ABSTRACT

Title of Dissertation: BAHAMIAN OOLITIC ARAGONITE SAND IMPACT ON WATER QUALITY AND MITIGATION OF PHOSPHATE AND PHOSPHORUS REMOVAL AND RECOVERY IN RECIRCULATION AQUACULTURE SYSTEMS

Steven R. Rodgers, Doctor of Philosophy, 2021

Dissertation directed by: Professor A. R. Place, University of Maryland Center for Environmental Science at the Institute of Marine and Environmental Technology

Recirculating aquaculture systems (RAS) require management of water conditions to ensure animal health and limit nutrient discharges. Oolitic aragonite sand (OAS) forms from whiting events off the coast of the Bahamian Islands is a sustainable, renewable and effective in controlling water quality. Cyanobacteria mediate the precipitation of aragonite by capturing CO_2, internally forming CO_3^{2-}, which reacts with Ca_{2+} in seawater forming $CaCO_3$ precipitations.

Studies in freshwater, brackish and marine waters maintained stable pH and alkalinities. Initially, OAS removed phosphate rapidly, slowing afterwards. The OAS removed phosphate at rates of 716, 705 and 215 mg PO_4/ kg OAS for freshwater, brackish and marine water, respectively. A system with daily P additions showed a removal capacity of 77.8 mg P /kg OAS. Treatment of phosphorus exposed OAS with 1.0% and 2.0% citric acid solutions show phosphate removals ranging from 17.3% to 93.5%. The citric acid increases the OAS surface area 1.66 times to 4.628 m^2/g OAS, confirmed by SEM. Microbiome analysis show similar bacterial phyla exist on the naïve OAS and the OAS used in different salinities.

Under anaerobic conditions, the control of system conditions were favorable for denitrification and anammox processes to occur. In freshwater, a loss of 215.8 gram of nitrogen (a loss of 90.5%) of the added nitrogen to the system occurred. In marine conditions, a loss of 253.04 g nitrogen, representing an 87.6% loss, occurred. Microbiome analysis identified phyla known to function as denitrifiers, though lacking known phyla for anammox bacteria. Losses of nitrogen in both salinities is likely due to denitrification, as oppose to anammox.

OAS in RAS holding Eastern and Pacific oysters, showed dissimilar responses. The water quality remained in acceptable ranges for oyster growth. The survival in Eastern oysters (≥80%) contrasted with the Pacific oysters (≤56%). Weight increases occurred only with the Eastern oysters. Both species shows increases in shell length, width and height, but unchanged or decreases in weight. Reduced somatic growth and limited shell development occurred, perhaps due stresses from nitrogen spikes in the systems. OAS shows no positive advantage with oyster growth.

BAHAMIAN OOLITIC ARAGONITE SAND IMPACT ON WATER QUALITY AND MITIGATION OF PHOSPHATE AND PHOSPHORUS REMOVAL AND RECOVERY IN RECIRCULATION AQUACULTURE SYSTEMS

by

Steven R. Rodgers

Dissertation submitted to the Faculty of the Graduate School of the

University of Maryland, College Park, in partial fulfillment

of the requirements for the degree of

Doctor of Philosophy

2021

Advisory Committee:
Professor A. R. Place, Chair
Professor A. Lazur
Professor D. E. Terlizzi
Assistant Professor C. Burge
Associate Research Professor E. Schott

Preface

Ever since I can remember, I have been interested in aquatic environments and fish. This early fascination lead me to a Bachelor's Degree in marine biology. Soon after college, I started in a technical position at a water quality laboratory near York, PA. With gained experience, I moved onto a positon at an Environmental Engineering firm in Harrisburg, PA. The idea of earning a Master degree in marine biology excited me, and I had a good friend, Mark, that was pursuing this path already. With that, I moved to Florida to attend the Florida Institute of Technology where I earned my Master's degree in 1992. The following year I started with COMB which latter morphed into the Institute of Marine and Environmental Technology. Over the years, I interacted and assisted many researchers in their work, prompting me to toy with the idea of working towards a PhD. At the age of 45, I started with my first class in the fall of 2010 and continued to the culmination with this dissertation. I hope others find this work insightful, leading to improvements in management of recirculating aquaculture systems. Dr. Place, Dr. Burge and Ernest Williams have been instrumental in providing guidance and reviewing of my developing dissertation. With their experience and input, my draft dissertation transformed into a professional document that I am proud to present to the public. I am deeply thankful for all of their assistance in helping me to refine my dissertation. I truly appreciate all of their effort, time and support.

Dedication

To my wife, Sharon, and my children, Derik, Courtney and Allison. Thanks for allowing me the time to pursue this achievement. I know the time spent away from all of you has been challenging over the years. I do appreciate your understanding or at least your tolerance in my studies. I love you all and dedication this dissertation to you.

Acknowledgements

I would like to thank my advisor Dr. Place for all of his help and believing in me from the start. Thanks to my committee members Drs. Lazur, Burge, Terlizzi and Schott for their support and guidance. Drs. Woods, Love and Summerfelt who provide direction early on in my pursuit. I am grateful to my co-workers Chris Tollini, Ernest Williams, John Stubblefield, Kayla Fairfield, Robert Bennington, Kevin Bishop, Jorge Gomerjurado, Odi Zmora and Justine Bedard for their assistance with the project and support. Thanks to the graduate students Natalie Rivlin, Tori Agnew and Mariah Kachmar in their help with data collection and husbandry. The technicians in the water quality lab, Kim Gaeta and Nick DeMetrick quickly analyzed samples and were supportive as well. Thanks to the ARC interns Benjamin Kuffel, Oliva Wikstrom, Emmanuel Chong Qui, Rebekah Skrivanek, and Alex Nadobny whom helped with all sort of measurements and maintenance. Thanks goes to Patrick Hudson of True Chesapeake Oyster Company for providing Eastern oysters. Thanks for Lucas Sawyer of Hog Island Oyster Company for supplying Pacific oyster. The MEES Program Coordinator, Hang Nguyen, and MEES Director, Dr. Timothy Canty, were instrumental in assisting with extensions, deadlines, forms and most all positive support. I am grateful for funding received from the IMET MEES Student Enhancement Stipend and the University of Maryland's Dean's Fellow Grad.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

CF – Canister Filter

cm/s – centimeters per second

FSF – Fluidized Sand Filter

GBWP - George Barley Water Prize

HRT – Hydraulic Retention Time

g/m2/hr – grams per square meter per hour

IMTA - Integrated Multi-Trophic Aquaculture

lpm/m2 – liters per minute per meter squared

LSI – Langelier Saturation Index

m/s – meters per second

mg/l – milligrams per liter

mg/g – milligrams per gram

mm - millimeter

mM - millimolar

mts – metric tons

OAS – Oolitic Aragonite Sand

RAS - Recirculating Aquaculture System

SNDPR - Simultaneous Nitrification Denitrification and Phosphorous Removal

TSS - Total Suspended Solids

µm – micrometer or micron

µM – micromolar

Chapter 1: Dissertation Introduction

Aquaculture is an expanding contributor to the food supply and particularly of the seafood supply chain, while addressing sustainability and minimizing their environmental footprint (Ahmed et al., 2019, Boyd et al., 2020). A growing sector in aquaculture is the development and use of recirculated aquaculture systems (RAS) (Figure 1.1). RAS systems have a number of advantages over traditional pond, raceway and net pen aquaculture. These advantages incorporate environment controls, improved growth rate, standardized harvest cycles, low water use, high densities, scale ability, close to markets, use of high valued species and reduction of nutrient releases (Zohar et al., 2005, Timmons & Ebeling, 2010, Tal et al., 2009, Davidson et al., 2016). Increased use of integrated aquacultures, which involves combination of species or plants for efficient utilizations of resources and the use of RAS is the direction aquaculture must move to provide sustainable, environmentally friendlier solutions to meet growing food production needs (Ahmed et al., 2019). Integrated Multi-Trophic Aquaculture (IMTA) may be the key

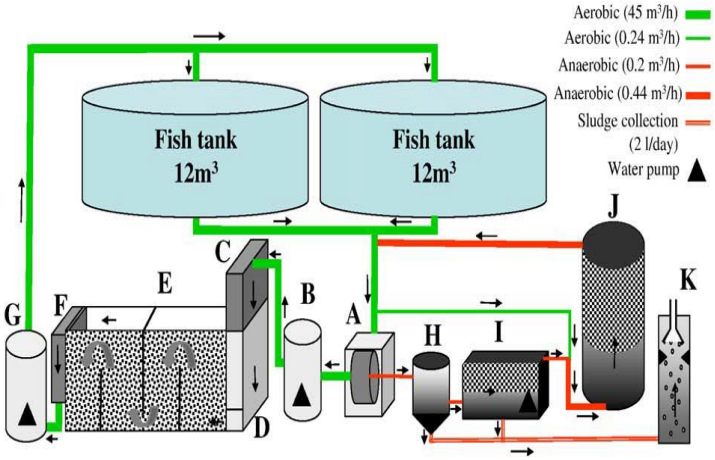


Figure 1.1 An intensive recirculating aquaculture system at the Institute of Marine and Environmental Technology in Baltimore, MD. Components include: A. drum filter, B. pump sump, C. CO_2 stripper, D. protein skimmer, E. biofilter, F. low head oxygenator, G. return sump to tanks, H. sludge collection tank, I. sludge digestion tank, J. denitrification unit, K. methane generator. Image courtesy of Tal et al. 2009.

enhancement to aquaculture to maintain its sustainability (Chopin et al., 2012, Troell et al., 2009). Using the effluent water from RAS as a resource to generate additional sellable products instead of wasted in discharges is an efficient approach (Chopin et al., 2012). Chopin, (2006) comments that IMTA is a simplified ecosystem, by including a suspension-feeding organism and an extractive algae species to consume a portion of the nutrients released from net pen operations. By harnessing additional trophic levels represented by bivalves, expansion of IMTA can be achieved (Chopin, 2006). Even though IMTA developed with the idea to curb nutrients impacts for net pen aquaculture, lessons learned from this application may be suitable for RAS. In European countries there is a push for increased use of RAS due to environmental and sustainability concerns (Badiola et al., 2012).

