rate controlling step (Wang and Guo, 2020).

$$q_t = k_{WM}\sqrt{t} + C \qquad (3)$$

Where k_{WM} (mg g^{-1} $min^{0.5}$) is the rate constant and *C* (mg/g) is a constant associated with the thickness of the boundary layer. If the constant *C* is equal to zero, meaning that a plot of $t^{0.5}$ versus q_t is a straight line that passes through (0, 0), intraparticle diffusion is the rate limiting step (Qiu *et al.,* 2009). If not, adsorption is controlled by multiple processes, and the plots will often show two distinct linear sections that represent film and intraparticle diffusion, respectively (Bizi, 2020; An *et al.,* 2020).

2.4.1.2. Adsorption reaction model

The Elovich model is given in Equation 5. This model was first developed to model the adsorption of gas onto solids through chemisorption, though more recently has been applied to the adsorption of pollutants from aqueous solutions (Qiu *et al.,* 2009).

$$\frac{dq}{dt} = \alpha e^{-\beta q_t} \qquad (4)$$

This model is an empirical model so it does not have distinct physical meanings, though it was developed with the assumption that the surface of the adsorbent is heterogeneous. Therefore, the Elovich model can indicate that the adsorbent is highly heterogeneous, meaning that the adsorbent possesses many

different types of binding sites with different binding energies (Kumar *et al.,* 2019). The Elovich model also indicates that chemisorption may be a dominating phenomenon (Riahi *et al.,* 2017).

2.4.2. Adsorption equilibrium models

2.4.2.1. Langmuir

The most common models used to fit phosphate adsorption data are the Langmuir and Freundlich isotherms. The Langmuir isotherm assumes that there is a 1:1 stoichiometry between the absorbate and the adsorbent binding sites, meaning that adsorption is a monolayer process (Chung *et al.,* 2015). The Langmuir model allows for the maximum adsorption capacity to be obtained from the data. The model followed by its linear form are given in Equations 5a and 5b.

$$q_t = \frac{q_{max} * K_L * C_e}{1 + K_L * C_e} \qquad (5a)$$

$$\frac{1}{q_t} = \frac{1}{K_L q_{max}} \left(\frac{1}{C_e}\right) + \frac{1}{q_{max}} \qquad (5b)$$

Where q_{max} is the adsorption capacity (mg g^{-1}), K_L is the Langmuir adsorption constant (L mg), and C_e is the equilibrium concentration (mg L^{-1}) (Langmuir, 1918).

2.4.2.2. Freundlich

The Freundlich isotherm is not restricted to a 1:1 stoichiometry between the absorbate and the adsorbent. This model does not allow for the maximum adsorption capacity to be calculated from the data, so this parameter is calculated experimentally by observing the kinetic adsorption data. The model and linear form are given in Equations 6a and 6b.

$$q_t = K_F * C_e^{\frac{1}{n}} \qquad (6a)$$

$$\log(q_t) = \log(K_F) + \frac{1}{n}\log(C_e) \qquad (6b)$$

Where K_F is the Freundlich adsorption constant ([mg g^{-1}][L mg^{-1}]$^{-1/n}$), and *1/n* is the unitless Freundlich adsorption intensity parameter (Freundlich, 1906). The adsorption intensity parameter is an indicator of the heterogeneity of the surface of the adsorbent; a value of *n* greater than 1 means that the sorbent is more heterogeneous (Lesmana *et al.,* 2009).

2.4.3. Breakthrough curve models

Most aquaculture wastewater treatment systems are operated as flow through systems; therefore, it is important to evaluate the performance of the adsorbent in a column system. This was done by analyzing the breakthrough curve data, or the percentage of phosphate removed from solution over time. The Thomas and Clark models were fit to the data to determine the best model for predicting the behavior of the adsorbent in a flow-through system.

2.4.3.1. Thomas

The Thomas model, given in Equation 7, is one of the most widely used models for predicting breakthrough curves, and this model assumes that the experimental batch data obeys the Langmuir isotherm. Theoretically, a well-suited Thomas model fit could indicate that diffusion is not a rate-limiting step (Xu *et al.,* 2013).

$$\frac{C_t}{C_0} = \frac{1}{1+\exp\left(\frac{k_{Th}q_e m_s}{Q} - k_{Th}C_0 t\right)} \qquad (7)$$

Where k_{Th} is the kinetic constant (mL min^{-1} mg^{-1}) and q_e is the adsorption capacity (mg g^{-1}). Many papers analyze adsorption data using the Thomas, Adams-Bohart, and Yoon-Nelson models, often comparing the fit of each model to their data. However, when the equations are simplified, these models are all mathematically equivalent and the different model parameters are identical (meaning that a regression fit with the three models will yield the same result), so it is not meaningful to compare between the three (Chu, 2020; Juela, 2021); thus, the Thomas model is satisfactory for the breakthrough curve analysis.

2.4.3.2. Clark

The Clark model uses the mass transfer coefficient in combination with the Freundlich isotherm to define a relation for the breakthrough curve in the following way (Hanbali *et al.,* 2014):

$$\frac{C_t}{C_0} = \left(\frac{1}{1+Ae^{-rt}}\right)^{\frac{1}{n-1}} \qquad (8)$$

Where *n* is the Freundlich constant (unitless), and *A* (unitless) and *r* (d^{-1}) are the Clark model parameters that do not have distinct physical meaning. A high R^2 with the Clark model indicates that adsorption is a multi-site process as defined in the Freundlich isotherm.

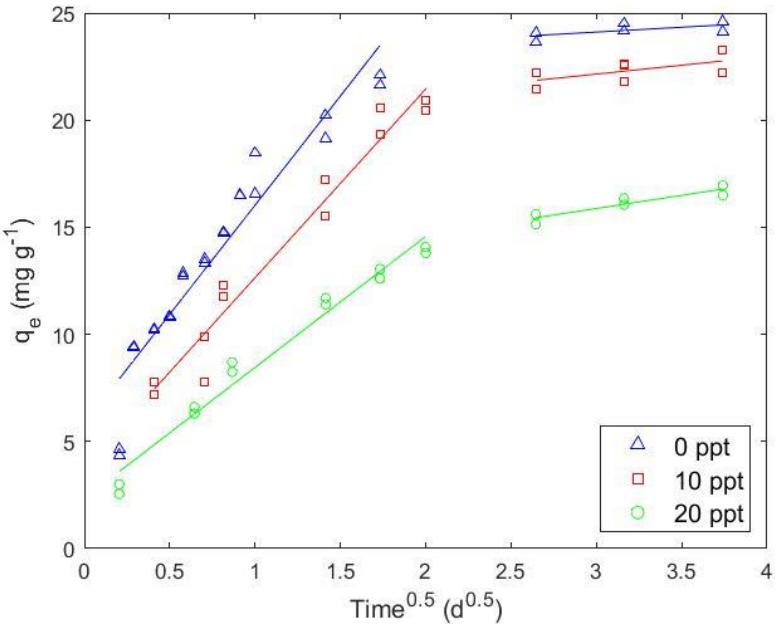
2.5. Results and Discussion

2.5.1. Adsorption kinetic results

2.5.1.1. Kinetic results for initial DI tests

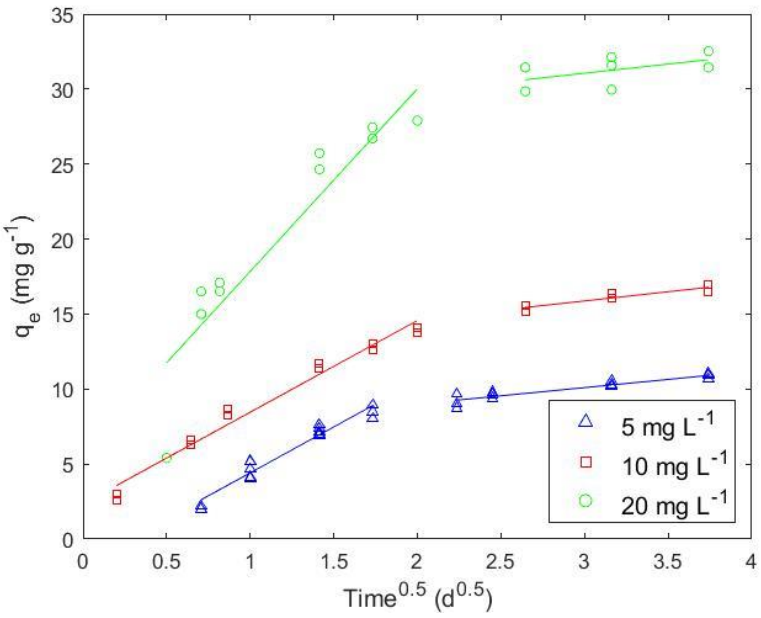
All kinetic parameters were found using linear or nonlinear regression analyses, and the diffusion coefficients and Elovich parameters can be found in Table 2.1. The Weber-Morris model showed a high value of the intercept *C* for all tests. This indicates that intraparticle diffusion is not the only rate-limiting step; instead, multiple steps control the process. The relative contributions of film and intraparticle diffusion were visualized by segmenting the plots of $t^{0.5}$ versus q_t into two sections, the first representing film diffusion and the second representing intraparticle diffusion. These are shown in Figure 2.1. The R^2 were very high for the film diffusion segments, all 0.93 or above. The film diffusion rate constant, $k_{WM\text{-}F}$, decreased with increasing salinity, indicating that the transport of the adsorbate through the liquid film was faster at lower salinities. This rate constant did not change with a concentration increase from 5 to 10 mg L^{-1}, though it doubled with a concentration increase to 20 mg L^{-1}. This may indicate that film diffusion is more affected by salinity at low PO_4^{3-} concentrations, but more affected by PO_4^{3-} concentration than salinity when the PO_4^{3-} concentrations are high. These results are similar to that of activated carbon,

which have shown increasing slopes of the initial segment of the Weber-Morris model with increased adsorbate concentration (Bizi, 2020). The very low slopes of the intraparticle diffusion segments in all tests indicate a slow rate of diffusion into the pores of the adsorbent, regardless of salinity or concentration, though the slopes increase slightly with higher salinities. This may be due to higher proportions of phosphate adsorbed more quickly at lower salinities—meaning that higher proportions of phosphate were available to adsorb with time in higher salinities.



a

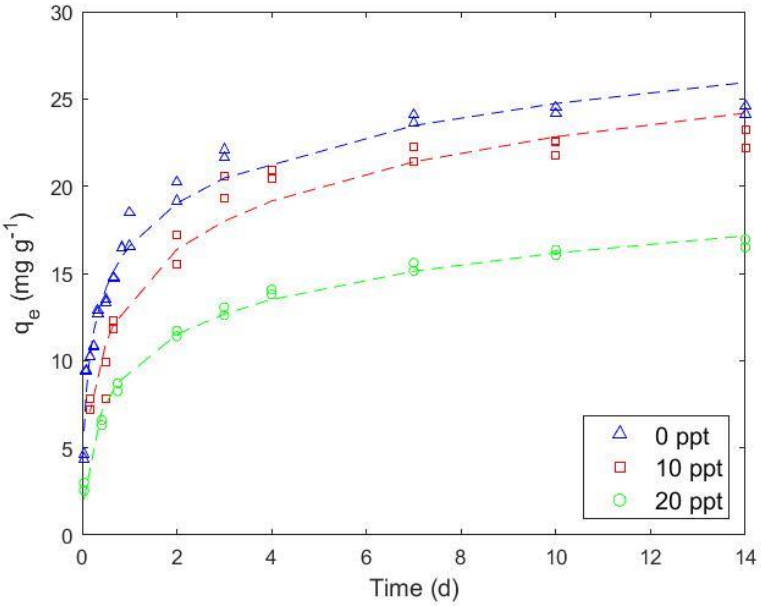
Figure 2.1. Effect of changing a) salinity and b) phosphate concentration on Weber-Morris model fit. Lefthand regressions correspond to film diffusion, righthand regressions correspond to intraparticle diffusion. Background parameters: a) C_0 = 10 mg L^{-1}, b) salinity = 20 ppt



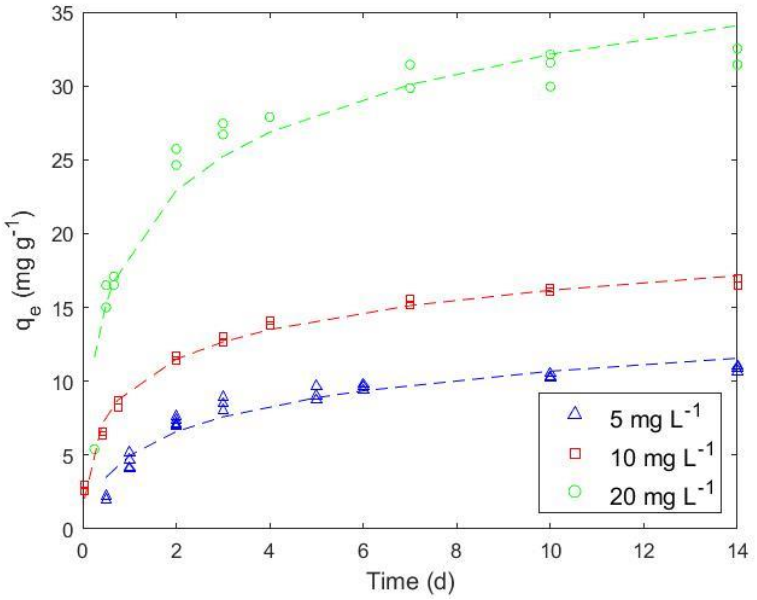
b

Figure 2.1. (continued)

The Elovich model also fit the data well for all tests, and the nonlinear regressions are presented in Figure 2.2. This may indicate that chemisorption is the dominating binding mechanism, meaning that a chemical bond forms between the adsorbate and the adsorbent binding site.



a



b

Figure 2.2. Effect of changing a) salinity and b) phosphate concentration on Elovich model fit.

Background parameters: a) C_0 = 10 mg L^{-1}, b) salinity = 20 ppt

The likely explanation for the decrease in PO_4^{3-} removal with salinity is that sulfate (SO_4^{2-}), bicarbonate (HCO_3^-), and other negatively charged ions may compete with the negatively charged phosphate ions for adsorption sites. Sulfate has been shown to be more competitive with phosphate than other anions, decreasing phosphate adsorption capacity (Liu *et al.,* 2015). At 10 and 20 ppt salt, the sulfate concentrations are two and three orders of magnitude larger than the phosphate concentrations, at 660 and 1320 mg L^{-1}, respectively. Therefore, the adsorbent still shows great affinity for phosphate, as the removal percentages only decreased from 83 to 57 with an increase from 0 to 20 ppt salt. It has been shown that aluminum oxide often has a high affinity for phosphorus (Tanada *et al.,* 2003; Pan *et al.,* 2017), which is supported by our results.