With all of the improvements achieved by RAS, the continued recycling of the water does pose challenges requiring management in order to provide an optimal, low stress environment for fish and invertebrate growth (Timmons & Ebeling, 2010). Ahmed et al (2019) point to some long-standing concerns with aquaculture regarding land and water use, habitat destruction, ecosystem functions and source of environmental nutrients. Some of the ongoing RAS water quality concerns and potential environmental influences are pH and alkalinity control and nitrogen and phosphate levels within systems and effluent streams (Timmons & Ebeling, 2010, Gichana et al., 2018).

A key limitation to continued marine aquaculture growth with RAS is the treatment and disposal of marine solids (Christianson et al., 2016, Sharrer et al., 2007). Formation of solids in the RAS occur by uneaten feeds, feces, and bacterial growths (*Manual on Effluent Treatment in Aquaculture: Science and Practice Aquaetreat-Improvement and Innovation of Aquaculture Effluent Treatment Technology*, n.d., Cripps & Bergheim, 2000). Timmons & Ebeling (2010) report that 25% of the feed fed to fish generate the total suspended solids (TSS) portion of the effluent stream on a dry weight basis. Feeding generates solids, which require removal from the culture tanks by mechanical filtration (Cripps & Bergheim, 2000). Maintenance of high quality water conditions should focus on TSS removal in order to avoid increasing biochemical oxygen demand because of prolonged solids leaching (Chen et al., 1997, Chen et al., 2002). With marine effluents, the resulting isolated solids often need to be disposed of in landfills, due to the accumulated salts, which render them unusable for land application. Addressing of the disposal of marine sludge due to local regulations and financial cost are issues for aquaculture farm management (Sharrer et al., 2007). Zhou et al. (2014) have shown that the Pacific oyster *Crassostrea gigas* and the blue mussel *Mytilus galloprovincialis* are able to remove TSS from aquaculture wastewater at a rate 3.73±0.27 and 2.76±0.20 times, respectfully, higher than just sedimentation alone. Zhou et al. (2014) showed settled solids on the bivalve culture units were significantly lower in organic matter and C and N concentrations as compared to controls. The authors suggest that filter feeding bivalves like oysters and mussels have the potential to reduce suspended solids in aquaculture effluents (Y.

Zhou et al., 2014). Application of RAS solids in anaerobic digestion processes and as a carbon source in denitrification removes accumulated solids from RAS, achieving solids reduction and nitrogen removal (Mirzoyan et al., 2010, Suhr et al., 2013).

The balance of pH and alkalinity are essential in providing stable water conditions for biological filtration to control total ammonium nitrogen and nitrite as well as for efficient CO_2 stripping (Summerfelt et al., 2015, Skov, 2019). Automatic dosing of sodium bicarbonate can control pH and alkalinities; one must consider that a fault in the system can lead to stressful water quality conditions quickly (Summerfelt et al., 2015).

Phosphorus is an essential element in living organisms, residing in bones, nucleic acids, ADP/ATP and proteins (Smil, 2000). The industrial production of phosphate has been pivotal in the expansion of increased food production starting in the nineteenth century and continuing to the present (Smil, 2000). Typically, phosphorus is in low supply in freshwater, brackish and coastal waters, but increases in concentrations can produce eutrophic conditions swiftly due to rapid growth and death of aquatic plants and algae (Smil, 2000). Elevated levels of phosphorus and nitrogen in waterways can lead to hypoxia zones, harmful algal blooms, and human health issues and negatively influence local economies (*The Issue | Nutrient Pollution | US EPA*, n.d.). Phosphorus content in sewage treatment facilities can range from 10-25 mg P/l, subsequent to aeration during trickling filter treatment to phosphates (Smil, 2000).

Historically, oversight of nutrient releases into the environment is limited, as with non-point nutrients on the Eastern Shore of the Chesapeake Bay (Staver et al., 2001). The state of Virginia had regulated agriculture fertilization rates only on a nitrogen basis and not phosphorus in the application of municipal sewage sludge and poultry manure, leading to excess phosphorus amounts beyond the plants requirements, which result in polluted water bodies (Staver et al., 2001, Land, 2012). As an improvement to this shortsightedness, the Virginia Administrative Code 4VA50-85-140 currently requires a nutrient management plan for nutrient amendments such as manure and biosolids onto agricultural fields, which account for nitrogen, phosphate and potash levels (Virginia Administrative Code Title 4. Conservation And Natural Resources Agency 50. Virginia Soil And Water Conservation Board Chapter 85. Nutrient Management Training and Certification Regulations, n.d.). In order to reduce the excessive nutrients releases into the Chesapeake Bay watershed, the author urges bans of these fertilizers on fields (Land, 2012). In support of this point, Smil (2000) calls attention to: erosion due to urbanization and farming, fertilizing agriculture fields with organic matter, high phosphorus containing sewage sludge and use of inorganic fertilizers, as human generated sources of phosphorus accelerating release in the environment.

The aquaculture discharges can be a source of phosphorus into the environment. As an example, flow through rainbow trout raceway units in Southern, Idaho typical have individual flows of 946 – 10,200 l/min (True et al 2004a). Measurements of total phosphorus concentrations of 0.09 ±0.01 mg P/l with 62% dissolved and 38% from

solid particles may occur in these flows (True et al., 2004b). In the studied farms, measured raceway velocities were below the rate that prevents settlement of solids (0.1-0.6 m/s). Therefore, a portion of the solids remaining in the culture area likely lead to increase dissolved phosphorus in the effluent instead of remaining on solids making removal less difficult (True et al., 2004b). The impact of aquaculture effluent can develop a persistent storage reserve in the environment leading to long-term eutrophication conditions after the phosphorus additions have stopped or decreased. An investigation of sediment cores extracted from the Sancha reservoir in southwest China, reveal high concentrations of phosphorus forms within sediment layers corresponding to periods when high amounts of phosphorus inputs from aquaculture operations in the lake occurred (Jia et al., 2015).

In order, to capture phosphorus from sources and the environment, mitigation efforts employ a number of methods and technologies, each with their own limitation. Improvement of fish feeds by inclusion of plant source replacements for fishmeal, the availability of phytase to increase phosphorus retention and meeting the minimal phosphorus requirement for the species being culture result in decreases of phosphorus levels in aquaculture wastewater discharges (Lazzari & Baldisserotto, 2008). Enacting of phosphorus effluents limits on aquaculture operations by the use of Total Maximum Daily Load regulations are also productive (Sibrell & Kehler, 2016). The use of flocculants can precipitate between 70- 90% of the contained phosphorus within sewage treatment facilities (Smil, 2000). In rainbow trout culture tanks, at the Northeast Fishery Research Center in Pennsylvania, Sibrell & Kehler

(2016) demonstrated the use of aluminum and iron oxide containing residuals from acid mine drainage neutralization efforts as a low cost material for phosphorus removal using a fixed bed process. The work shows phosphorus removal rates between 50-70% with an average phosphorus concentration of 0.064 mg/l in the influent flow (Sibrell & Kehler, 2016). The mine drainage residual material proved capable of regeneration with a sodium hydroxide method, albeit with limitations (Sibrell & Kehler, 2016). Demonstrations of the ability of bentonite clay pillared with polyhydroxy aluminum was capable of removing 11.85 mg/g of phosphorus from aquaculture discharges of varying salinities but less effective at higher salinities (Kumararaja et al., 2019). The use of aluminum and lanthanum compounds for phosphorus treatment in freshwaters has become increasingly common. Regular monitoring of the environmental concentrations of aluminum and lanthanum is important in avoiding an unintentional human health risk (D'Haese et al., 2019). Development of cross-linked polymeric hydrogels by Kioussis et al. (1999) demonstrated their ability to remove phosphate from low salinity aquaculture waters. The hydrogels show a capacity to remove as much as 47 mg/g of hydrogel and represent a possible aquaculture solution for phosphorus removal (Kioussis et al., 1999).

Removal of phosphorus from discharges is best accomplished when it is in particulate form. Experimental results show a phosphorus reduction of 11% in effluent from a flow through aquaculture operation occurred using reticulate foam media (True et al., 2004a). The foam removed particulate phosphorus solids in the effluent flow to less

100 µm with a head loss of 150 mm. The flow of flow-through systems is 47 and 800 greater than pond or RAS production systems, respectively (True et al., 2004a). The proposed incorporation of foam filtering in flow-through raceway operations must take into consideration the maintenance efforts required for the treatment.

Phosphorus is a limited resource. In contrast with the importance of phosphorus to living organisms, crop production and the environment, the planets biomass contains minor amounts of this element (Smil, 2000). The cycling of phosphorus between living organisms and the soils is highly efficient, though eventually this phosphorus is lost through transportation to the oceans by water flows (Smil, 2000). Rock deposits are the source for phosphorus, though a large measure of the element often become quickly fixed and unavailable for plant growth (Smil, 2000). Most of the natural sources of phosphorus in rock and sediments are not exploitable for commercial extraction (Smil, 2000). China, the United States and Morocco produce about 2/3 of the world's phosphate rock, a raw source for phosphorus (Husemann et al., 2018). The known deposits of phosphate rock are finite with expectations ranging from 50 to 130 years of remaining supplies at projected usage (Husemann et al., 2018).

One needs to understand the chemistry of the carbonate system to appreciate its effect on the pH and alkalinity on RAS. The carbonate system incorporates carbon through a series of tightly interlinked equations in the forms of carbon dioxide (CO_2), carbonic acid (H_2CO_3), bicarbonate ions (HCO_3^-) and carbonate ions (CO_3^{2-}), which are temperature, pH and salinity dependent (Timmons & Ebeling, 2010, Zeebe, 2012, Boyd et al., 2016).

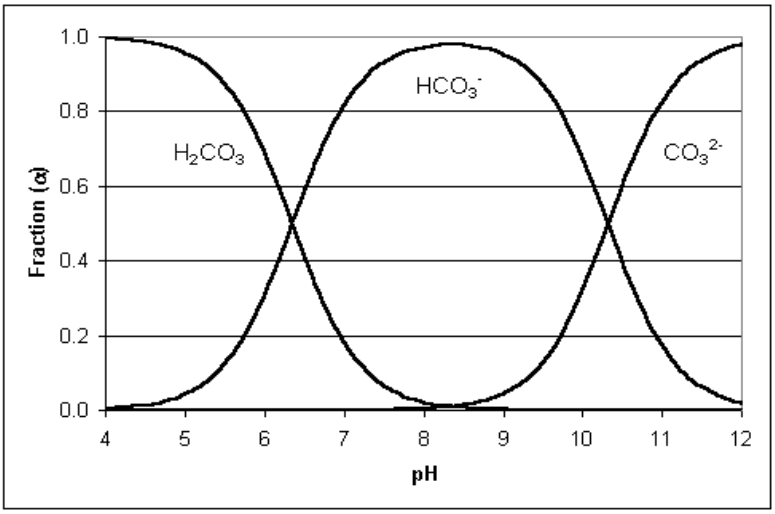


Figure 1.2 Graphical representation of the carbonate cycle showing pH influence on carbonic acid, bicarbonate ions and carbonate ions. Source: http://ion.chem.usu.edu/~sbialkow/Classes/3650/Carbonate/Carbonic%20Acid.html

Figure 1.2 represents the continuum of change between carbonic acid, bicarbonate ions and carbonate ions at varying pH values. The conversion of organic matter derived from animal feeds in RAS generate hydrogen ions by bacterial mediation by the nitrification process that lowers the water pH (Ebeling, 2000, Timmons & Ebeling, 2010). In RAS, the control of alkalinity through the addition of bicarbonate ions or other means buffers the water from rapid swings in pH, which can precede to toxic unionized ammonium in elevated pH conditions leading to stress and possible mortalities (Ebeling, 2000, Timmons & Ebeling, 2010, Boyd et al., 2016).

A promising filter medium for removing phosphate/phosphorus from phosphorus enriched water sources is aragonite, which is a natural forming and sustainable resource. The authors first termed the aragonite grains as ooid or oolite structures consisting of a nucleus and up to a few layers of aragonite crystals (Newell et al., 1960) (Figure 1.4). They are typically 2 mm in diameter or less and accrete around a nucleating fragment in dynamic environments. Newell et al. (1960) noted that the oolitic aragonite contained organic matter and hypothesized the sand stemmed from a recrystallization or precipitation process. Aragonite exhibits physical properties that mark it is a superior $CaCO_3$ resource over mined calcite. Aragonite has an orthorhombic crystal structure, adsorbs phosphorus, a surface area of >1.82 m^2/g, a high zeta potential and a higher micro porosity than calcite (Kamennaya et al., 2012). The physical structure of oolitic aragonite has a greater porosity than calcite or dolomite due its crystal structure (Cherkas et al., 2018). Its needle-like crystals with high aspect ratios act as fillers for the improvement of mechanical properties of paper and polymer materials (Figure 1.3). Aragonite is also a good biomedical material, because it is denser than calcite and could be integrated, resolved, and replaced by bone or shell.

Figure 1.4 Aragonite ooids found on the Grand Bahamian Bank, Bahamas.

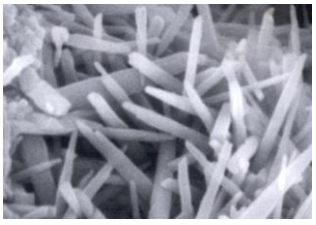


Figure 1.3 Aragonite needle-like structure.

Shearman et al. (1970) also reported the observation of concentric layer formation on aragonite oolites from the Persian Gulf. Margolis & Rex (1971) using light and

scanning electron microscopy reveal the porous structure of oolitic aragonite grains formed by both boring algae and the needle-like crystals of the aragonite. Mitterer (1972) suggested the organic material embedded in the $CaCO_3$ assemblage might be a nucleus, eluding to the possibility that oolitic aragonite particles may form on this and precipitate. Edgcomb et al. (2013) in studying the oolitic sands of Highborne Cay in the Bahamas report observation of endolithic borings in the grains, which are habitats for various bacteria.

The creation of aragonite has been discuss for decades with multiple concepts purported. Whitings are events that form water-borne aragonite particles in mostly tropical marine waters viewable by aerial images as large white areas in the water (Robbins et al., 1997, Bustos-Serrano et al., 2009, Larson & Mylroie, 2014). For decades, observed whitings have perplexed investigators who worked to elucidate the causes for this natural phenomenon (Bustos-Serrano et al., 2009). Newell et al. (1960) postulated CO_2 enriched cool water washing into the shallows by tidal action; initiate aragonite formation due to existing super-saturated aragonite conditions. Work conducted by Shinn et al. (1989) point to two causes of whiting formations: those caused by sediment resuspension after storm events and those formed by conditions causing precipitation.

In review of research on whitings in the Bahamas and the Dead Sea, Friedman (1993) concluded that different triggers are involved in initiating the events. Robbins and Blackwelder (Friedman, 1993) reply to comments by Friedman that evidences does not completely support only an abiotic formation of whitings and suggests that further

work in biological origins of whitings is needed. Morse et al. (2003) proposed whitings formed by carbonates adhering to resuspensions of sediments rather than direct precipitation based on reaction kinetics of calcite. Dierssen et al. (2009) noticed evidence of phytoplankton association with whitings from measurements of chlorophyll concentrations. Dierssen et al. (2009) provide evidence of Langmuir currents as being a possible means to resuspend sediments into the water column due their reach from surface waters to bottom sediments and current velocities. The trigger for whiting is suggest to be the Florida Current bring $CaCO_3$ supersaturated water over the Great Bahama Bank (Purkis et al., 2017). Larson & Mylroie (2014) suggest there exist two different types of whiting events: large light colored whitings in deep bank water lasting 2 days and a smaller type, found in shallow water, darker in appearance and with a duration of one day. The authors propose a new model of whiting formations. The model reconciles older ^{14}C and young ^{7}Be isotopes within the whiting particles. The model suggests tidal water pumped through sediment filled Blue Holes in the Bahamas transport CO_2 laden water toward the surface waters, which form super saturated conditions leading to precipitation of aragonite. The $CaCO_3$ forms onto a nucleus of organic material representing the source of the ^{7}Be. Bahamian anglers report of smaller, near shore whiting formations attributed to disturbances of bottom sediment by fish as causing the whitings. In consideration of large whiting events, Shinn et al. (1989) used multiple methods to determine if there were fish presence within whiting events. They were not able to locate fish schools and concluded that resuspension of bottom sediments by activity of fish schools do

not account for most of the whiting events in the Bahamas. Reported whitings remain visible up to 45 hours, Dierssen et al. (2009) points out that any fish activity resuspensions likely dissipate within 6 hours, meaning they could not be the sole initiation action to maintain these conditions.