Another parameter affected by bicarbonate addition is the pH. This parameter was not controlled throughout the adsorption experiments, as it would be unlikely to be adjusted in practice. The pH was also measured at the end of each experiment, and none of the samples had significant changes in pH over the course of the test. The pH can affect phosphate competition with other ions by changing the speciation; for example, the dominant form of phosphate at neutral pH is $H_2PO_4^-$, while HPO_4^{2-} dominates at pH above 7.2 (Hinsinger, 2001). Inorganic carbon in solution will be present in different forms at different pH; the dominant forms are CO_2 at pH < 6.3, HCO_3 at 6.3 < pH < 10.3, and CO_3^2 at pH > 10.3 (Marcandalli *et al.,* 2021). Therefore, at neutral pH, where most RAS are maintained (Patillo, 2014), the inorganic carbon will be predominantly HCO_3^- with some CO_2. The negatively charged HCO_3^- may compete with phosphate for binding sites, but the neutral CO_2 will not. However, as pH increases, there will be more HCO_3^- and CO_3^{2-}, the latter of which is more competitive with phosphate due to its higher charge. Sulfate may compete with phosphate for binding sites at any of these pH levels. This can help to explain the decreasing adsorption capacity in higher salt solutions.

Another possible contributor to the decrease in adsorption capacity at high salt concentrations is that the binding sites on the adsorbent surface are affected by the higher pH caused by the increase in bicarbonate. The pH_{PZC} (point of zero charge) is the pH at which the surface charge on the adsorbent is zero; above the pH_{PZC} the surface charge will be negative, and below it will be positive (Crittenden *et al.*, 2012). The pH_{PZC} for aluminum oxide is approximately 9.1 (Crittenden *et al.,* 2012). As the phosphate ion is negatively charged, it is reasonable that higher pH (or less positive net surface charge) will allow for less bonding between the RhizoSorb® and the phosphate ions.

Table 2.1. Kinetic parameters for different concentrations and salinities

			Weber-Morris									**Elovich**		
			Film Diffusion			Intraparticle Diffusion			Original Unsegmented Fit					
C_0 (mg L^{-1})	**Salinity (ppt)**	**pH**	k_{WM-f} (mg L^{-1} $d^{-0.5}$)	C_f (mg L^{-1})	R^2	k_{WM-i} (mg L^{-1} $d^{-0.5}$)	C_i (mg L^{-1})	R^2	k_{WM} (mg L^{-1} $d^{-0.5}$)	C (mg L^{-1})	R^2	α	β	R^2
10	0	6.0	10.19	5.84	0.96	0.45	22.76	0.65	4.98	9.56	0.90	369.0	0.281	0.97
10	10	8.0	8.81	3.84	0.97	0.83	19.66	0.62	4.69	8.20	0.91	102.4	0.241	0.92
10	20	8.7	6.11	2.34	0.99	1.23	12.20	0.93	3.79	4.68	0.94	72.89	0.342	0.99
5	20	8.8	6.12	-1.71	0.93	1.09	6.82	0.54	2.50	2.77	0.90	14.61	0.377	0.93
20	20	8.7	12.18	5.66	0.97	1.22	27.40	0.93	6.18	12.60	0.88	149.1	0.173	0.92

2.5.1.2. Effect of salinity and concentration on removal

Table 2.2 shows the PO_4^{3-} removal percentages at the end of each kinetic test. The test with the highest removal percentage was the 10 mg L^{-1} test with no salt added. As salinity increased from 0 to 20 ppt, the removal percentages decreased from 83% to 57%, indicating a lower adsorption capacity at higher salinity and pH. As PO_4^{3-} concentration increased from 5 mg L^{-1} to 20 mg L^{-1}, the removal percentage decreased from 67% to 53%. This indicates that at lower concentrations, a higher percentage of the total

PO_4^{3-} may be removed. This is a common trend in adsorption (Mekonnen *et al.,* 2021; Banerjee and Chattopadhyaya, 2017; Barca *et al.,* 2012), as the binding sites with higher affinity will be saturated first, and only the binding sites with weaker affinity will be available to bind the remainder of the PO_4^{3-}.

Table 2.2. Removal rates for kinetic tests of different concentration and salinity

C_0 (mg L^{-1})	Salinity (ppt)	pH	Removal (%)
10	0	6.0	83
10	10	8.0	77
10	20	8.7	57
5	20	8.8	67
20	20	8.7	53

An adsorbent dose of 0.33 g L^{-1} was used for the kinetic tests. The removal percentages with this adsorbent dose and PO_4^{3-} concentration are comparable to other existing PO_4^{3-} adsorbents that performed tests with similar parameters. A study by Younes *et al.* (2019) on phosphate adsorption by glauconite showed an approximate 85% removal rate for a dose of 0.25 g L^{-1} and an initial concentration of 5 mg L^{-1}, which is comparable to the results of the RhizoSorb® in freshwater. Another study by Fetene and Addis (2020) on pumice showed a removal efficiency of 73% for a dose of 2 g L^{-1} and an initial concentration of 3 mg L^{-1}. This is lower than the removal rate exhibited by RhizoSorb in a test using a smaller amount of adsorbent (0.33 g L^{-1}) and a higher phosphate concentration (5 mg L^{-1}) (i.e., less favorable adsorption parameters). This indicates that the RhizoSorb® is comparable or better than some other adsorbents in terms of capacity. However, an important point to note is that the RhizoSorb® took a very long time to approach saturation: around four days. Often, in batch experiments, equilibrium is reached within a few

hours (Liu *et al.,* 2015; Yousefi *et al.,* 2018; Riahi *et al.,* 2017; Xu *et al.,* 2009). Therefore, this adsorbent may function better in systems where contact time can be extended.

2.4.1.3. Effect of salmon wastewater on removal

Removal percentages were compared for salmon wastewater and DI water to elucidate the effects of wastewater constituents. Unfortunately, the bicarbonate concentrations were not available for the salmon wastewater, though the pH was given and is highly dependent on bicarbonate concentration. NCWMAC does not buffer their pH, and it generally stays between 6.5 and 7.5. These removal rates are presented in Table 2.3.

Similar to the DI tests, the salmon wastewater tests exhibited a decrease in PO_4^{3-} removal at higher salinities. However, the wastewater tests help to indicate the relative influences of salt, as the same pH in the wastewater samples may indicate a consistent bicarbonate concentration. Therefore, the differences in removal between the two water types may be attributed to the differences in anion competition, most likely sulfate. At the same pH, the removal rates decreased by 10% with a salinity increase from 0 to 20 ppt. This is a smaller decrease in removal than the decrease exhibited by the DI tests where the pH increased with salt (20%).

There were substantial differences in the two water types at each respective salinity. First, the removal efficiency was decreased in the salmon wastewater at 0 ppt as compared to the DI water. However, the removal efficiency was higher in the salmon wastewater than the DI at salinities of 7 and 33 ppt. A possible explanation for these results is that the salmon wastewater contains organic matter that may have bonded to the adsorbent and contributed to polymer bridging along with the salt (Brewer *et al.,* 2021), which would increase adsorption capacity in the saltier salmon wastewater tests. Overall, these results are promising for the use of this adsorbent for RAS water, as they show that the removal

percentages may be higher in RAS conditions. Furthermore, the wastewater obtained from the RAS facility for this study had approximately 0.5 mg PO_4 L^{-1}, but was adjusted up to 10 mg L^{-1} for testing. Therefore, the removal percentage may further be increased in true RAS water due to the lower PO_4^{3-} concentrations, as shown by the original DI tests.

Table 2.3. Kinetic parameters for RAS wastewater compared to DI

Water Type	Salinity (ppt)	pH	Removal (%)
WW	0	7.5	75
	7	7.5	67
	33	7.5	65
DI	0	6.0	82
	7	7.5	63
	33	8.8	62

Background concentration: C_0 = 10 mg L^{-1}

2.5.2. Adsorption equilibrium results

The data obtained from the batch tests fit both the Freundlich and the Langmuir models, though the Freundlich isotherm was a slightly better fit overall. Parameters can be found in Table 2.4, and the linear fits for each model can be found in Figure 2.3. Aluminum oxide often exhibits close R^2 for both the Freundlich and Langmuir isotherms (Gaayda *et al.,* 2021; Zhu *et al.,* 2018), so these results are not unusual.

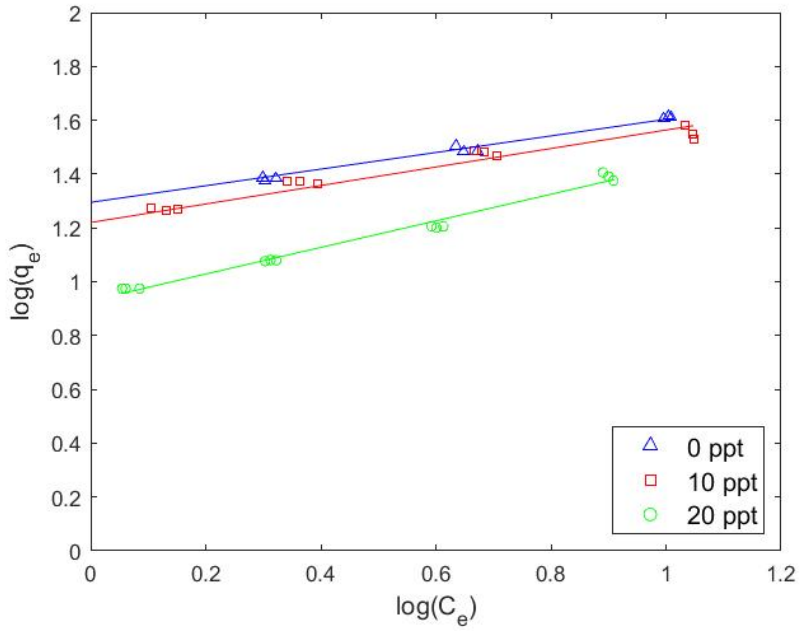
Adsorption capacity decreases as salinity and pH increase. The better Freundlich fit indicates that adsorption is likely a multilayer process; however, a definitive statement cannot be made due to the high R^2 of both the Freundlich and Langmuir models. As the salinities and pH increased, the Freundlich constant K_F decreased. The intensity parameter, *1/n,* decreased with increasing salinity and pH, indicating that that

the heterogeneity of the adsorbent decreases with increasing salinity. This can be explained by the increasing pH and ion competition. As the pH gets closer to the pH_{PZC}, the aluminum oxide will have a smaller positive surface charge; additionally, changes in speciation will result in more competition between phosphate and other ions. Therefore, the number of available binding sites for PO_4^{3-} adsorption decreases with increasing salinity and pH. The Langmuir isotherm provides the adsorption capacity in mg g^{-1}, making Q_{max} a good parameter for comparison. Overall, these results fall within typical values of Q_{max} for phosphate adsorption capacity of activated carbon, which range from ~7 to 95 mg g^{-1}) (Wang *et al.,* 2012; Zhong-liang *et al.,* 2011; Najmi *et al.,* 2020; Ouakouak and Youcef, 2016).

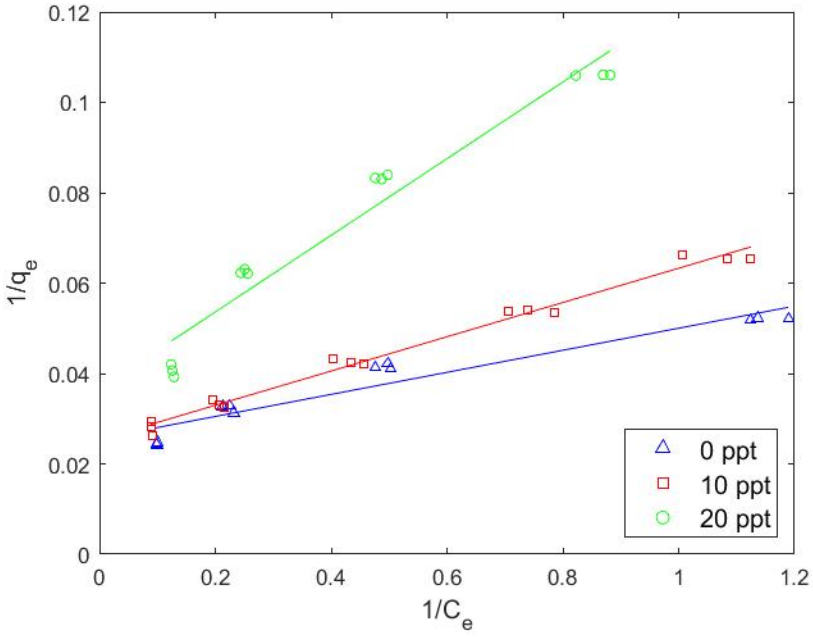
Table 2.4: Freundlich and Langmuir adsorption parameters for different salinities

Salinity	**pH**	**Freundlich**			**Langmuir**		
(ppt)		K_F (mg g^{-1})(mg L^{-1})$^{-1/n}$	*n*	R^2	K_L (L mg^{-1})	Q_{max} (mg g^{-1})	R^2
0	**6.1**	19.72	3.167	0.99	1.049	39.05	0.96
10	**7.5**	17.16	3.134	0.98	0.670	39.40	0.99
20	**8.7**	8.20	1.914	0.99	0.432	27.27	0.97

Background parameters: C_0 = 20 mg L^{-1}



a



b

Figure 2.3. a) Freundlich and b) Langmuir isotherms for different salinities, performed in triplicate.

Neutral pH is ideal for most aquaculture species, and system pH should be kept as close to neutral as possible to keep the percentage of toxic unionized ammonia nitrogen low (Pattillo, 2014). Therefore, the pH of the water treated with this adsorbent will most likely be fairly close to neutral at all times, keeping the number of active sites high and optimizing the RhizoSorb® adsorption capabilities.

2.5.3. Breakthrough curves

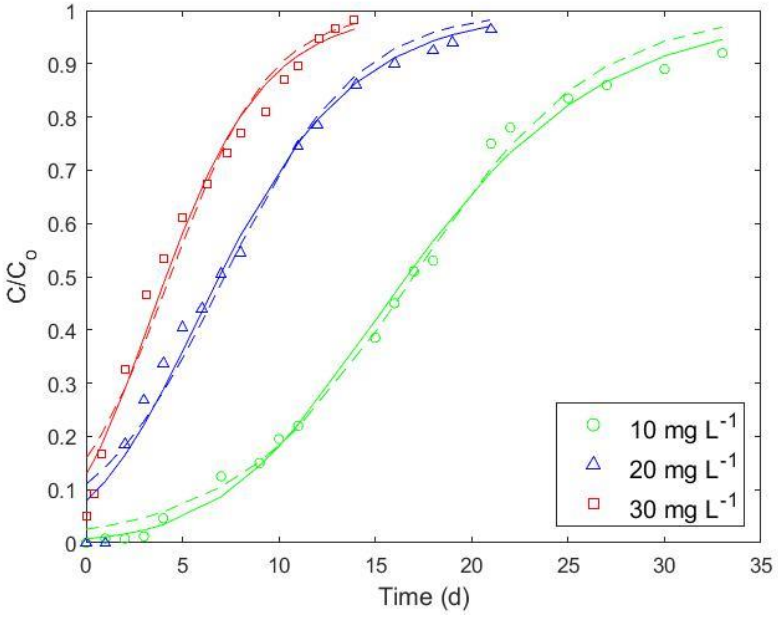
The results of the column tests are shown in Figure 2.4. Column parameters can be found in Table 2.5.