Kamennaya et al. (2012) layout the current accepted understanding of aragonite formation from whiting events as cyanobacteria mediated precipitations. Cyanobacteria are able to capture CO_2 and store internally to concentrations 1000-fold over the medium in which they reside. The dissolved CO_2 forms H_2CO_3 in solution, which reacts and forms HCO_3^- and CO_3^{2-}. These two ions ultimately react with Ca^{2+} generating $CaCO_3$. Cyanobacteria produce exopolymeric substances, which can function as a nucleation particle. $CaCO_3$ formation occurs on the cell surface of Cyanobacteria by generation of localized alkaline conditions and concentrations of CO_3^{2-}. The exopolymeric substance on the surface of the cyanobacteria act as the nucleus with the CO_3^{2-} binding with Ca^{2+} forming $CaCO_3$, which precipitates (Kamennaya et al., 2012). The $CaCO_3$ develop as layers on the surface of the cyanobacteria, which it sheds as whitings (Kamennaya et al., 2012).

Robbins et al. (1997) using NASA photographs (1965-1993) by low-earth orbit observations, observed 888 whiting events that clustered on the Great Bahama Bank at coordinates of 25°N, 78°50'W, indicating a majority of occurrences are at specific locations. Robbins et al. (1997) observed some whiting events on photographs that remained visible up to 7 and even 11 days after first sighted, showing a significantly longer period than previously reported.

Using NASA photographs of whiting events, the authors show that April and October months had the highest frequency of occurrence, pointing to spring and fall as the seasonal periods of whitings, though they do occur year round (Robbins et al., 1997). Purkis et al. (2017) supports a strong seasonality to whiting events with 70% occurring during the winter months between November and the end of April, contrary to the accepted idea that the summer provides the optimal conditions.

As a measure of the productivity of the whitings, Shinn et al. (1989) conducted cruises through ongoing whiting events showing carbonate sediment concentrations averages of 10 mg/l within the whitings, compared to 1.5 mg/l outside of the event. Shinn et al. (1989) measured settling rates of particles within the whitings at a rate as high as 34 $g/m^2/hr.$, theorizing that whitings are actively producing sediments continuously. The measured time to settle particles from whitings or other suspended bottom sediments take about six hours. With this short length of time to settle, Shinn et al. (1989) would expect the events would be short and not lasting for days as observed.

The harvesting of aragonite is one of the few renewable natural resources that shows little ecological impacts with its removal. Robbins et al. (1997) estimate the Grand Bahamian Bank in the Bahamian produces on average 1.4 million metric tons of aragonite per year. Around Ocean Cay, an island between Andros Island, Bahamas and Miami, FL along the Straits of Florida, produce an estimated amount of oolitic aragonite from whitings of 266,000 mts to 2,310,000 mts annually (Turrell, Hall & Associates Inc. Marine & Environmental Consulting – April 2012). The estimation is

Ocean Cay holds between 50 and 100 billion tons of oolitic sands (Turrell, Hall & Associates Inc. Marine & Environmental Consulting – April 2012). It can be argued that oolitic sands from the Ocean Cay in the Bahamas is such a resource that has yet to be tapped for its full potential as a substrate for environmental mitigation. Millero et al. (2001) report that aragonite and calcite can bind phosphate at levels of 1.66 and 0.65 mg PO_4 per gram of aragonite or calcite, respectively, showing an aragonite binding phosphate capability of 2.5 time than of calcite (Millero et al., 2001). Millero et al. (2001) attributed the differences in phosphate binding to the disparities with bind sites on the two $CaCO_3$ forms. Desorption experiments on phosphate-saturated aragonite and calcite show aragonite is able to desorb a greater portion of bound phosphate than that of calcite (Millero et al., 2001). Aragonite is able to absorb a greater concentration of phosphate, as temperatures increase in seawater (Millero et al., 2001). In contrast, aragonite-binding increases as salinities decrease (Millero et al., 2001). With the presence of Mg^{2+} and Ca^{2+}, the aragonite adsorption increases in seawater acting as a possible bridge between the aragonite and PO_4 (Millero et al., 2001). Work by Khan et al. (2021) support the bridging theory of aragonite to Ca^{2+} to $P04^{3-}$ proposed by Millero et al. (2001) showing this as the mechanism for phosphorus removal by aragonite. Using bivalve shells from food waste sources, Khan et al. (2021) generated aragonite by a unique process of high temperature (900°C), magnesium chloride and calcium oxide solutions and carbon dioxide. Khan et al. (2021) showed the generated aragonite was capable of removing about 97% of phosphorus from P solutions of ≤1 mg/L within 10 hours, offering a

sustainable treatment for low concentration phosphorus removal. In work using synthesized fusiform aragonite, Xu et al. (2016) shows it has a capacity at pH ≥6.0 to remove >90% of phosphate at concentrations ≥16.5 mM.

Aragonite and $CaCO_3$ products saturated with phosphates possess an ability to be renewed. Jiang et al. (2017) used an alkali produced calcium-silicate composite from waste glass and discarded shells to form an absorbent with the ability to remove phosphate from solutions with an absorption capacity of 120 mg/g of product. In addition, the authors demonstrated the release of the captured phosphate from the composite using a 2% solution of citric acid for a recovery of 96% and suggested its use as an agricultural fertilizer (Jiang et al., 2017). In a recent paper supporting citric acid release of phosphate, Li et al. (2021) used a novel electro-chemical dolomite process to remove phosphate and ammonium from water. Following this, the authors exposed the used dolomite to a 2% citric acid solution, which released, a reported, 100% of the removed P and N within minutes (X. Li et al., 2021). The ability of releasing bound phosphate from a calcium based composite by citric acid presents a plausible method for phosphate/phosphorus removal and reuse from aragonite.

Oolitic aragonite sand is an untapped resource useful as a microbial refugium for a bacterial consortium able to perform important mitigation functions such as nitrification and denitrification. Its large surface area per volume ratio, buffering action to maintain pH and alkalinity levels and binding capacity for phosphorus characterize aragonite as a promising means to alleviate nutrient issues both in

aquatic and terrestrial settings. These inherent properties of aragonite sand and sustainability advocate it as a prospective substrate for water quality control in RAS. A major obstacle to developing marine and freshwater aquaculture is its interaction with the environment. The advantage of recirculating aquaculture systems is their inherent capability to effectively manage, collect and treat nutrient wastes that accumulate during fish growth, which is a key factor in the development of this technology as a mainstream environmentally sound production system for marine fish. Considering the ever-growing legislative measures to reduce pollution from fish production operations, the success of fish farming activities will depend largely on their ability to reduce their adverse environmental impacts. If appropriately designed and operated, RAS can meet this challenge while being economically competitive. Although aquatic animals generally show low toxicity to nitrates, over time concentrations in RAS can escalate as a product of the nitrification cycle. Elevated nitrate concentrations can affect the immune systems, increase mortality, inhibit growth and effect the osmoregulatory systems in animals (van Rijn et al., 2006, Timmons & Ebeling, 2010). Management of nitrates in aquaculture operations have been historically by water exchanges, though discharge limitations and the need to conserve water is motivating the use of denitrification methods on RAS recirculating waters and discharge (Sauthier et al., 1998, Singer et al., 2008, Timmons & Ebeling, 2010). The use of denitrification to reduce nitrates to N_2 gas by heterotrophic or autotrophic bacteria ensure the removal of nitrogen from RAS to the atmosphere (Timmons & Ebeling, 2010). The process needs to occur in anaerobic conditions

with a carbon source for bacterial growth (Timmons & Ebeling, 2010). Using a upflow granule pack vertical column reactor, Sauthier et al. (1998) cautions that denitrification reactors requires stable total organic carbon/nitrogen ratio and a continuous carbon supply to maintain activity and avoid large negative swings in redox-potential and the formation of harmful sulfide levels. Development of computer controlled denitrification systems, which monitor dissolved oxygen, oxidation reduction potential, pH and control carbon dosing and flow rates can produce nitrate levels <5 mg/l in a commercial scale RAS (Lee et al., 2000). Management of denitrification conditions prevent nitrite and hydrogen sulfide production (Lee et al., 2000). A literature search revealed nitrate removal rates by denitrification vary greatly (1-166 mg NO_3-N/l/h), likely due to differences in the denitrification reactor, operating conditions, source water conditions, carbon sources, etc. (van Rijn et al., 2006). In a freshwater application, nitrogen removal rates of 670 – 680 g N/m^3/day were measured in an up flow denitrification reactor with floating plastic media showing no differences between carbon sources of methanol, acetic acid, molasses and Cerelose™ (hydrolyzed starch) (Hamlin et al., 2008). In order to reduce the chance of leaking residual organics back to culture water from liquid carbon sources in the denitrification reactors, Singer et al. (2008) employed cotton wool at a C/N ratio of 0.82 g cotton/ g of nitrate N, which resulted in nitrate levels of < 10 mg/l.

Using endogenous carbon (i.e. fish feces) for denitrification, a nitrate removal rate of 26.9% using a fluidized sand with hydraulic retentions time of 15 minutes occurred

(Tsukuda et al., 2015). The authors postulated the nitrate removal rate would increase by applying a longer retention time and ensuring enough carbon is present for denitrification activity to continue (Tsukuda et al., 2015).

Sulfur based denitrification can occur using elemental sulfur and developed biofilms (Y. Wang et al., 2016). Biofilm formations sufficient for denitrification required 35-50 days making it slower than carbon based denitrification applications (Y. Wang et al., 2016). Implementation of sulfur-based denitrification may be limited due to nitrite production and strict management requirements (Y. Wang et al., 2016). A negative effect of sulfur-based denitrification that would need close monitoring in RAS aquaculture is the acidity produced by the reactor resulting in pH decreases in the effluent (Zou et al., 2016).

Incorporation of denitrification systems on large scale commercial operations are likely to be adopted to reduce animal stress from improvements to water quality than savings on operating cost initially, though cost of denitrifications systems and their operation will improve over time (van Rijn et al., 2006).

The current studies aims to exploit the attributes of sustainably harvested oolitic aragonite sand by applying it as biofiltration, water quality modifier, phosphate remover and as an aid for growth of oysters. Utilization of four approaches occurred with aragonite in highlighting its ability to be an effective component for a RAS.

In chapter 2, oolitic aragonite sand is evaluated as a substrate for biofiltration and as well as a phosphate remover in RAS systems. This involves placing oolitic aragonite sands in small-scale standard canister filters connected to three reservoirs filled with

fresh, brackish and marine waters. Throughout the project, assaying of nutrient levels occurred. To repeat the study, fluidized sand filters, which are a more efficient system for particles of this size, held the OAS. Preliminary experiments have found that the sands are completely biocompatible for colonization by nitrifying bacteria, i.e. reduction of ammonia completely to nitrate and observation of a 15% reduction in circulating phosphate. In addition, as expected, alkalinity was increased and maintained in all three salinities. In order to show the response of aragonite sand to daily phosphorus additions, the researcher used a scaled up system using oolitic aragonite filled fluidized sand filter, following the small-scale trials. The study applied citric acid to phosphate exposed oolitic aragonite sand for testing the removal of bound phosphate. Upon completion of the scaled up work, the surface area analysis of sand occurred, before and after a citric acid treatments.

Chapter 3 investigates whether the OAS provides appropriate conditions for development of denitrifying bacteria (anaerobic conditions) to remove nitrate in freshwater. This work seeks to evaluate the use of OAS as a medium in a fluidized sand filter (FSF) in establish conditions conducive for denitrification and anammox activities. The study focused how an oolitic aragonite sand filter on an anaerobic system handles repeated nitrogen additions with respect to the difference forms of nitrogen present, ultimately showing removal of nitrogen from the freshwater setup, signifying occurrence of denitrification and possibly anammox activity.

Understanding the need to limit nitrogen built up in the environment and the requirement for aquaculture effluents to be low in nitrogen, the objective of chapter four was to repeat the same work of chapter three, but with marine water.

With the understanding of the importance of oyster aquaculture, seeing the efforts being applied to RAS systems and considering our experiences in oyster husbandry, chapter five studied the use of oolitic aragonite sand to stabilize water quality and possibly improve growth performance in Pacific and Eastern oysters housed in RAS systems. The experimental objectives of this work was to determine if oysters are able to survive and show growth in a simple RAS, determine if culture with oolitic aragonite sand has a positive effect on growth and determine if these improvements occurred with both the Eastern and Pacific oysters.

Chapter 2: RAS Phosphate and Phosphorus Removal and Recovery Using Oolitic Sand (Calcium Carbonate – Aragonite)

2.1 Abstract

Aquaculture is an expanding contributor to the food supply with recirculating aquaculture systems, RAS, being a sustainable and environmental sensitive approach to a reliable high quality protein source. With all of the improvements achieved by RAS, the continued recycling of the water does pose challenges requiring management in order to provide an optimal, low stress environment for fish and invertebrate growth. RAS operations must manage water quality concerns such as pH, alkalinity, nitrogen and phosphorus for the aquatic inhabitants as well as limiting nutrient loads in any discharges from the system. A promising filter medium is oolitic aragonite sand, OAS. OAS is a sustainable, renewable, natural occurring and effective medium in controlling water quality in RAS. Formation of OAS occurs within whiting events in the warm tropical waters off the coast of the Bahamian Island Andros. The production of the OAS around Ocean Cay on the Grand Bahamian Bank generate large, used reservoirs of OAS annually. Testing of OAS in systems with canister filters (CF) and fluidized sand filters (FSF) with freshwater, brackish and marine waters show stable pH readings of 8.02 ±0.16 (CF), 8.09 ±0.09 (FSF) and stable or increasing alkalinities typical for RAS. The OAS removed phosphate rapidly over the first four days continuing at a slower rate afterwards. The OAS show capacities to remove phosphate at rates of 716, 705 and 215 mg PO_4/ kg OAS for freshwater, brackish and marine water, respectively. Using 0.57-m^3 system

with daily P additions showed a phosphorus removal capacity of 77.8 mg P /kg OAS. Treatment of phosphorus exposed OAS with 1.0% and 2.0% citric acid solutions show phosphate removals ranging from 17.3% to 93.5% across all treatments. Surface area analysis show the citric acid exposed OAS increased its surface area 1.66 time to 4.628 m^2/g OAS. SEM images confirm increased surface roughness and voids. Microbiome analysis show similar bacterial phyla exist on the naïve OAS and the OAS used in different salinities, varying by percentage.

2.2 Introduction

Aquaculture discharges can be a source of phosphorus into the environment. As an example, flow through raceway units in Southern, Idaho typical have individual flows of 946 – 10,200 l/min (True et al., 2004b). Measurement of total phosphorus concentrations of 2.91 ± 0.32 µM with 62% dissolved and 38% from solid particles have occurred in these flows (True et al., 2004b). In the studied farms, measured raceway velocities were below the rate that prevents settlement of solids (0.1-0.6 m/s). Therefore, a portion of solids remaining in the culture area for the rainbow trout likely lead to increased dissolved phosphorus in the effluent (True et al., 2004b). The impact of aquaculture effluent can develop a persistent storage reserve in the environment leading to long-term eutrophication conditions after the phosphorus additional have stopped or decreased. An investigation of sediment cores extracted from the Sancha reservoir in southwest China, reveal high concentrations of phosphorus forms within sediment layers corresponding to periods when high

amounts of phosphorus inputs from aquaculture operations in the lake occurred (Jia et al., 2015).

In order, to capture phosphorus from sources and the environment, mitigation efforts employ a number of methods and technologies, each with their own limitation. Improvement of fish feeds by inclusion of plant source replacements to fishmeal, the availability of phytase to increase phosphorus retention and meeting the minimal phosphorus requirement for the species being culture result in decreases of phosphorus levels in aquaculture wastewater discharges (Lazzari & Baldisserotto, 2008). Enacting of phosphorus effluents limits on aquaculture operation by the use of Total Maximum Daily Load regulations are also productive (Sibrell & Kehler, 2016). The use of flocculants can precipitate between 70- 90% of the contained phosphorus within sewage treatment facilities (Smil, 2000). In rainbow trout culture tanks, at the Northeast Fishery Research Center in Pennsylvania, Sibrell & Kehler (2016) demonstrated the use of aluminum and iron oxide containing residuals from acid mine drainage neutralization efforts as a low cost material for phosphorus removal using a fixed bed process. The work shows phosphorus removal rates between 50-70% with an average phosphorus concentration of 2.07 µM in the influent flow (Sibrell & Kehler, 2016). The mine drainage residual material proved capable of regeneration with a sodium hydroxide method, albeit with limitations (Sibrell & Kehler, 2016). Demonstrations of the ability of bentonite clay pillared with polyhydroxy aluminum was capable of removing 11.85 mg/g of phosphorus from aquaculture discharges of varying salinities but less effective at higher salinities (Kumararaja et al., 2019). The

use of aluminum and lanthanum compounds for phosphorus treatment in freshwaters has become increasingly common. Regular monitoring of the environmental concentrations of aluminum and lanthanum is important in avoiding an unintentional human health risk (D'Haese et al., 2019). Developed cross-linked polymeric hydrogels by Kioussis et al. (1999) demonstrated their ability to remove phosphate from low salinity aquaculture waters. The hydrogels show a capacity to remove as much as 47 mg/g of hydrogel and represent a possible aquaculture solution for phosphorus removal (Kioussis et al., 1999).