Table 2.5: Column parameters for various flow rates and masses of adsorbent

Inside diameter of column (cm)	Mass of Adsorbent (g)	Height (cm)	Flow rate (mL min^{-1})	Contact Time (min)
1.1	1.5	1.0	0.4	2.38
	2.0	1.7	0.4	4.04
			1.0	1.62
			1.5	1.08
	2.5	2.4	0.4	5.70

As shown in the kinetic and equilibrium experiments, the adsorbent needs a long contact time for the most efficient removal, much longer than some other adsorbents. The time to column exhaustion (i.e., the time at which all binding sites are occupied, and C/C_0 approaches 1) decreased with higher

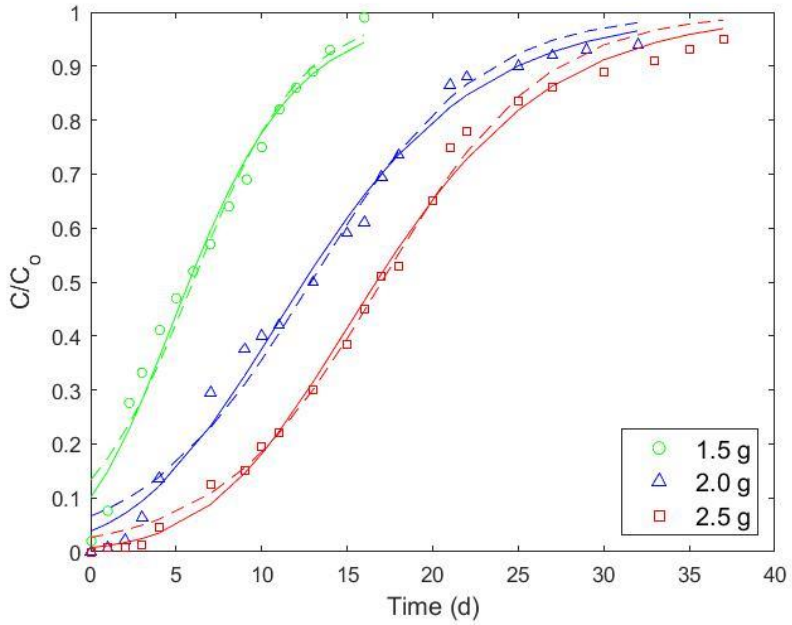
concentrations of phosphate (Figure 2.4a), lower masses of adsorbent (Figure 2.4b), higher flow rates (longer contact time) (Figure 2.4c), and higher salinity (Figure 2.4d).



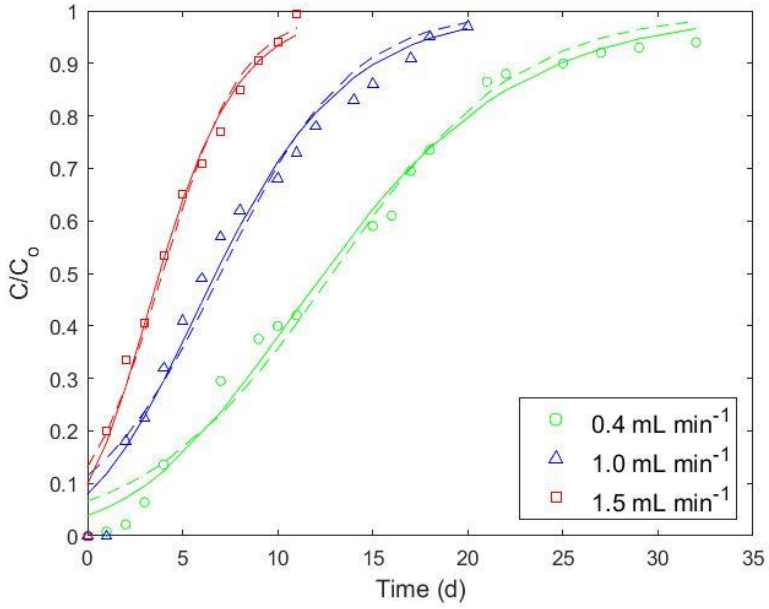
a

Figure 2.4. Effect of changing a) PO_4 concentration, b) mass of sorbent, c) flow rate, d) salinity; solid lines: Clark model fit, dotted lines: Thomas model fit.

Background parameters: a) m_s = 2.5 g, Q = 0.4 mL min^{-1}, o/oo = 0 ppt; b) C_0 = 10 mg L^{-1}, Q = 0.4 mL min^{-1}, o/oo = 0 ppt; c) m_s = 2.0 g, C_0 = 10 mg L^{-1}, o/oo = 0 ppt; d) m_s = 2.0 g, C_0 = 10 mg L^{-1}, Q = 0.4 mL min^{-1}

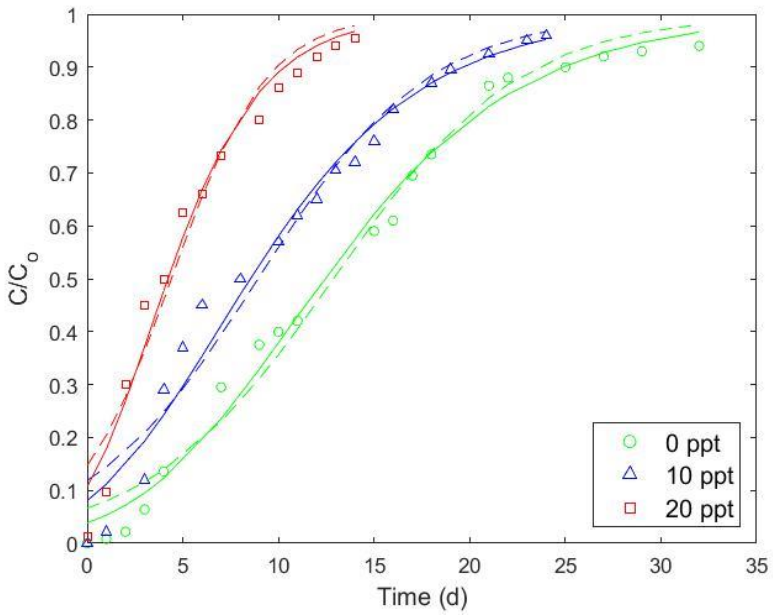


b



c

Figure 2.4. (continued)



d

Figure 2.4. (continued)

The decrease in time to reach exhaustion with higher concentrations of PO_4^{3-} agrees with the trend in the kinetic tests where the removal percentage decreased as the concentration increased; therefore, this should be expected. Lowering the mass of adsorbent decreases the overall amount of available binding sites, allowing for less total phosphate to be adsorbed. Additionally, lowering the mass of adsorbent effectively decreased the contact time by decreasing the volume of the column through which the solution passed. The kinetic tests showed the relative influences of film and intraparticle diffusion; without sufficient contact time, the PO_4^{3-} ions are unable to make their way through the liquid film layer and into the particle. This increases the amount of PO_4^{3-} in the effluent water. This is similar to the effect that the increase in flow rate exhibits on the column; as the contact time is decreased, there is less opportunity for film diffusion and binding. Finally, the decrease in exhaustion time for columns with higher salinities is similar to the effect of salinity in the batch tests. This may be due to ion competition as the salt is added, or the influence of pH on the binding sites and the heterogeneity of the adsorbent.

The Clark and Thomas models were fit to the column experiment data using nonlinear regression analysis, and these results are shown in Table 2.6. The unitless Clark parameter *A* increased with increasing mass of adsorbent, and decreased with increasing phosphate concentration, flow rate, and salinity. The parameter *r* decreased as the adsorbent mass was increased from 1.5 g to 2.0 g and stayed relatively constant when the mass of adsorbent was increased from 2.0 g to 2.5 g. As the phosphate concentration, flow rate, and salinities increased from 0 to 30 mg L^{-1}, 0.4 to 1.5 mL min^{-1}, and 0 to 20 ppt, respectively, *r* increased. The good fit to the Clark model indicates that adsorption is a multi-layer process, in agreement with the Freundlich isotherm fit.

Table 2.6. Clark and Thomas parameters for breakthrough curves

Column Parameter				**Clark**			**Thomas**		
m_s (g)	**C_0 (mg L^{-1})**	**Q (mL min^{-1})**	**Salinity (ppt)**	A	r (d^{-1})	R^2	k_{Th}	q_e (mg g^{-1})	R^2
1.5	**10**	**0.4**	**0**	0.968	0.252	0.98	0.031	24.21	0.97
2.0	**10**	**0.4**	**0**	1.622	0.158	0.99	0.021	39.06	0.98
2.5	**10**	**0.4**	**0**	3.282	0.160	0.99	0.021	41.19	0.99
2.5	**20**	**0.4**	**0**	1.119	0.231	0.98	0.015	34.82	0.97
2.5	**30**	**0.4**	**0**	0.833	0.314	0.98	0.013	31.65	0.97
2.0	**10**	**1.0**	**0**	1.110	0.236	0.98	0.029	53.06	0.97
2.0	**10**	**1.5**	**0**	0.979	0.387	0.98	0.048	44.80	0.98
2.0	**10**	**0.4**	**10**	0.741	0.176	0.98	0.023	27.07	0.96
2.0	**10**	**0.4**	**20**	0.637	0.320	0.98	0.040	13.34	0.96

* Greyed area indicates changing variable for a set of experiments.

2.6. Conclusions

This study revealed that RhizoSorb® has potential to serve the RAS wastewater treatment process, though efficiency will be affected by some process parameters such as salinity, pH, bicarbonate, contact time, and PO_4^{3-} concentration. The segmented Weber-Morris model showed that there is a clear influence of both film and intraparticle diffusion during the adsorption process. The good fits of the Elovich model and the Freundlich isotherm indicate that binding may be caused by chemisorption, though further work to investigate binding energies is required to make a conclusive statement. In salty solutions, there may be competition with other ions, reduced binding sites due to the changing pH, or a combination of the two. However, RhizoSorb® shows high selectivity for phosphorus in kinetic, equilibrium, and column tests. Additionally, the PO_4^{3-} removal rates in true salmon RAS wastewater showed that this adsorbent may function well, in particular, as part of RAS wastewater treatment. Additional work may show desorption parameters of the RhizoSorb® and further characterize it for its use as a slow-release fertilizer.

CHAPTER 3

EFFECTS OF SALINITY CHANGES ON NITRIFICATION RATES OF NITRIFYING BIOFILTER MEDIA

3.1. Abstract

In recirculating aquaculture systems (RAS), where only a small percentage (typically around 10%) of the system water is exchanged per day, ammonia-nitrogen produced by fish must be converted to the less toxic nitrate-nitrogen before recirculation. Nitrifying biofilters can be sensitive to changes in environment, including changes in salinity. However, acclimation to a small amount of salinity before transition to a higher salinity may help biofilters recover from these changes more quickly. A series of experiments was performed on both freshwater and brackish (3 ppt) biofilters to assess their ability to recover nitrification activity after an abrupt change in salinity (3, 20, and 33 ppt), based on the ammonia oxidation rates and nitrate production rates. Tests were run for a two-week period in which the nitrification rates were monitored. The brackish-adapted biofilters showed a small recovery in nitrification after a shift to 20 ppt, while the freshwater biofilters did not; however, neither the freshwater nor brackish biofilters adapted to 33 ppt. Illumina sequencing of the V4 region of the 16s rRNA gene from community DNA was performed for microbial community analysis. Sequencing revealed that, while the shifts in salinity inactivated the nitrifying community, the heterotrophic communities in the biofilms were affected by the salinity changes at a higher proportion. A series of longer tests could identify the long-term effects of salinity changes on freshwater and brackish biofilters.

3.2. Introduction

Recirculating aquaculture systems (RAS) have become an increasingly popular source of high-quality protein production in recent years, with strong potential to supplement traditional, water-

intensive agriculture methods. In RAS, only ~10% of the total system water is exchanged per day (Davison, 2019). Thus, to maintain the water quality required for healthy growth, the recirculated water must be treated to remove or transform the fish waste products. When fish consume proteins in food, some of the nitrogen is excreted through the gills in the form of ammonia (Lazzari and Baldisserotto, 2008). Most aquatic species cannot tolerate more than 1.0 mg L^{-1} of total ammonia nitrogen (TAN) in water; ideally, the concentration for long-term exposure should be kept under 0.05 mg L^{-1} (Gao *et al.*, 2020). It is imperative that the ammonia-nitrogen be converted to its less toxic form, nitrate, before the RAS water is recirculated or discharged. This conversion, nitrification, is a two-step process where ammonia (NH_4^+) is converted to nitrite (NO_2^-), and then to nitrate (NO_3^-) (Skoyles *et al.,* 2020), in an aerobic environment. The microbes that perform these conversions in biofilters grow in biofilms on plastic microbeads or other filter media. These microbes, also called nitrifiers, are sensitive to changes in their environment, such as changes in pH, temperature, and salinity. Changes in salinity of RAS are commonplace when raising anadromous species such as Atlantic salmon, and this has proven to be a hinderance to nitrifying biofilters (Kinyage *et al.,* 2019).

Many studies have been performed to assess nitrifying biofilters' resistance to changes in their environments, highlighted in depth by Navada and Vadstein (2022). It has been shown that biofilms may be more resilient to changes in salinity if they have previously experienced a shift in salinity (Navada *et al.,* 2020), and osmotic stress preparation has been shown to improve biofilters' resistance to salinity changes. However, osmotic stress preparation and recovery of freshwater biofilters may take weeks or months, bringing added expense. One method of salinity acclimation that has not yet been investigated in depth is the potential to run freshwater systems at a low level of salinity before shifting to a higher level. Many types of fish, including salmon, eels, bass, and flounder, are able to tolerate a large range of salinity over their lifetimes (Wurts, 1998). If the fish raised in a particular RAS can withstand a low level of

salinity, maintenance at this salinity may assist the biofilters in their transition to higher levels of salinity later. This differs from osmotic stress preparation in that the biofilters are continuously operated at a very low salinity rather than exposed to a moderate level of salinity, brought back down to freshwater, and then fully transitioned to the high level of salinity. Operation of an RAS at any salinity level comes with a higher cost than operation with freshwater (Moran, 2010), so potential benefits of this method should be observed in the short-term. Therefore, this study investigates whether biofilters continuously operated at a low level of salinity (3 ppt) are better equipped in the short-term to handle abrupt changes in salinity than fully freshwater biofilters.