Experimental results show a phosphorus reduction of 11% in effluent from a flow through aquaculture operation occurred using reticulate foam media (True et al., 2004a). The foam remove solids in the effluent flow to less 100 um with a head loss of 150 mm. The filtered solids removed particulate phosphorus. The flow of flow-through systems is 47 and 800 greater than pond or RAS production systems, respectively (True et al., 2004a). The proposed incorporation of foam filtering in flow-through raceway operations must take into consideration the maintenance efforts required for the filter material.

A promising filter medium for removing phosphate/phosphorus from phosphorus enriched water sources is aragonite, which is a natural forming and sustainable resource. The creation of aragonite has been discuss for decades with multiple concepts purported. Whitings are events that form water-borne aragonite particles in mostly tropical marine waters viewable by aerial images as large white areas in the water (Robbins et al., 1997, Bustos-Serrano et al., 2009, Larson & Mylroie, 2014).

For decades, observed whitings have perplexed investigators who worked to elucidate the causes for this natural phenomenon (Bustos-Serrano et al., 2009). Newell et al. (1960) postulated CO_2 enriched cool water washing into the shallows by tidal action; mitigate aragonite formation due to existing super-saturated aragonite conditions. Work conducted by Shinn et al. (1989) point to two causes of whiting formations: those caused by sediment resuspension after storm events and those formed by conditions causing precipitation.

Oolitic aragonite sand is a natural resource that appears to be a stable renewable and provides buffering capacity for pH and alkalinity control and show signs making it useful as in phosphorus removal. This work will focus on using this material as a phosphorus removal media, pH and alkalinity control in aquaculture effluents and other phosphorus rich water sources.

The objective of this work is to:

- Assess the ability of oolitic aragonite sand to modify water quality and diminish phosphate/phosphorus from different salinity waters.
- Define the response of oolitic aragonite sand to repeated additions of phosphorus.
- Describe the recharging effect of citric acid on oolitic aragonite sands.
- Determine effect of citric acid on the sand particles.

2.3 Material and Methods

2.3.1 Oolitic Aragonite Sand in Canister Filter Arrangement

The Aquaculture Research Center (ARC) facility of the Institute of Marine and Environmental Technology in Baltimore, MD (https://imet.usmd.edu/) was the location of the work. The Sandy Cay Development Company Ltd, Nassau Bahamas (4 George St. Mareva House Nassau Bahamas) was the source of the oolitic aragonite calcium carbonate sand (OAS).

Figure 2.1 Internal compartment of Fluval® 406 canister filter holding 1000g of oolitic aragonite sand, as weighed on a scale.

The experiment utilized the Fluval® 406 canister filters (CF) with 1000 grams of OAS placed in a single compartment section of the filter (Figure 2.1). Due to the fine size of the sand particles, polyester fiber filter material, approximately 8 mm thick (Pentair, www.pentair.com), was placed below and above the sand layer to contain the sand within the filter compartment. The canister filters were installed on three separate 90-gallon fiberglass tanks (Gemini Fiberglass, model FRT-90) containing 297 liters of water. Placement of each supply and return line from the filters were identically at the end of each tank (Figure 2.2).

Freshwater, brackish and marine water sources came from recirculating aquaculture systems (RAS) holding tilapia (*Oreochromis niloticus*), European Sea Bass

(*Dicentrarchus labrax*) and nurse sharks (*Ginglymostoma cirratum*), respectively. Consecutively run CF treatments occurred in triplicate, referred to as Trials 1, 2 and 3.

Flow rates in liters per minute were calculated using the formula: Flow Rate (lpm) = (timed volume, l/ time, s) x 60. Measurement of flows occurred in triplicate with results averaged for the flow rate. The ability to view the tank bottom determined the water clarity. A scale was used with a value of 0 meaning the tank bottom was clearly visible, 0.5 the bottom could be seen, but not clearly and 1 if the bottom was not able to be viewed. Placement of a section of black plastic sheeting over the top of each tank was to reduce evaporation and prevent possible water from entering by nearby systems.

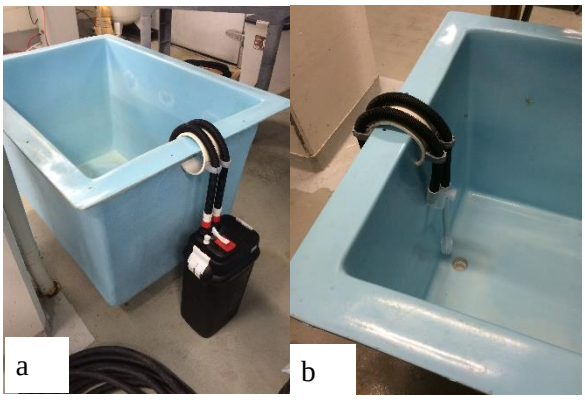


Figure 2.2 Canister Filter mounted on outside of 90-gallon fiberglass tank (a). Supply and return lines positioned in tank (b).

In order to access a possible explanation for the cause of the cloudiness conditions in the experimental waters of the canister and fluidized filter experiments, the Langelier Saturation Index value were calculated. The Langelier Saturation Index (LSI) is a calculation, which incorporates water temperature, pH, total dissolved solids, calcium concentration and alkalinity using the formulas:

LSI = pH - pHs
pHs = (9.3 + A + B) – (C+D)
A =(Log10[TDS] – 1)/10

B = 13.12 x Log10(°C + 273)
C = Log10[Ca2+]
D = Log10[alkalinity as CaCO3]

In order to simplify the LSI number generation, calculations used an online Langelier Saturation Index calculator (cleanwaterstore.com/resource/calculators/langlier). The water temperature, pH and alkalinity values were from water quality records. Calculation of the total dissolve solids (TDS) values were from the measured salinity values. Conversion of salinity data to conductivity occurred by use of an online salinity conversion calculator (hamzasreef.com/Contents/Calculators/SalinityConversion.php). Conductivity values were then changed to TDS using the equation y= 421.62x – 1.9946, were x = conductivity and y = TDS. Development of the TDS formula occurred by plotting data from a conductivity to TDS conversion table (corrosion-doctors.org/Cooling-Water-Towers/Index-Lang-TDS) and fitting an equation to the data. Since no calcium measurements existed for the experiments, use of the last calcium measurement from the source waters occurred for all calculations. Comparison of the LSI values occurred between cloudy and clear water conditions for all three salinities in the canister filter and fluidized sand filter trials.

2.3.2 Oolitic Aragonite Sand in Fluidized Filter Arrangement

Figure 2.4 Image of the fluidized sand filter filled with gravel and 1000 g of oolitic aragonite sand and mounting position on end of fiberglass tank during a trial using brackish water.

For the fluidized sand filter (FSF) method, the study used the V2 Bio1500 Fluidised Sand Filter model. The filter contained a layer of inert gravel (approximately 160 ml) on the bottom and 1000 grams of aragonite sand added on top of the gravel (Figure 2.4). A submersible pump (Danner Supreme Aqua-Mag) connected to each FSF fluidized the sand bed and recirculated the water. The pump was place on the tank bottom next to the end wall of the tank (Figure 2.3). Adjusted flow rates to match each other.

Figure 2.3 Image of fluidized sand filter placement, pump with control valve position and connection (left side) and filter return (right side) for a marine trial.

Sand bed expansion was determined by measuring the difference between the height of the sand bed with flow and that without flow to calculate percent increase. The percentage of sand bed expansion = ((sand height with flow – sand height without flow)/ sand height without flow) * 100. As with the canister filter arrangement, consecutively run treatments were in triplicate. By measuring the difference in distance between the water surface and the top edge of the tank at the start and end of the experiment, water loss during the course of the

experiment was calculated. By subtracting the weight of the remaining sand from the starting sand weight, the weight of sand loss was calculated.

2.3.3 Water Quality Analysis for Canister and Fluidized filter Experiments

The water laboratory for National Aquarium in Baltimore, MD (aqua.org) conducted all water testing. Water samples over the course of the experiment, were collected directly from treatment tanks. Water was analyzed for salinity, pH, NH_4^+, NO_2^-, alkalinity, PO_4^+ and NO_3. The water laboratory referenced the following methods from the manual Standard Methods for the Examination of Water and Wastewater, 21st Edition to analyze the parameters (2510, 4500- H+, 4500- NH_3, 4500- NO_3, 2320, 4500- P and 4500-NH_3/4110, respectively). An alcohol thermometer positioned in each tank determined daily temperatures.

2.3.4 Oolitic Aragonite Sand Arrangement Used in the George Barley Water Prize (GBWP)

The system design was composed of a Solar Components Corporation (solar-components.com) tank of 700-liter volume (30” diameter x 5’ high, item# 11012). The reservoir tank was raised approximately 9 cm above the floor with wood supports. A flexible tubing connected the bottom center mounted ¾” (19 mm) tank bulkhead fitting with a circulation pump (PAN World Magnetic Pump Model NH-

Figure 2.5 Image of the two-tank layout and fluidized sand filter filled with oolitic aragonite sand for the George Barley Water Prize trials.

50PX) provided circulation. A FSF (MacClean Water Treatment System, Model# 3P971, Rated Service Flow 5 gpm (18.9 lpm), Cuno Water Treatment Churubusco, IN, USA) received water from the pump outlet (Figure 2.5). On the outlet of the pump, between the pump and FSF, installation of a sampling port occurred (Figure 2.6).

Placing gravel in the bottom of the FSF distributed in-flowing water. As used in the previous CF and FSF experiments, 20 kg of raw OAS filled the FSF.

The reservoir tank received the FSF effluent by a connection between the pump outlet and the top of reservoir tanks. The return flow entered the reservoir at the surface of the water creating a circular rotation due to its position at the side of the tank. Deionized water (568 liters) filled the reservoir tank. A second reservoir tank was set up identical as the first tank and positioned next to it. The second tank consisted of the tank, valve and bottom fitting connections. Filling of the second tank in

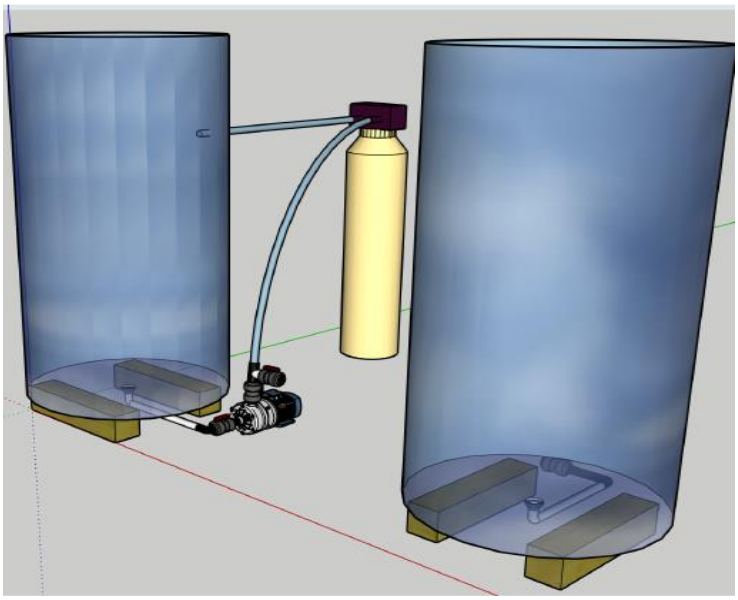


Figure 2.6 Drawing of two tanks, fluidized sand filter, circulation pump and connections for George Barley Water Prize test system.

advanced, allowed the project to continue in the first tank with the FSF. Measurement of the flow rate into the first tank from the FSF followed the same method used for the previous CF and FSF experiments.

The above-discussed system treated 150 gallons (568 liters) daily to remove phosphorus over a 2-week period, in the GBWP Trial. Using a 1000 ppb stock solution (SCP Science, scpscience.com), the reservoir tank was spiked with phosphorus. A 42.585 ml aliquot of the phosphorus stock solution was added to the reservoir system daily to make a 75 ppb total phosphorus concentration in the system for week one. This system operated with daily phosphorus additions for one week. Switching to the second tank on week two, involved moving the pump, filter and plumbing to tank two. New deionized water filled this tank and the daily additions of stock solution increased to 124.93 ml to make a concentration of 220 ppb phosphorus in the system. This system operated under these conditions for one week. The media in the FSF remained unchanged. Collection of water samples occurred as outlined in the GBWP Stage 2 Entry Guide (barleyprize.org, Table 2-1 Sampling frequency and location as outlined by George

Barley Water Prize Stage 2 guidelines.). The laboratory ALS in Jacksonville, FL (alsglobal.com) analyzed all samples. A Chain of Custody Form accompanied all samples shipped to ALS.

Two consecutive trials occurred with the experiment with a brief period between to thoroughly clean and rinse the system, as well as perform a 2% citric acid treatment on the OAS. At the completion of the two-week period, the flow rate was measure again.

Table 2-1 Sampling frequency and location as outlined by George Barley Water Prize Stage 2 guidelines.

Sampling Schedule
1 blank from week 1: deionized water sample, taken before concentrated with phosphorus
1 blank from week 2: deionized water sample, taken before concentrated with phosphorus
14 daily inflow samples
14 daily outflow samples
2 random inflow samples from week 1 as duplicates to be tested
2 random outflow samples from week 1 as duplicates to be tested
2 random inflow samples from week 2 as duplicates to be tested
2 random outflow samples from week 2 as duplicates to be tested
1 QA outflow sample from Week 1
1 QA outflow sample from Week 2

2.3.5 Optimization of citric acid concentration for removal of phosphate and phosphorus from oolitic aragonite sand

In testing the removal of phosphate from OAS, the use of different concentrations of citric acid solutions occurred. For each source per citric acid concentration, two samples were tested. The sand samples were dried in a drying oven at 105ºC for >2 hours. After cooling two 10.0 g samples from each sand source, placed in 250 ml

Erlenmeyer flasks, and covered with Parafilm®. Making of a 2% citric acid solution use double distilled water and reagent grade citric acid powder. An addition of a 50 ml volume of the citric acid solution occurred for each flask, covered and placed on a shaker. An innOva™ 4300 Incubator Shaker, New Brunswick Scientific unit set at 180 rpm, 20ºC and 30 minutes was used. After the agitation period, the flasks were decanted, and the volume measured. Washing of the sand with two 50 ml of double distilled water occurred and analyzed as separate washes. The sand and flasks dried in a drying oven at 105ºC overnight, placed in a desiccator to cool and weighed. The calculated percent loss of sand was between the initial weighing and the finial weighing. Repeat of the same procedure occurred with a 0.2% and 1% citric acid solution using sands from the third trial of the freshwater treatment, estuarine treatment and marine treatment CF and unused sand.

2.3.6 Treatment of OAS used in GBWP trials with a citric acid for removal of phosphate

Upon completion of the GBWP experiment trial 1, the used sand in the FSF was citric acid washed using a 2% solution. An Ace Roto-Mold 60 Gallon Full Drain Inductor Tank & Poly Stand Set - 24.5" dia. x 55" H (Item #: 11715; US Plastics Corporation, usplastic.com) was positioned beside the fluidized sand as a citric acid reservoir. The citric acid reservoir contained 15 gallons (56.8 l) of a 2% citric acid solution made with deionized water. After mixing for 75 minutes before use to ensure complete dissolution of the reagent circulation in the citric acid tank, the started treatment

lasted 1 hour with flow through the FSF. Sampling of the collected citric acid solution occurred and the rest drained. Refilling of the rinsed citric acid tank occurred with 20 gallons (75.7 l) of deionized water and started circulation through fluidized sand filter for 1 hour. Sampling occurred once the time elapsed. The rinse was repeated a second time with new deionized water and sampled. The deionized water filled reservoir tank circulated through the fluidized sand filter for 24 hours before being sampled for phosphate analysis and drained. Filling of the reservoir one tank with deionized water occurred for the start of GBWP trial 2. Trial two used the same sand. The repeat of the citric acid wash occurred on trial 2, as in trial 1. At the start and end of each treatment, measured flows rate occurred.

At the completion of the GBWP experiment, removal of the sand and gravel within the fluidized sand filter to a perforated, pre-weighed bucket occurred with filter material laid over the drain holes. The sand was allowed to dewater in the bucket, transferred approximately ¾ of the sand to flat plastic trays for dying. To dry the sand, placement of the bucket and trays occurred in a fume hood unit for 6 days. Frequent mixing of the sand over this time allowed evaporation of entrained water. Separation of the gravel from the sand occurred with a sieve. Once dried, the remaining sand, bucket and filter material was weighted, and the initial bucket and filter material weight subtracted. Calculation of the percent loss of sand occurred from the difference with the starting amount.

2.3.7 Surface area measurements of oolitic aragonite sand before and after citric acid treatments.

Raw OAS and citrate treated sand was sent for surface area measurements. Quantachrome Instruments LabQMC (labqmc.quantachrome.com) using Single Point and Multi-Point BET Surface Area Analysis (protocol 06000-3N) on a QuadraSorb Station 2 instrument, version 7.01, conducted surface area determination. The analysis involves the adsorption of nitrogen gas on the surface of the particles and calculations based upon the Brunauer, Emmett and Teller (BET) theory.

2.3.8 SEM

Scanning electron microscopy occurred on naive OAS and citric acid treated sand. Specimen were scattered onto aluminum stubs with conductive carbon adhesive tabs (Ted Pella, Inc., Redding, CA) and coated with 10 to 20 nm of platinum/Palladium in a sputter coater EMS 150T ES (Electron Microscopy Sciences, Hatfield, PA). SEM images occurred with a scanning electron microscope Quanta 200 (FEI. Co. Hillsboro, OR) at 7.5 KV and 81 pA beam current.