To assess the reactions of the biofilters to the shifts in salinity, both nitrification performance and microbial community composition should be considered. The main categories of ammonia-transforming microbes found in RAS biofilter media are ammonia oxidizing archaea (AOA), ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), complete ammonia oxidizers (comammox), and anaerobic ammonia oxidizers (anammox) (Hupeden *et al.*, 2020; Skoyles *et al.,* 2020). The first two categories, AOA and AOB perform the first step in the nitrification process ($NH_4^+ \rightarrow NO_2^-$), while the second step is performed by NOB ($NO_2^- \rightarrow NO_3^-$). Comammox bypass the two-step process and convert ammonia to nitrate, while anammox convert ammonia to nitrogen gas in the presence of nitrite (Hupeden *et al.,* 2020). Studies have shown that the community compositions of biofilters differ with salinity (Navada *et al.,* 2020; Gao *et al.,* 2020; Khangembam *et al.,* 2017; Hupeden *et al.,* 2020; Roalkvam *et al.,* 2020).

In order to assess shifts in microbial communities, DNA extraction and sequencing are commonly performed. There are several steps that go into extracting DNA. First, the cells must be broken open to release the DNA. This is usually performed by adding a lysis solution and vigorously mixing the samples, often with some form of microbeads that will physically break open the cells. Next, the lysate is removed from the sample, and the DNA in the solution is separated from any insoluble particles and other cell

components by centrifugation or filtration. Then, the DNA is bound to a selective material such as a resin or a silica membrane (Sajali *et al.,* 2018), which is then washed with one or more solutions to remove proteins, salts, and other compounds that may have been introduced or co-purified during the previous extraction steps. The DNA can then be released from the membrane or resin by washing the DNA off with nuclease-free water or an elution buffer. This buffer is commonly Tris-EDTA (TE), which contains a pH buffering agent (Tris) and a metal-binding agent (EDTA) that helps to prevent degradation. The DNA is then ready for downstream applications such as PCR amplification and sequencing.

Microbial community analysis is often done by amplifying one specific gene from whole community DNA obtained as described above, then submitting the amplicons for high throughput sequencing. The 16S rRNA gene is present in all bacteria, making it a useful gene for comparison of different types of microbes. There are nine variable regions in the 16S rRNA gene which have varying levels of conservation (Bukin *et al.,* 2019). More conserved regions (or regions with less change between different species) can only distinguish major differences in taxa, while less conserved regions may indicate changes at the species level. The 16S rRNA gene is also useful because it is commonly studied, meaning that there is a large amount of information available for consultation and comparison, and alignment algorithms available to align and classify the taxonomy of sequences, enabling community comparisons among samples.

3.3. Materials and Methods

3.3.1. Experimental setup

Media from a freshwater nitrifying biofilter was obtained from the University of Maine Cooperative Extension Diagnostic and Research Laboratory's Aquatic Animal Health Lab in Orono, Maine. Before the experiments began, the biofilter used for the experiments was taken from a RAS system with

several tanks containing freshwater salmon parr. In the first round of experiments, four different salinities were tested: 0 ppt (freshwater), 3 ppt, 20 ppt, and 33 ppt (full strength seawater). The 3 ppt salinity was chosen as the arbitrary low level of salinity, while the 20 ppt was chosen as the middle-ground salinity between 3 ppt and full-strength seawater. Each salinity was tested in triplicate, so four sets of three buckets with 6 L of water at their respective salinities and 2 L of biological media were transferred directly from the freshwater RAS biofilter. Each newly transferred biofilter was aerated using air stones connected to an air pump or central air from the lab to maintain approximately 9 mg L^{-1} of dissolved oxygen in each bucket. The buckets were opaque and lids were placed over each one to ensure minimal photolytic interference.

Each filter was spiked daily with ammonium chloride (NH_4Cl) to achieve a daily initial NH_4-N concentration of 10 mg L^{-1}. For every gram of ammonia-nitrogen that is converted to nitrate-nitrogen, 7.05 g of alkalinity as $CaCO_3$ is required (Timmons *et al.,* 2018). This ratio was used to calculate the amount of alkalinity needed for each biofilter to account for the daily amount of oxidized ammonia; thus, each filter was also fed with 5.92 g of baking soda ($NaHCO_3$) per g of N oxidized to supply the microbes with a carbon source and to buffer pH. The daily feedings and chemical testing were performed by removing 10% of the water (0.6 L) from each biofilter. The removed water was tested for ammonia and nitrate concentrations along with pH (as described below), and an equivalent volume of well water at the test salinity was added to the biofilters with the required dry masses of NH_4Cl and $NaHCO_3$ to bring the NH_4-N concentration to 10 mg L^{-1} and supply sufficient buffering capacity. The water temperature was maintained at 16 ^oC throughout the test periods.

The second round of experiments was performed by adjusting 18 L of media from the freshwater RAS biofilter to 3 ppt salinity and allowing the media to fully recover and reach steady state over a period of 30 days. At the end of the adjustment period, the newly acclimated 3 ppt biofilters had reached

complete nitrification of 10 mg N L^{-1} d^{-1}. The same ratio of water to media was used for the brackish biofilters as the freshwater biofilters (6:2). The biofilters were maintained and operated with the same conditions as the freshwater biofilters (as listed above). All experiments were run for 14 days, as this has been reported to provide enough time to observe changes in biofilter microbiomes (Hupeden *et al.,* 2020).

3.3.2. Chemical analysis

The pH in each biofilter was monitored with a YSI probe, while NH_4-N and NO_3-N concentrations were tested using Vernier Ion-Selective Electrodes. The ammonia probe was calibrated with standards of 1 and 10 mg NH_4-N L^{-1}, while the nitrate probe was calibrated with standards of 10 and 100 mg NO_3-N L^{-1}. The ammonia oxidation rate (r_A) and the nitrite oxidation rate (r_N) were calculated using a mass balance of the ammonia-nitrogen and the nitrate-nitrogen in each biofilter. The theoretical maximum oxidation rate of ammonia- and nitrate-nitrogen, r_{max}, was 10 mg N L^{-1} d^{-1}.

3.3.3. DNA extraction and sequencing

Samples of biofilm were taken from the biofilters at the start and finish of each experiment. Approximately 0.05 g of (wet) biofilm was scraped off the plastic media from each filter with a sterile spatula and DNA was extracted using a Qiagen® DNeasy PowerSoil Kit® according to the manufacturer's instructions. The concentrations of the samples were all 5-8 ng/µL; therefore, a two- or four-fold (depending on the original concentration) ethanol precipitation was performed to increase the concentration of each sample to >10 ng/µL. This was performed by adding one-tenth volume of 3M sodium acetate (pH of 5.3) to the samples, then adding 2.4 volumes of 100% ethanol and mixing. Samples were placed in a -30 ^oC freezer overnight, then centrifuged and washed twice with 70% ethanol. The pellet was eluted by adding TE buffer to make up each sample to >10 ng/ µL. PCR amplification, library preparation and sequence determination were performed by Novogene Corporation using universal

bacterial primers 515F (GTGYCAGCMGCCGCGGTAA) and 806R (GGACTACNVGGGTWTCTAAT). Paired-end Illumina NovaSeq 2 x 250 reads was performed on the variable V4 region of the 16s rRNA gene. The forward and reverse reads were trimmed to 230 bp to avoid deterioration in quality at the beginnings and ends of the reads. FASTQ files were used to upload the data to R, and the package Divisive Amplicon Denoising Algorithm (DADA2) (Callahan *et al.,* 2016) was used for dereplication, inference, merging and chimera removal. The sequence variants were then assigned taxonomy based on a Silva training set (Callahan *et al.,* 2016), and sequences identified as chloroplasts and mitochondria were removed before further analysis. Sequence reads will be placed in the National Center for Biotechnology Information (NCBI).

3.3.4. Sequence data analysis methods

Once sequences have been assigned taxonomy using an established database, analyses can be performed. Alpha and beta diversity are two different measures that are often used to assess the similarity of samples. Alpha gives information about the species richness of the sample, which is the estimate of the total number of different taxa within a given sample. Beta diversity is the similarity (or lack thereof) of taxa between different samples. These statistics are useful for determining changes in community with changes in environmental variables.

The alpha diversities of the samples were quantified with observed OTUs as well as Shannon's Diversity Index. A non-metric multidimensional analysis (nMDS) was performed in order to assess the difference between the samples. Furthermore, a canonical coordinate analysis (CCA) was performed to further understand the influencing factors in phylogenetic distance between the samples. The R package DESeq2 (differential expression analysis) (Love *et al.,* 2014) was used to determine the taxa in higher abundance in the freshwater biofilters versus the brackish biofilters. Feature prediction using the R

package dplyr (Wickham *et al.,* 2022) was used to find genera that were statistically different between sets of samples. Feature prediction was also used to indicate taxa that were more likely to be in individual samples. A significance level of 0.05 was used for all tests except for the DESeq analysis, which used a significance level of 0.01.

3.4. Results and Discussion

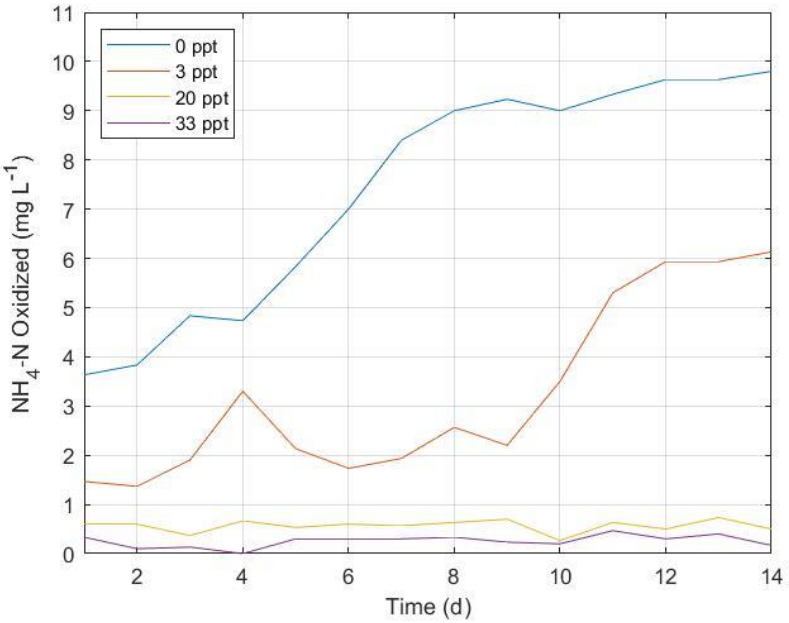
3.4.1. Nitrification rate recovery

3.4.1.1. Nitrification recovery in freshwater-adapted biofilters

The results of the nitrification rate recovery tests on the freshwater media are presented in Figure 3.1. The pH of all biofilters stayed between 8.1 to 8.2 throughout the duration of the tests. Figure 3.1a shows the results of the daily ammonia-nitrogen oxidation, while Figure 3.1b shows the results of the daily nitrate production. The biofilters that were maintained at 0 ppt served as the control. In all tests, the ammonia oxidation and nitrate production rates were very similar and showed almost identical reactions to the shifts in salinity, indicating that there was not a significant accumulation of nitrite (which was not monitored). This can be inferred because nitrite-N oxidation and nitrate-N production have a 1:1 molar ratio—meaning that an accumulation of nitrite would show a decrease in nitrate production. Initially, around 4 mg NH_4-N L^{-1} d^{-1} was oxidized in the freshwater biofilters, reaching the r_{max} of 10 mg L^{-1} d^{-1} (equal to the total amount of ammonia added) after about 12 days. This lag time before complete ammonia oxidation is most likely attributed to the effects of a shift in environment and the 10 mg/L ammonia concentration used in the experiment. The source biofilter before the start of this test was continuously exposed to approximately 0.5 mg L^{-1}. The ammonia oxidation in the control biofilters seems to have recovered slightly more quickly to the shift in environment than the nitrite oxidation, as the ammonia

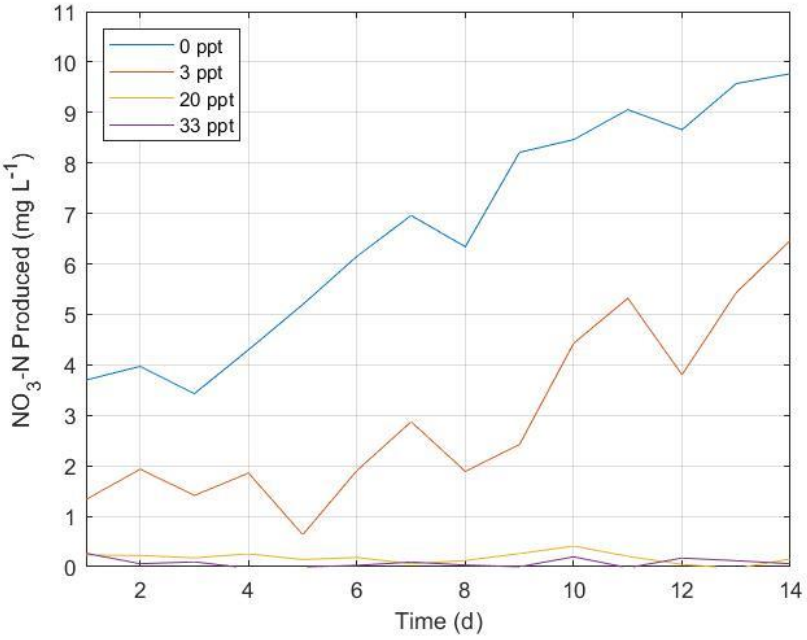
oxidation reached approximately 9 mg L^{-1} d^{-1} at day 8 while the nitrite oxidation was approximately 6 mg L^{-1} d^{-1} at this point.

Nitrification in the biofilters was initially depressed by the transfer to 3 ppt. After a lag time of about 4 days, the ammonia oxidation rate per day began to increase over time. By the end of the test, the biofilters transitioned to 3 ppt were nitrifying approximately 65% of the ammonia per day as the amount nitrified by the freshwater biofilters. Again, the ammonia oxidation recovered slightly faster than the nitrite oxidation. The biofilters transitioned to 20 and 33 ppt, however, did not recover at all from the abrupt change in salinity, performing negligible nitrification each day.



a

Figure 3.1. a) Ammonia oxidation and b) nitrate production at each salinity for freshwater biofilters.



b

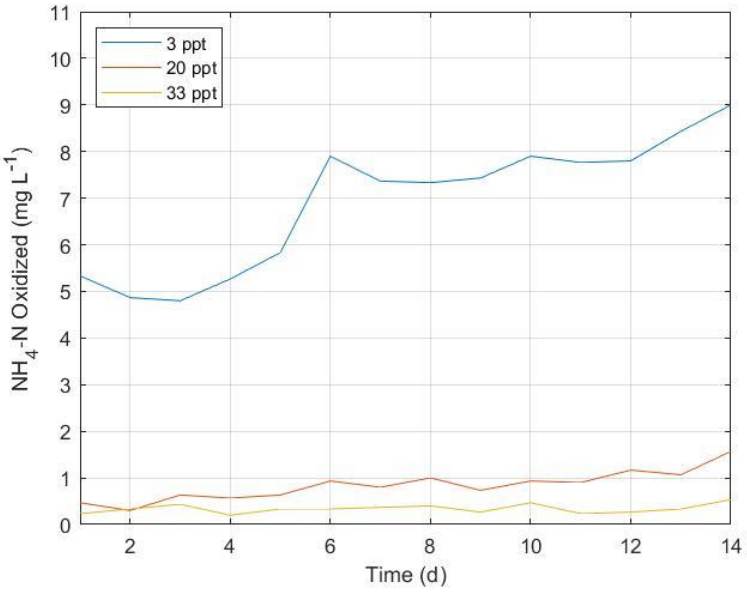
Figure 3.1. (continued)

3.4.1.2. Nitrification recovery in the low salt-adapted biofilters

The results of the tests using biofilter media adapted to low salt concentrations are presented in Figure 3.2. The biofilters acclimated to and maintained at 3 ppt served as the control for this set of tests. During the acclimation period of 30 days, the biofilters were fed with the same ratio of water to ammonia and baking soda to achieve an initial ammonia concentration of 10 mg N L^{-1} d^{-1}, which was consumed entirely each day. Theoretically, the transfer from the maintenance phase to the control biofilters at the start of the second test should have shown 10 mg N L^{-1} of ammonia oxidized daily. However, at the start of the test, the control biofilters oxidized about 5 mg NH_4-N L^{-1} d^{-1}. This was slightly higher than the controls of the freshwater test which only oxidized about 4 mg NH_4-N L^{-1} d^{-1} at the start. One possible explanation for this is that the 100% water exchange at the beginning of the second experiment shocked

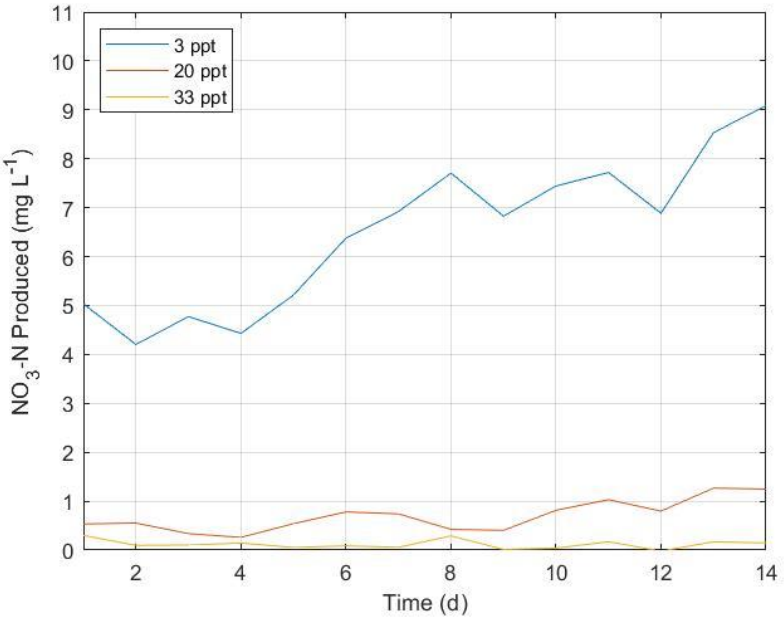
the nitrifiers into low productivity at the beginning of the test, as they had experienced only a 10% daily water exchange during the maintenance period.

Similar to the results of the freshwater tests, the ammonia oxidation recovered more quickly than the nitrite oxidation in the control biofilters, and the biofilters did not recover with a shift to 33 ppt. However, the biofilters transitioned to 20 ppt did show some recovery over the two-week test, consuming about 1 mg NH_4-N L^{-1} d^{-1}. At the end of the test, the 20 ppt biofilters fully oxidized about 11% of the amount of ammonia consumed by the control biofilters. These results indicate that the biofilters operated at 3 ppt may be more resilient to an abrupt increase in salinity than freshwater biofilters. This may indicate a change in the microbiome between the freshwater and the brackish biofilters, investigated in the following section.



a

Figure 3.2. a) Ammonia and b) nitrite oxidation at each salinity for brackish biofilters.



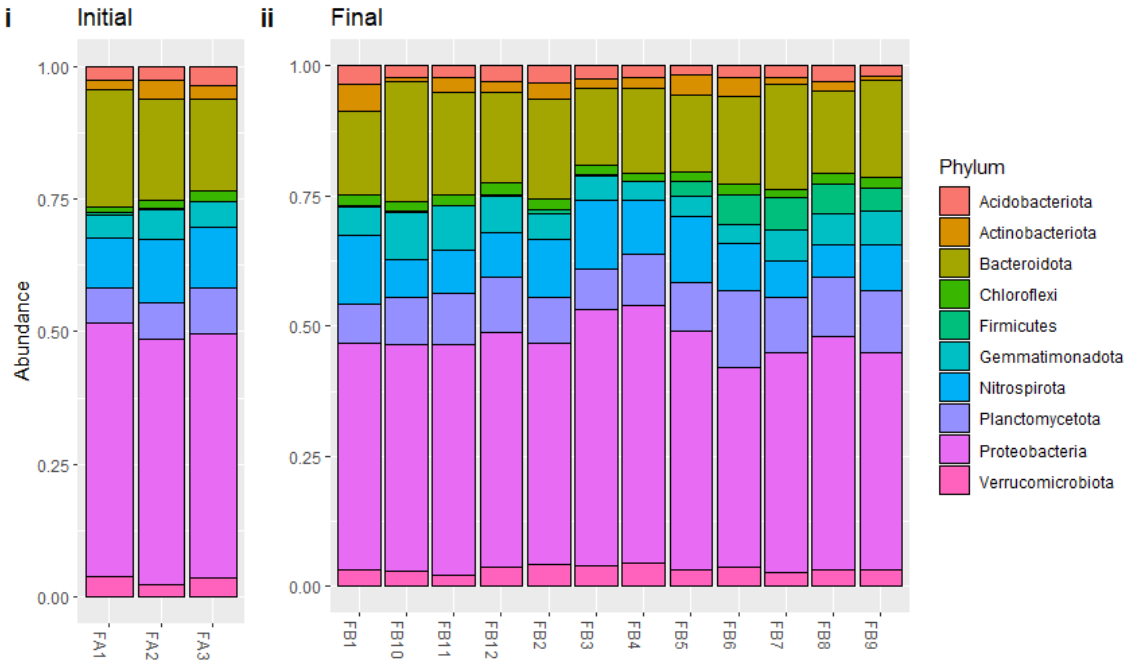
b

Figure 3.2. (continued)

3.4.2. Microbial community analysis

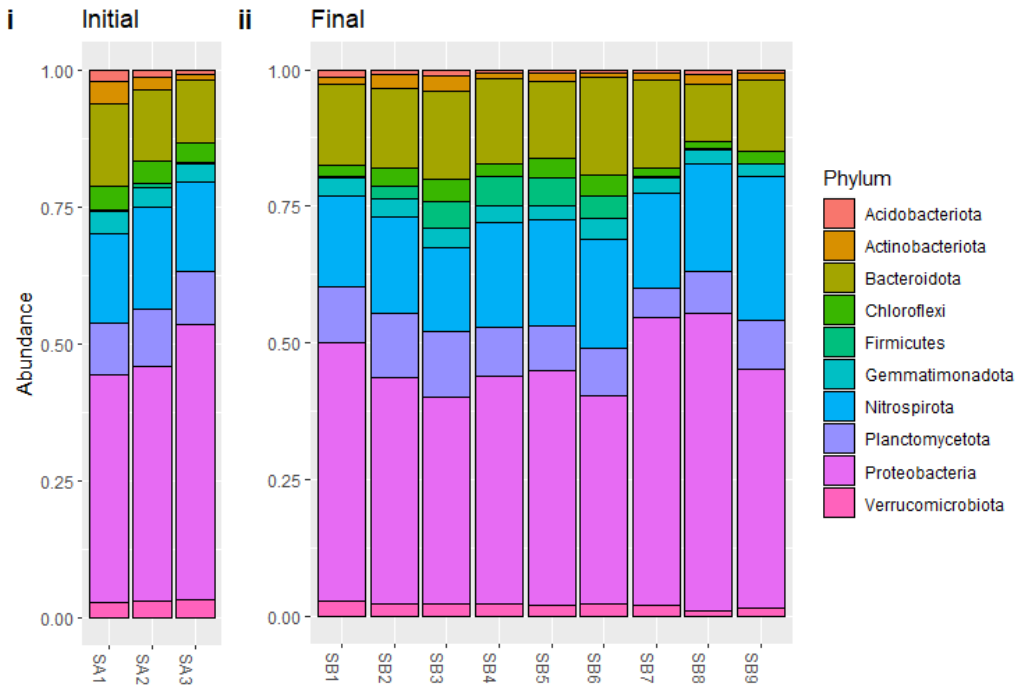
There were 4589 unique OTUs in the sample set, with 82 shared taxa making up the core microbiome. The most abundant phylum in all samples, at about 43% of the total, were classified as *Proteobacteria,* followed by *Bacteroidota,* at about 16%, and *Planctomycetota,* at around 10%. The rest of the most abundant phyla were made up of *Acidobacteriota, Actinobacteriota, Chloroflexi, Firmicutes, Gemmatimonadota, Nitrospirota,* and *Verrucomicrobiota*. Nitrifying genera made up about 27% of the total reads. In several of the following figures, the samples have been designated shorthand names. FA1-3 corresponds to the initial freshwater samples, while FB1-3, FB4-6, FB7-9, and FB10-12 correspond to the final samples at 0 ppt, 3 ppt, 20 ppt, and 33 ppt, respectively. SA1-3 corresponds to the initial brackish

samples, while SB1-3, SB4-6, and SB7-9 correspond to the final samples at 3 ppt, 20 ppt, and 33 ppt, respectively. The phylum distribution in each sample is shown in Figure 3.3.



a

Figure 3.3. Phylum distribution for a) freshwater and b) brackish biofilters.



b

Figure 3.3. (continued)

3.4.2.1. Bacterial diversity

Tables 3.1 and 3.2 present the bacterial diversity using both Shannon's Index and evenness. Initially, there were 699-798 OTUs in the freshwater biofilters. After the two-week long test, the number of observed OTUs in the control spanned from 571-1005. In the 3 ppt biofilters, this number spanned from 540-887 at the end of the test. At the beginning of the brackish tests, the number of OTUs ranged from 583-1005. After the two-week test, the 3 ppt biofilters serving as the control had 497 to 899 OTUs. The 20 ppt biofilters had a relatively similar number of OTUs in both the freshwater and the brackish tests (485-600 and 456-511, respectively). The 33 ppt biofilters had a noticeably higher number of OTUs in the freshwater tests, though this could be attributed to the inhibition of activity in those biofilters resulting in

little change in the community profile as seen in the NMDS ordination (Figure 3.5). Visualization of the species richness in Figure 3.4 shows greater richness in the freshwater adapted biofilters than the brackish-adapted biofilters.

As a whole, the freshwater biofilters had higher evenness than the brackish biofilters. Evenness corresponds to the similarity of abundance of the species within the sample; therefore, the freshwater samples showed higher species similarity, potentially indicating a more stable population.

Table 3.1. Alpha diversity and evenness of freshwater biofilter samples

Stage	Test Salinity	Observed # OTUs	Richness (Shannon's)	Evenness
Initial	0	699	4.804	0.734
	0	798	4.850	0.726
	0	734	4.779	0.724
Final	0	1005	5.011	0.725
	0	931	4.897	0.716
	0	571	4.715	0.743
	3	887	4.890	0.720
	3	562	4.513	0.713
	3	540	4.430	0.704
	20	595	4.707	0.737
	20	600	4.790	0.749
	20	485	4.406	0.712
	33	697	4.745	0.725

	33	792	4.806	0.720
	33	727	4.796	0.728

Table 3.2. (continued)

Table 3.2. Alpha diversity and evenness of brackish biofilter samples

Stage	**Test Salinity**	**Observed # OTUs**	**Richness (Shannon's)**	**Evenness**
Initial	3	699	4.760	0.689
	3	798	4.468	0.673
	3	734	4.473	0.702
Final	3	1005	4.574	0.673
	3	931	4.144	0.661
	3	571	4.196	0.676
	20	887	4.403	0.709
	20	562	4.269	0.685
	20	540	4.075	0.666
	33	595	4.264	0.691
	33	600	4.180	0.683
	33	485	3.836	0.617

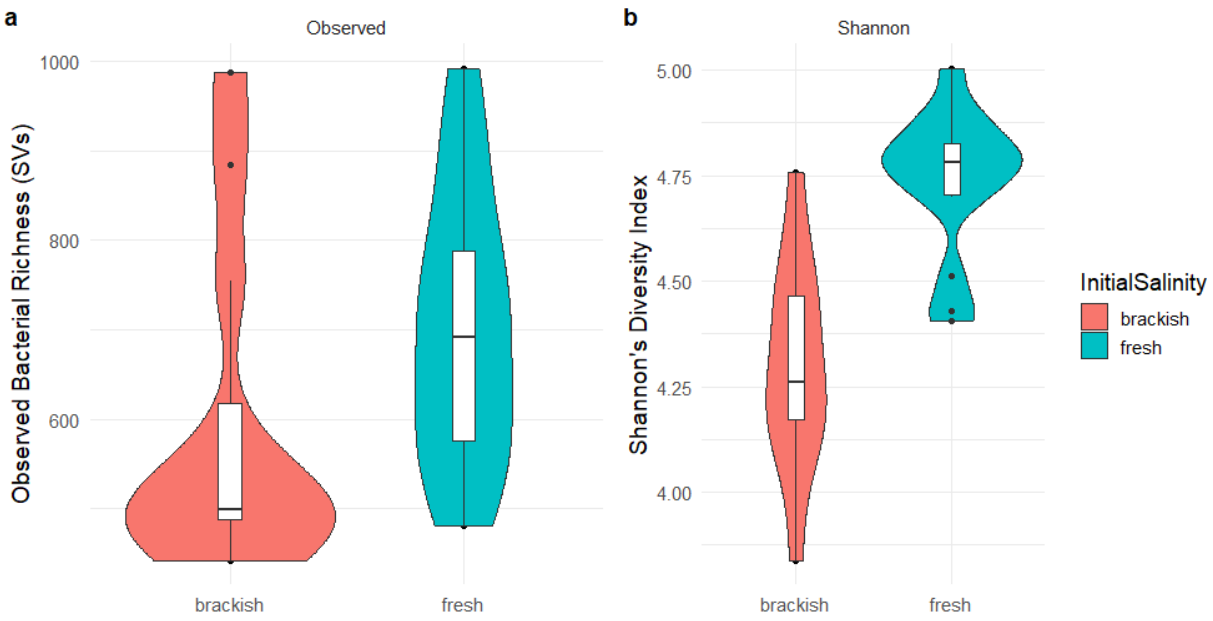


Figure 3.4. Bacterial richness by a) observed SVs and b) Shannon's diversity index.

3.4.2.2. Ordinations and phylogenetic distance

Differences between samples were represented using the Bray-Curtis dissimilarity index using non-metric multidimensional scaling (NMDS) as shown in Figure 3.5. The analysis shows that the samples are somewhat clustered based on their phase (freshwater or brackish), stage (initial or final sampling), and salinity, although similarly treated samples do not cluster perfectly. The ANOVA test performed on the model showed that all three variables had a significant influence of microbial community (p-values < 0.05), with type of biofilter (freshwater or brackish) as the most significant factor.

The initial samples of the freshwater biofilters were clustered together. After the two-week test, the 33 ppt biofilters showed the greatest similarity to the initial biofilters, which could indicate that inhibition of microbial activity at full seawater essentially prevented changes to the community structure. This lack of activity is supported by the lack of observable nitrification in these samples. The 20 ppt

biofilters were clustered together near the same area. The final communities from the biofilters maintained at 0 ppt and the biofilters shifted to 3 ppt, showed a larger spread, indicating higher dissimilarity between the replicates, or a more varied response to the salinity change.

Two of the three initial brackish samples were clustered separately from the rest of the data, and the 3 ppt samples at the end of the experiment, had a larger spread than the final 0 ppt samples for the freshwater experiment. The 20 and 33 ppt biofilters were clustered together next to each other near the 20 ppt biofilters from the freshwater test. The 20 ppt biofilters from both phases were clustered near each other, showing that the communities were not drastically different.

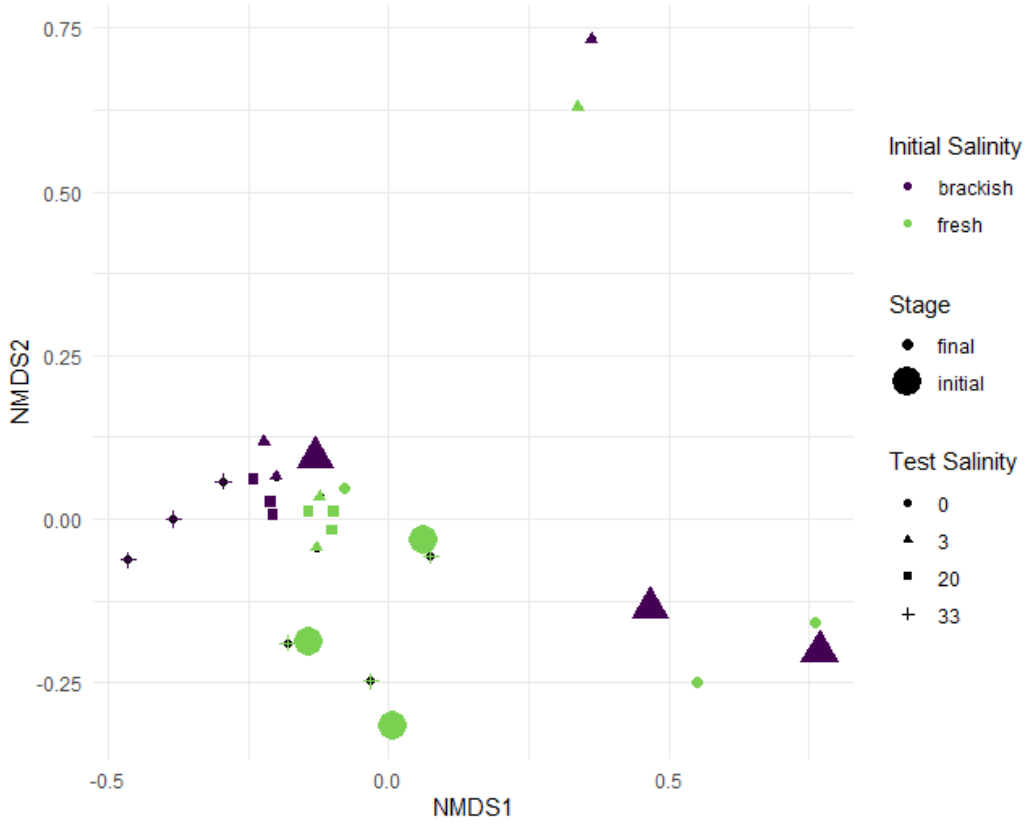


Figure 3.5. Non-metric multidimensional scaling analysis using Bray-Curtis dissimilarity (run 20 stress 0.13).

A canonical coordinate analysis (CCA) using Bray-Curtis dissimilarity was performed on the data to further investigate the similarities between the samples, shown in Figure 3.6. The model was formed using initial salinity, stage (initial or final sample), and test salinity as variables. An ANOVA test was performed on the model as a whole, and the results showed that the model is significant; however, an additional ANOVA test on the variables showed that only initial salinity and test salinity are significant. Evidently, there is a stark divide between the freshwater biofilters and the brackish biofilters. Furthermore, there is a clear gradient upwards as salinity increases for both types of biofilters. The only divergent clustering is the initial freshwater samples that are clustered near the 20 and 33 ppt filter clusters. The change in freshwater samples from the start to the end of the experiment is likely due to a combination of the increased ammonia concentrations used in the tests relative to the concentration experienced by the biofilters before the start, and the lack of organic matter and other fish waste constituents included in the water fed to the biofilters during the experiment.

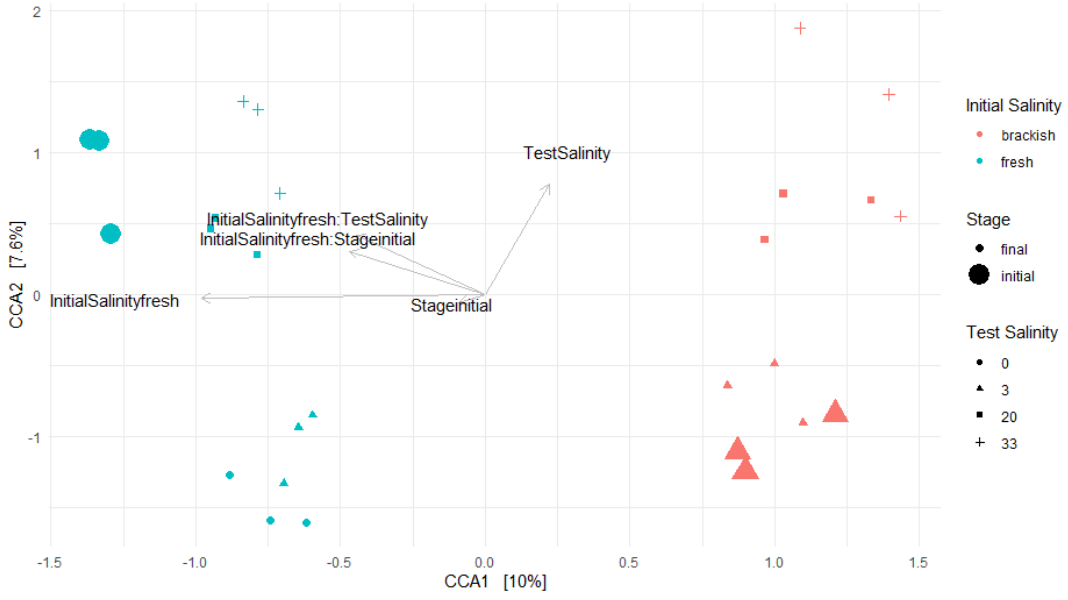


Figure 3.6. Canonical coordinate analysis (CCA) plot of all biofilter samples, arrows show influence of variables on clustering.

A cluster dendrogram is presented in Figure 3.7. Similar to ordination plots, dendrograms are another way of visualizing the distance, or similarity, between samples. There is a clear divide between the freshwater and the brackish biofilters, though grouping of replicates at each test salinity are not perfect. This shows that the month-long maintenance period was long enough to form a stable community of brackish biofilm, but both two-week long experiments were too short to establish clear differences in community between each set of biofilters. The most notable outlier in the freshwater test is one of the biofilters transitioned to 3 ppt (FB6), as the other two replicates are clustered together near the initial samples and the control samples. There were not any major outliers in the saltwater tests, though all the samples were clustered closer together than the samples in the freshwater tests, indicating less overall change, which could be due to greater acclimation to lower organic matter in the starting biofilter materials.

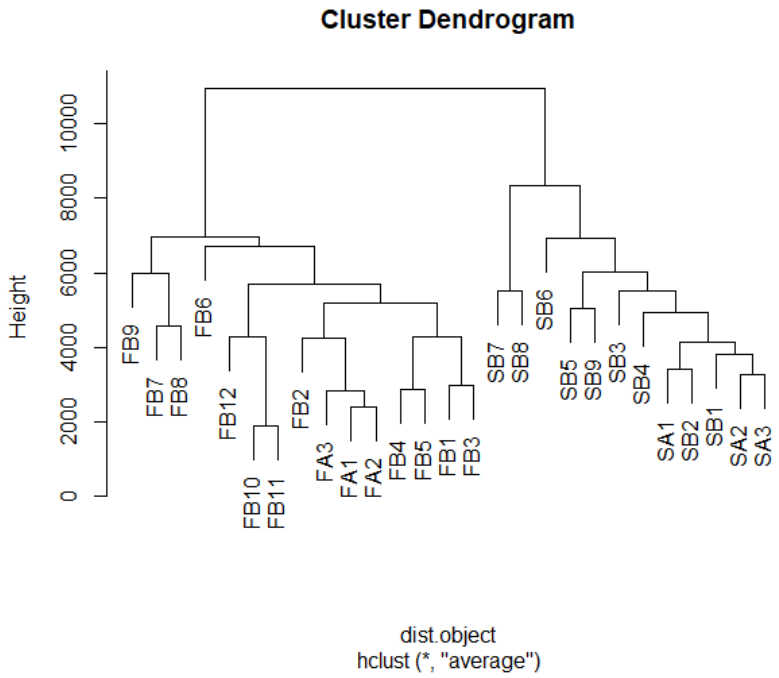


Figure 3.7. Cluster dendrogram showing distance between samples.

3.4.2.3. Nitrifying bacteria composition

The ammonia oxidizing and nitrite oxidizing sequences were analyzed separately from the rest of the dataset in order to assess the nitrifying communities in the biofilters. As previously mentioned, the five types of nitrifying bacteria include AOB, AOA, NOB, anammox, and comammox bacteria. Several of the nitrifying bacteria could only be classified to the family level as *Nitrosomonadaceae.* The primary AOBs classified to genus level were *Nitrosomonas,* while the primary NOBs were *Nitrospira.* There were no AOAs nor anammox bacteria detected in the sequences. The genus *Nitrospira* has been shown to contain both NOB and comammox; however, phylogenetic analysis based on the 16S rRNA gene is not able to distinguish between the two (Sun *et al.,* 2020). Therefore, the nitrite-oxidizing population will be referred to as NOB, though it should be noted that there could be some comammox *Nitrospira.*

For the AOB, there were 81 sequences classified to the *Nitrosomonadaceae* family, and 22 sequence variants were able to be classified to genus level as *Nitrosomonas.* The most prevalent AOB was a sequence variant (SV) of *Nitrosomonas* that was not classified to species level. The second most abundant, however, was classified as *Nitrosomonas aestuarii*, which has been found to be the dominant AOB in several marine biofilters (Hupeden *et al.,* 2020; Ma *et al.,* 2020). None of the other AOB genera (at proportions higher than 0.5% of the total) were able to be classified to the species level. It has been theorized that *Nitrosomonas* are often absent or in low abundance in freshwater nitrifying biofilters (Bartelme, 2017); however, other studies have found that AOB populations in freshwater biofilters are dominated by *Nitrosomonas* (Khangembam *et al.,* 2017; Hupeden *et al.,* 2020). The other known AOB genera, *Nitrosospira* and *Nitrosococcus,* were not present in this study at proportions higher than 0.5% of the total reads. Therefore, ammonia oxidation can be attributed largely to *Nitrosomonas sp.* in both the freshwater and brackish biofilters.

The NOB had fewer unique OTUs classified at the family level, at 29. The family *Nitrospiraceae* made up 22 of these sequences, all 22 of which were classified as *Nitrospira*. The most prevalent NOB throughout the biofilters was classified to the species level as *Nitrospira defluvii*. This species of *Nitrospira* has been found to be the dominant NOB in freshwater and brackish biofilters in one study (Hupeden *et al.,* 2020). None of the other NOB genera were classified to the species level. Two other known NOB genera, *Nitrobacter* and *Nitrotoga,* were not present at proportions higher than 0.5% of the total reads, while *Nitrococcus* and *Nitrospina* were absent entirely form the sequences. Therefore, nitrite oxidation can be attributed largely to *Nitrospira sp.,* with *Nitrospira defluvii* as the most common species. *Nitrospira defluvii* has been found to grow well in high DO environments, similar to the levels maintained in these tests (Mehrani *et al.,* 2020).

3.4.2.4. Relative abundance and community percentages

Pairwise comparisons using the R package DESeq were used to investigate the genera that changed most in the biofilters. The DESeq figures show the significance of the different taxa based on one factor such as initial salinity or stage (initial or final sampling) with the significance levels indicated by the distance away from the center line. The sizes of the points indicate the relative abundance of the significant taxa.

Figure 3.8 presents the analysis between all the biofilters, compared by initial salinity. There were 72 taxa determined to have significant changes between the two experiments. Interestingly, the two major genera of nitrifiers, *Nitrosomonas sp.* (AOB) and *Nitrospira sp.* (NOB), the species that were in highest abundance that showed a significant change, and were in much higher abundance in the brackish biofilters, despite the slower nitrification recovery in the brackish test for the final 3 ppt and 20 ppt

biofilters, and lack of recovery altogether in the 33 ppt biofilters. A more specific analysis of the abundance of the nitrifiers can be found in Table 3.3.

Figure 3.8. Differential abundance between Experiment I (freshwater) biofilters and Experiment II (brackish) biofilters (microbes likely to be significantly more abundant in freshwater on left, 3 ppt on right).

Further investigation into the percentages of nitrifying communities is presented in Table 3.3, which shows the percentage of sequence reads in each sample that could be attributed to nitrifying genera. As a whole, the brackish biofilters had a higher percentage of nitrifying consortia than the freshwater samples. Furthermore, within the brackish samples, the percentage of all six nitrifying genera was the highest in the 33 ppt biofilters, which could indicate that the heterotrophs were more likely to die off. The AOB and the NOB showed similar trends in proportion between samples.

Table 3.3. Percentage of total reads per sample for top 6 nitrifying genera (able to be classified to genus level and at >0.5% of total reads), average of the three biofilters at each salinity

Family	**Genus sp.**	**Freshwater biofilters (%)**					**Brackish biofilters (%)**			
		Initial	**Final**				**Initial**	**Final**		
			0 ppt	**3 ppt**	**20 ppt**	**33 ppt**		**3 ppt**	**20 ppt**	**33 ppt**
Nitrospiraceae	*Nitrospira defluvii*	2.18	3.60	2.68	1.43	2.05	6.06	6.58	7.34	10.71
Nitrosomonadaceae	*Nitrosomonas sp.*	0.64	1.19	1.31	0.98	1.87	4.49	5.40	4.09	6.66
Nitrospiraceae	*Nitrospira sp.*	3.33	2.12	2.37	2.09	1.98	2.40	2.18	2.40	3.46
Nitrosomonadaceae	*Nitrosomonas aestuarii*	1.18	1.81	3.12	3.95	1.59	1.20	1.78	1.60	1.79
Nitrospiraceae	*Nitrospira sp.*	0.55	0.63	0.71	0.59	0.56	0.61	0.57	0.57	1.09
Nitrosomonadaceae	*Nitrosomonas sp.*	0.36	0.72	0.96	0.49	0.30	0.63	0.60	0.50	0.95

An interesting difference between the freshwater and brackish tests is the difference in their initial nitrifier proportions, particularly in *Nitrospira defluvii* and the most abundant *Nitrosomonas sp.* The proportion of *Nitrospira defluvii* nearly tripled between the starts of the freshwater test and the brackish tests, while the proportion of *Nitrosomonas sp.* increased nearly eightfold. Both *Nitrospira sp.* and *Nitrosomonas sp.* are often associated with human wastewater, which have much higher effluent N concentrations (Mehrani *et al.,* 2020; Lehtovirta-Morley, 2018). The high ammonia concentrations added in these tests may have influenced their increased proportion from the start of the freshwater tests to the start of the brackish tests The higher proportions of nitrifiers at the beginning of the brackish test could help to explain the higher initial nitrification rates in the brackish 3 ppt biofilters relative to the freshwater 0 ppt biofilters, though the theoretical maximum nitrification rates were not achieved in the brackish biofilters. This could indicate that, while there may have been a higher proportion of nitrifiers in the brackish biofilters, they performed nitrification at a slightly slower rate than the freshwater biofilters. However, the 100% water exchange was likely also a contributing factor.

The *Nitrospira defluvii* showed a large increase in relative proportion as salinity was increased in the brackish biofilters. This trend was not observed in the freshwater biofilters, in which the proportions of each nitrifier stayed relatively constant, though nitrification only occurred in the 0 and 3 ppt biofilters (Figure 3.1). This indicates that the microbes became mostly inactive or died after a transition to 20 or 33 ppt rather than changing in response to their new conditions. In the brackish biofilters, however, the relative proportion of nitrifiers increased, and nitrification had started to occur in the 20 ppt biofilters approximately halfway through the test (Figure 3.2). The increase in the percentage of nitrifiers in the brackish tests shows that the nitrifying community in the brackish biofilters responded better to a change in salinity, as they continued to grow, at least in proportion to the rest of the community. The lack of nitrification, however, shows that they were still inactive at the end of the experiment, so perhaps more

heterotrophic bacteria were lysed by the transition to saltwater. A longer test with other measures of microbial activity would need to be performed in order to determine this.

The purpose of these tests was to investigate whether brackish biofilters were better prepared for a shift in salinity in the short term. The DNA results show that the microbial community did have a higher proportion of nitrifiers, however the rate of nitrification did not reach 10 mg N L^{-1} d^{-1} in the 3 ppt test biofilters at the end of the two-week experiment, as was achieved in the freshwater experiment. This could indicate that the community needed more time to adapt to a more resilient state, or that flexibility comes at the cost of speed of the response. Once again, this could be investigated more in a longer experiment where the prior acclimation time could be varied.

The percentages in each sample of the major non-nitrifying community members are presented in Table 3.4. The most abundant OTU in all of the samples (including the nitrifying community) was a member of the family Microscillaceae which was not able to be classified to genus level, which was kept in the table due to its high abundance. However, the remainder of the table shows the taxa able to be classified to genus level. Several of the genera showed an overall decrease in proportion between the freshwater and brackish biofilters, such as *Pirellula sp.* and *Terrimonas sp.* The rest of the genera either stayed at similar proportions, or were increased in the brackish biofilters. This shows that the larger communities of heterotrophs in the biofilters may have been able to "bounce back" after the shift in salinity due to the larger number of members, while the less abundant species (<0.5% of the total reads) were lysed almost entirely.

Table 3.4. Percentage of total reads per sample for top 9 non-nitrifying genera (able to be classified to genus level and at >0.5% of total reads, 16 taxa were left out), average of the three biofilters at each salinity

Family	Genus	Freshwater biofilters (%)					Brackish biofilters (%)			
		Initial	Final				Initial	Final		
			0 ppt	3 ppt	20 ppt	33 ppt		3 ppt	20 ppt	33 ppt
Microscillaceae	*NA*	5.30	4.39	5.05	4.53	5.73	3.16	4.49	3.62	6.06
Phycisphaeraceae	*SM1A02*	1.95	1.71	2.82	2.55	3.16	3.23	3.88	2.34	3.91
Hyphomonadaceae	*Hirschia*	2.36	1.36	1.54	1.66	2.20	1.99	1.86	1.56	2.03
Pseudohongiellaceae	*Pseudohongiella*	1.79	1.16	1.37	1.40	1.50	0.76	0.92	0.88	2.00
Pirellulaceae	*Pirellula*	0.87	0.96	1.58	1.99	0.98	0.55	0.71	0.71	0.37
Chitinophagaceae	*Terrimonas*	1.12	1.17	1.03	1.45	1.21	0.41	0.47	0.42	0.41
Microscillaceae	*OLB12*	0.35	0.40	0.40	0.40	0.75	1.12	1.24	1.20	0.60
Comamonadaceae	*Hydrogenophaga*	0.51	0.40	0.75	0.55	0.59	0.61	0.63	1.22	1.62
Oceanibaculaceae	*Oceanibaculum*	0.56	0.38	0.37	0.38	0.74	0.66	0.57	0.48	1.40

3.4.2.5. Further pairwise comparisons

A DESeq analysis was performed on the initial and final freshwater samples maintained at 0 ppt to investigate which taxa were present at the start of the test that either did not survive the shift in ammonia concentration or increased in abundance over the two-week test. These results are presented in Figure 3.9. Several notable genera that were significantly more likely to appear in the initial biofilter samples were *Paraglaciecola*, *Maricaulis*, and an unclassified genus in the family Saprospiraceae. *Paraglaciecola* is often associated with marine algae (Wang *et al.,* 2020), so the move to a new freshwater filter explains the decrease in abundance over the two-week test. The family Caulobacter, to which *Maricaulis* belongs, generally contains species of bacteria that thrive in oligotrophic environments and consume dissolve organic matter (Abraham *et al.,* 2002). Members of Saprospiraceae are often found in RAS in which Atlantic salmon are grown, as they have been found in the gills (Slinger *et al.,* 2021). Therefore, the decrease in abundance of *Maricaulis* and Saprospiraceae can be explained by the lack of fish waste constituents and organic carbon added to the test. Furthermore, it is likely that only the most competitive bacteria survived the experiments. All of the taxa that were significantly more likely to be present in the final samples had relatively low sequence abundances (<50).

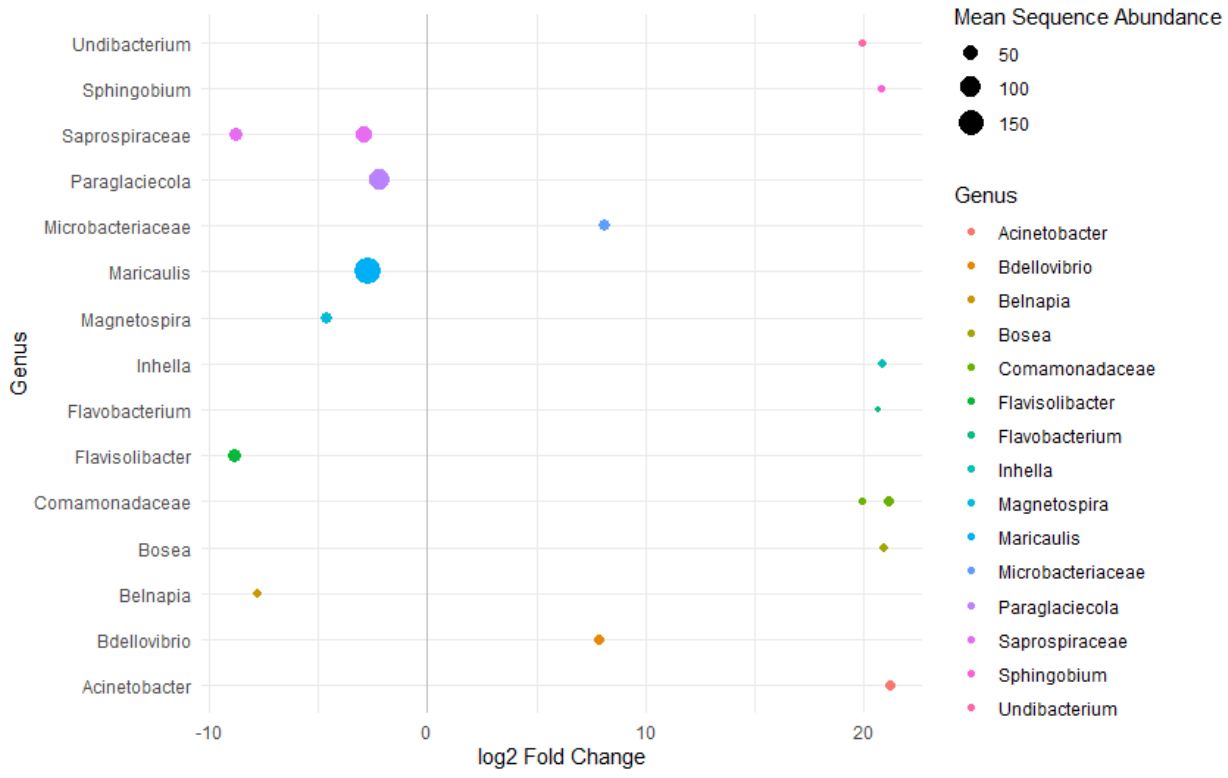


Figure 3.9. Differential abundance for initial freshwater samples and final freshwater samples maintained at 0 ppt.

Finally, a third DESeq analysis was performed in order to show the taxa that were significantly changed in the 3 ppt biofilters over the month-long period of acclimation to saltwater. For the purposes of this comparison over the 30-day period that stretched from the end of the first test to the beginning of the second test, the 3 ppt biofilters at the end of the freshwater test will be referred to as the initial 3 ppt biofilters, and the samples at the beginning of the brackish test will be referred to as the final 3 ppt biofilters.

The family of bacteria with the highest significant abundance in the initial 3 ppt biofilters was Sphingomonadaceae, Members of this family have been found in soils, corals, eutrophic waters, plant surfaces, and more; they are also chemoheterotrophic and often associated with the formation of biofilms

(Vaz-Moreira *et al.,* 2011). This helps to explain the decrease in abundance from the freshwater biofilters to the brackish biofilters, as the microbes were deprived of organic carbon. The second most abundant bacterium that was present in the initial samples but not the final samples was *Chthoniobacter sp.*, a genus which has been cultured from rye grass (Sangwan *et al.,* 2004). This was likely present in the fish feed and decreased due to the lack of addition of fish waste constituents. Another family that was more present in the initial samples for similar reasons is Vicinamibacteraceae, which is a family whose members often grow with sugars, organic acids, or nucleic acids (Huber and Overmann, 2018). These results confirm that heterotrophic microbes were more greatly affected by the experimental conditions than the nitrifiers.

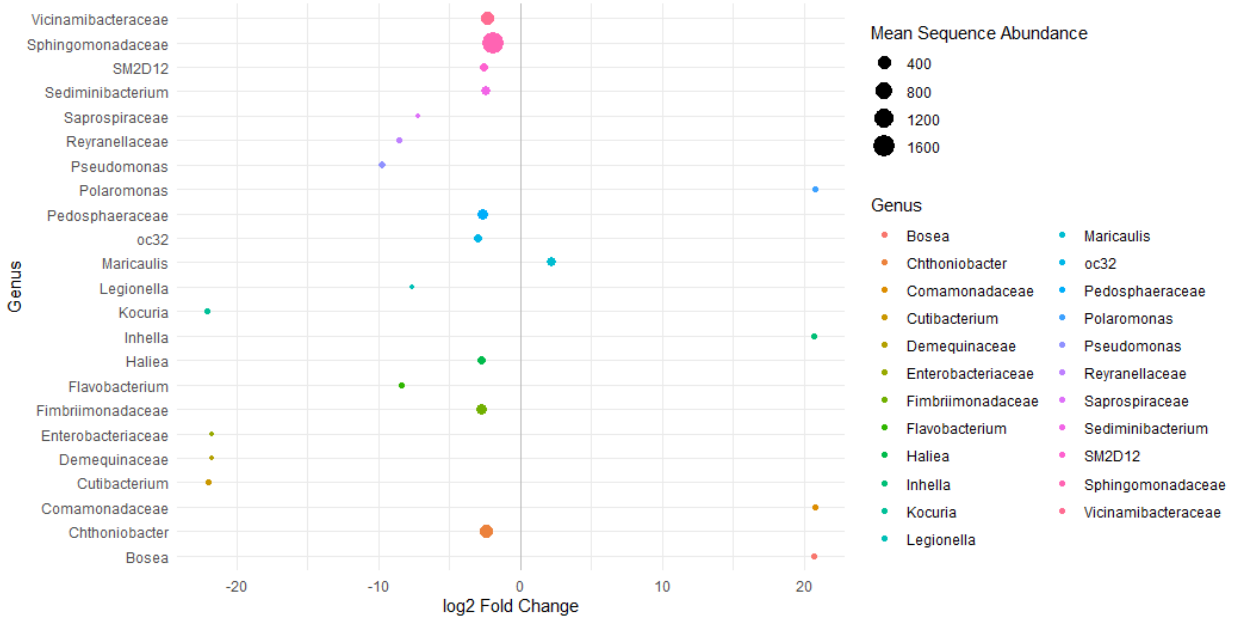


Figure 3.10. Differential abundance for final freshwater samples maintained at 3 ppt and initial brackish samples maintained at 3 ppt.

3.4.2.6. Feature prediction

Feature prediction is similar to DESeq in that it can give insight into the more abundant taxa in certain biofilters; however, rather than showing the significance level and sequence abundance, tiles are

colored for each taxon based on the likelihood that the particular taxa would be found in that sample. Feature prediction revealed some stark contrasts between the freshwater biofilters and the brackish biofilters, though several of the bacteria were only able to be classified to the Family level. Figure 3.11 shows the top 30 significant OTUs, compared between samples. Interestingly, none of the top 30 significant OTUs belonged to nitrifying genera. This further indicates that the lack of nitrification in the brackish biofilters may be attributed to inactivation of the nitrifiers rather than lysing.

The top eight sequence variants displayed on the graph were detected mostly in the freshwater biofilters. In particular, *Bdellovibrio sp.* was detected in all freshwater biofilters but detected in only one of the brackish biofilters. *Bdellovibrio sp.* is a type of predatory bacteria that preys on other gram-negative bacteria (Hobley *et al.,* 2006). This genus and similar microbes are known as *Bdellovibrio* and like organisms (BALOs), and they have been found in both freshwater and brackish RAS (Kandel *et al.,* 2014). While these bacteria may have applications against pathogens in aquaculture, they also may reduce biofilm structures (Mookherjee and Jurkevitch, 2022). The relative abundance of *Bdellovibrio sp.* in the freshwater biofilters decreased slightly as salinity increased in the experiment beginning from the freshwater population, and they were absent from the 3 ppt-adapted biofilters, which had more time to develop in response to experimental conditions. This indicates that this strain was not well suited to an abrupt change in salinity, and possibly the lack of added organic matter in the tests. This could be a significant factor as BALOs continue to be assessed for their potential for reducing the abundance of drug-resistant pathogens in RAS (Mookherjee and Jurkevitch, 2022; Waso *et al.,* 2021).

Several of the other bacteria with higher abundance in the freshwater biofilters able to be classified to genus level include *Iamia sp.*, *Turneriella sp.,* and *Flavobacterium sp.* There is little information on *Iamia sp.*, as it has only been found in sea cucumbers (Kurahashi *et al.,* 2009). *Turneriella* has only been found to contain one species, *Turneriella parva,* which has been associated with the gut of Tilapia

(Abdelhafiz *et al.,* 2021). *Flavobacterium sp.* are bacteria often associated with infections in fish; however, they can be found on the external surfaces of healthy fish as well (Verma and Rathore, 2015). Thus, it is likely that the lack of fish wastewater supplied to the biofilters during the tests resulted in the decrease of *Turneriella sp.* and *Flavobaterium sp.* between the freshwater tests and the brackish tests. Furthermore, replication or maintenance of the population of these microbes may have been hindered by more competitive bacteria in the biofilms.

The only bacteria that were significantly predicted to be of higher abundance in the brackish biofilters and able to be classified to genus level were *Mesorhizobium sp.* and *Nitrosomonas sp. Mesorhizobium sp.* have been classified as symbiotic bacteria that help to fix nitrogen in legumes, though they have also been found in freshwater RAS and are thought to improve water quality (Shan *et al.,* 2022). Interestingly, *Nitrosomonas sp.* was the only nitrifying OTU significantly predicted to be in higher abundance in the brackish biofilters. This shows that, while both *Nitrospira sp.* and *Nitrosomonas sp.* were resistant to the salinity changes, the *Nitrosomonas sp.* may have been more resilient, and are more likely to be of higher abundance in brackish biofilters.

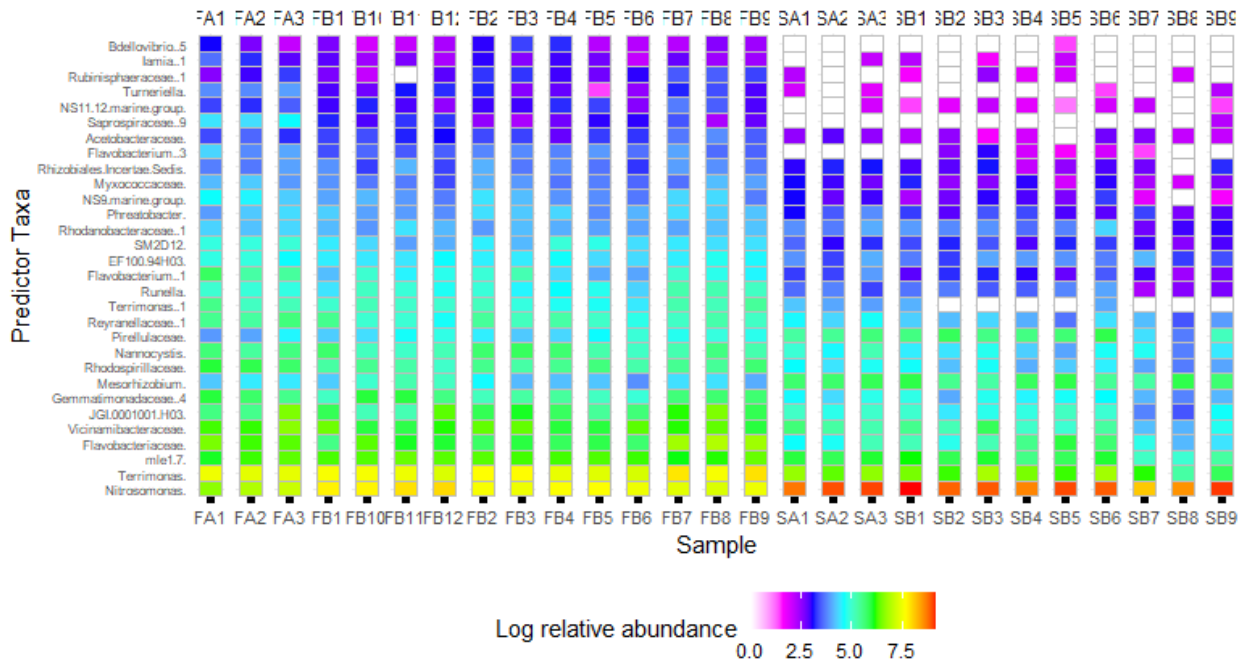


Figure 3.11. Feature prediction of taxa in each biofilter, top 30 most significant taxa.

3.5. Conclusions

The results of this bench-scale study showed that maintaining biofilters with a small amount of salinity can help them recover nitrification activity more quickly in response to a change in salinity, though the short-term benefits may not outweigh the cost of running a system with salinity. Freshwater biofilters did not recover nitrification activity within two weeks after a shift to 20 or 33 ppt salt, and their nitrifying communities were not significantly different after the two-week test. Brackish biofilters, maintained at 3 ppt for one month prior to a more substantial increase in salt concentration, were able to oxidize approximately 1 mg N L^{-1} d^{-1} ammonia 14 days after a shift to 20 ppt. The time scale of these experiments did not allow for nitrification recovery to be observed in the 33 ppt biofilters. Overall, there was a higher proportion of both AOB and NOB in the brackish biofilters than the freshwater biofilters. The dominant AOB found in all samples was *Nitrosomonas sp.*, while the dominant NOB was *Nitrospira defluvii.* Ordination plots showed that the samples did not cluster perfectly within replicates, though they were

significantly different based on initial salinity. Differential abundance plots and feature prediction showed a higher relative abundance of nitrifiers in the brackish samples, likely due to lysis of many of the heterotrophic bacteria present in the freshwater samples. While some nitrification recovery was shown in brackish biofilters shifted to higher salinities, a longer series of tests could possibly fully characterize the effects of acclimation to salinity and help to fully understand the microbial community dynamics.

CHAPTER 4

CONCLUSIONS

4.1. Summary

In summary, recirculating aquaculture systems have high potential to provide a sustainable seafood protein source to a major percentage of the world's population as the industry grows, and different process parameters should be investigated to make the system as efficient as possible. Nutrients are a major component of RAS wastewater, and these should be removed prior to discharge to avoid eutrophication in waterbodies receiving the discharge. Phosphorus can be removed from the effluent through adsorption, a process that binds the phosphorus to a solid and removes it from water. Ammonia can be transformed to nitrate by microbes, a process that involves the oxidation of ammonia to nitrite, and then nitrite to nitrate. When adsorption and nitrification are used in RAS, they can be very efficient at mitigating these nutrients and ensuring the health of the fish. However, many factors can influence these processes; in particular, the varying salinity levels required by anadromous fish such as Atlantic salmon can greatly affect the efficiency of nutrient mitigation. Therefore, the two *in vitro* studies in this thesis investigated the influence of salinity (and other factors) on phosphorus adsorption and biofilter nitrification.

The results of the adsorption study on the aluminum oxide-based adsorbent, RhizoSorb®, showed that salinity, pH, and coexisting ions all affect the adsorption process. For practical use in the RAS industry, these factors should all be considered during system design. As many treatment systems are more likely to use up-flow column treatment than batch treatment, the breakthrough column tests, are particularly helpful for making decisions about system design, as they showed the influence of different concentrations of phosphorus, salinity, masses of adsorbent, and contact time. These results are supplemented by the batch tests, which showed the influence of contact time and phosphate

concentration on the removal efficiency of the adsorbent. The time scale of these tests, measured in days rather than hours, showed that the adsorbent could be well applied to an RAS with low phosphorus concentration. However, exact process parameters should be determined based on the effluent P concentration and the flow rate of the water in a particular system. Finally, the pH of the water should be considered during system design, as the results of this study showed that the adsorbent may function better in water with lower pH. Fortunately, most systems are kept at a pH of around 7 for the health of the fish; therefore, the pH should be maintained as such before the water is treated with this adsorbent.

The results of the nitrification study on the different biofilter types showed that brackish biofilters may be slightly better equipped to deal with changes in salinity; however, it does not seem that the time scale of the tests performed in this study allowed for a stable shift in the microbial population. Other factors influenced the nitrification performance of the microbes, including the added ammonia concentrations. However, shifting from 0 ppt to 3 ppt showed a lag time of only about a week before the nitrification rate began to match that of the biofilters maintained at 0 ppt, and the nitrifying consortia did not display major changes. Moving from 0 to 20 ppt showed a complete inactivation of the nitrifying bacteria, though moving from 3 to 20 ppt showed a slight recovery of the nitrifiers by the end of the two-week long test; once again, the nitrifying consortia did not display major changes. Interestingly, the nitrifying consortia did show an increase in proportion between the freshwater biofilters and the brackish biofilters. This may be attributed to inactivation of the nitrifiers, and lysis of many of the heterotrophs that coexist in the biofilm during the month-long maintenance period during which the brackish biofilters were operated at 3 ppt. Practically, brackish biofilters may help to decrease the time in which salinity transitions can be made; however, the cost of operation of a 3 ppt system (as opposed to a freshwater system) may outweigh the benefits, as the 3 ppt brackish biofilters showed an increase in nitrification after approximately 10 days when transferred to 20 ppt. In order to make practical decisions about RAS

design, more work should be performed to effectively characterize the best way of transitioning biofilters to different salinity levels.

4.2. Future work

Each of the two projects reported in this thesis have potential for future work. The RhizoSorb® was investigated for its applicability to adsorb phosphorus from water in batch kinetic, batch equilibrium, and column tests. One area of influence that could still be investigated is the relative contributions of coexisting ions, pH, and wastewater constituents, by controlling the pH in both DI and wastewater experiments. Furthermore, the tests performed in this study were lab-scale, and a full-scale test could be performed to adequately observe the behavior of the adsorbent in scenarios with much higher flow rates, masses of adsorbent, and different contact times. Addressing the applicability of the phosphorus-saturated adsorbent was not within the scope of the study. Thus, future work could involve testing and measuring the desorption properties of the adsorbent in water or soil environments. Additionally, this adsorbent could be investigated for use in other areas. Human wastewater is high in nutrient concentration; therefore, this adsorbent could be applied here. Agricultural runoff is often not collected and treated, resulting in the major issues discussed in Chapter 1. If this runoff was collected and treated with RhizoSorb®, there is potential for the wasted nutrients to be collected and redistributed. This is another area that could be investigated in more depth.

The second study presented in this thesis investigated varying salinity conditions on nitrifying biofilters. The effects of RAS process parameters on biofilters is a relatively new area of research; most papers in this area have only been published within the last 5 years. Therefore, there are many facets of biofilter design that may be further studied. The goal of this study was to observe the short-term effects of salinity changes on freshwater versus brackish biofilters. The first factor that could be investigated

further is the effect of ammonia concentration on biofilter efficiency. The concentration of ammonia added to the biofilters each day was 10 mg L^{-1}, a much higher dosage than the 0.5 mg L^{-1} concentration fed to the biofilters before the start of the test. The freshwater control biofilter had a lag time of about 4 days before the nitrification rate per day began increasing. Varying the levels of ammonia concentration and assessing nitrification in freshwater or saline environments is an area of research that could be pursued.

Another factor that could be looked into more is the effect of fish waste constituents and organic carbon. The tests in this study were performed with freshwater and artificial saltwater, combined to make up appropriate salinities where necessary. Therefore, when the tests began, the biofilters were deprived of the dissolved organic carbon that comes with fish waste. This factor could be controlled in another experiment, and the effects of salinity changes on these biofilters could be further assessed. The results of such a study could be compared with the results of the study presented here, as previous studies have shown that biofilters deprived of organic carbon show resilience to increases in carbon concentration (Navada *et al.,* 2020). It may be possible that the resilience built by the lack of organic carbon could carry over and result in resilience to changes in salinity; however, tests would need to be performed to confirm or deny this hypothesis.

More factors that could be investigated in future studies include research into the rates of change of salinity on freshwater and brackish biofilters. Studies have addressed this for freshwater biofilters (Navada *et al.,* 2022), but brackish biofilters may potentially react differently to different rates of change in salinities. Temperature, pH, dissolved oxygen, or alkalinity content may also be varied in any of the above-mentioned tests to assess their effects on nitrifying biofilters.

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BIOGRAPHY OF THE AUTHOR

Eliza Costigan was born in Lewiston, ME on July 7, 1998. She spent most of her childhood in Raymond, Maine, before she moved to the Bangor area and attended John Bapst Memorial High School, from which she graduated in 2016. She attended the University of Maine and graduated in 2020 with a Bachelor's degree in Civil and Environmental Engineering, with a focus in Environmental Engineering and a minor in Business Administration. After graduating, she began her Master's degree in Environmental Engineering at the University of Maine in the summer of 2020. After receiving her degree, Eliza will be moving to Boston to pursue a PhD in Environmental Engineering from Northeastern University. Eliza is a candidate for the Master of Science degree in Civil and Environmental Engineering from the University of Maine in August 2022.