2.3.9 Microbiome Analysis of Oolitic Sands Exposed to Three Salinity Environments.

Weighing of triplicate oolitic sand samples from fresh, estuarine, and marine recirculating aquaculture systems as well as naive sands occurred into 100 mg aliquots and placed into the homogenization tubes from the Qiagen DNeasy powersoil kit (Germantown, MD). Homogenization of the twelve samples occurred for 30 seconds in an MPbio (Santa Ana, CA) FastPrep-24 at a power setting of five.

450 µl of supernatant were removed from the homogenization tubes and placed into a Qiacube (Qiagen) carrier holder. DNA extraction occurred using the protocol for the DNeasy powersoil kit on the Qiacube and the optional IRC step for PCR inhibitor removal. Remaining PCR inhibitors were removed by passing each sample through a Zymo Research Zymo-Spin IV-HRC column for 1 minute at 10,000 x g following removal of storage buffer. Amplifiability was tested for each sample using the bacterial 16S primers, 27F and 1492R (Suzuki 1995) and the 2x PCR mastermix from Promega (Madison, WI). Separation of PCR products occurred by electrophoresis on a 1% agarose TBE gel and imaged using ethidium bromide on a BioRad (Hercules, CA). Use of five ng DNA total occurred to generate the libraries as depicted in Figure 2.21. We sequenced V3-V4 region. It was a 2 X 300-cycle run. We10pM total pooled library to load the sequencer for sequencing. The gene-specific sequences used in this protocol target the 16S V3 and V4 region. Selection of sequences occurred from the Klindworth et al. (2013). Nucleic Acids Res 41(1) as the most promising bacterial primer pair. Illumina adapter overhang nucleotide sequences are added to the gene-specific sequences. The full-length primer sequences, using standard IUPAC nucleotide nomenclature, to follow the protocol targeting this region are:

> 16S Amplicon PCR Forward Primer = 5' TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGC WGCAG 16S Amplicon PCR Reverse Primer = 5' GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGG TATCTAAT CC

Data Analysis Pipeline for Aragonite Sand Microbiome

1. **Quality trimming**
    a. All read pairs quality trimmed using CLC Genomics Workbench (Qiagen, Hilden, Germany)
        i. Trimmed using quality score (limit = 0.05)
        ii. Trimmed ambiguous nucleotides (max. number = 2) iii. Minimum sequence length after trimming (n = 100 bp) iv. Orphan reads were discarded
2. **Merger of read pairs**
    a. All read pairs were merged using CLC Genomics Workbench
        i. Mismatch cost (n = 2)
        ii. Gap cost (n = 3)
        iii. Maximum unaligned end mismatches (n = 0)
        iv. Minimum score (n = 8)
    b. Unsuccessfully merged read pairs were excluded from downstream analyses
3. **Read normalization**
    a. Reads were normalized across samples
        i. ~697,000 merged reads/sample which is the lowest read per sample count. OTUs were picked using the open reference OTU method in QIIME

OTU picking method – uclust. Identity threshold (n = 0.97)
Reference database - Silva123_QIIME_release
Percent subsample (n = 0.001)
Prefilter percent identity (n = 0.0)
Minimum failure threshold (n = 100000)
vii.Minimum cluster size (n = 2)

Taxa summaries

Reporting of relative abundances of taxonomic groups were for all samples using QIIME. Reporting of taxonomies were for levels 2 through 6.

Alpha diversity measures

Calculated alpha diversity metrics were from constructed BIOM OTU table (output from open reference OTU picking). Alpha diversity metrics: Goods coverage, chao1, observed_otus, simpson, simpson_e, PD_whole_tree, observed_species. Multiple rarefaction occurred on OTU tables to generate average rarefaction plots.

Beta Diversity
Beta diversity measures calculations occurred from constructed BIOM OTU table (output from open reference OTU picking). Generation of weighted and unweighted 2-D and emperor PCoA plots occurred for all samples.

2.3.10 Statistical Analysis

Use of the statistical program R-Studio (version 3.6.2) to compare means between replicates and treatments in all three studies using One-Way ANOVA occurred. Where detected differences in ANOVA analyses existed, a Tukey, post-hoc test elucidated were the differences occurred. Calculated averages include ± standard deviation where appropriate.

2.4 Results

2.4.1 Oolitic Aragonite Sand in Canister Filters

2.4.1.1 Water Conditions

The flow rate through the CF averaged 14.8 lpm (± 0.4 std. dev.) for the first trial. The cross-sectional, open area in the CF compartment was measured to be 61.3 cm_2, resulting in an average cross-sectional flow rate across the three salinities of 2,410 ±36.21 lpm

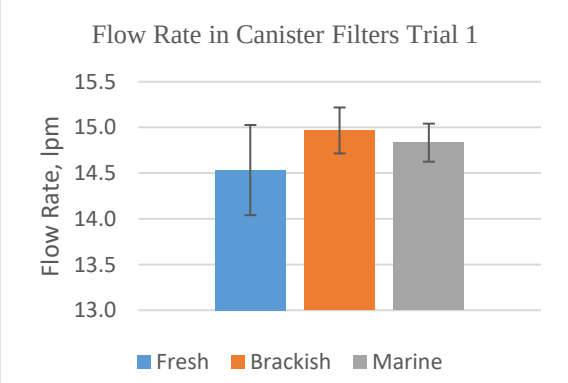


Figure 2.7 Flow rate through Fluval 406 canister filters with 1000 grams of oolitic aragonite sand in fresh, brackish and marine water during trial 1. Error bar represent standard deviation.

per m_2 and a velocity of 4.02 ±0.06 cm/s. The flow between salinities were consistent and not significantly different (ANOVA [F (2, 6) =1.267, p=0.348]), (Figure 2.7). The clarity varied according to the salinity of treatment water. The fresh water treatment remained cloudy for 12 days in the second trial but continued for the duration of the first and third trials. Brackish and marine treatments showed short periods of cloudiness initially, followed by clear water. The brackish water trials cleared on the second day, with the marine trial clearing on day 5 (Figure 2.8)

The clarity data shows there are differences in the cloudiness between the three salinities ANOVA [F (2, 212) =97.256, p<0.05]. A Tukey, post-hoc test, revealed that the cloudiness in the freshwater treatment is significantly different from the brackish and marine treatments (p< 0.05).

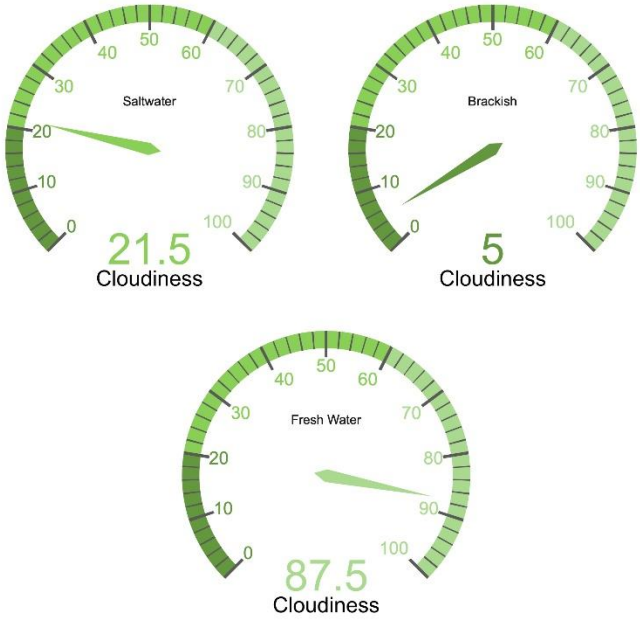


Figure 2.8 Treatment clarity during the three canister filter trials (T1, T2, and T3) Scale: 0 meaning the tank bottom was clearly visible, 0.5 the bottom could be seen, but not clearly and 1 the bottom was not able to be viewed. Clarity Value for 20-day period. Scales measure percent time system remained cloudy.

2.4.1.2 Temperature

The CF starting temperatures varied according to the source water. The overall average temperature among all CF trials is 21.4°C ± 1.6.

2.4.1.3 Water Quality

The CF salinities remained stable throughout the trials in all treatments. The differences seen between trials within a treatment is due to the starting salinities as received from the source tank. The pH of all treatments and trials ranged from 7.40 to 8.36, with an average ±standard deviation of 8.02±0.16

Table 2-2). The differences seen between trials within a treatment is due to the starting pH as received from the source tank.

Table 2-2 Average pH ±standard deviation for each treatment during canister filter trials.

Treatment	pH ± standard deviation
Freshwater	7.97 ±0.17
Brackish	8.01 ±0.22
Marine	8.08 ±0.08

The alkalinity of the three freshwater trials show increases from the starting concentrations with an initial rapid increase followed by slower, leveling-off of concentrations. Trials 1, 2 & 3 had changes between minimum and maximum values of 0.38, 0.54 and 0.31 mM alkalinity as CO_3^{2-}, representing 37%, 61% and 22%

increases from starting values, respectively. Differences exist between trials, ANOVA [F (2, 25) =12.442, $p<0.05$]. There are differences between trials 1 and 3, 2 and 3, (Tukey post-hoc, $p<0.05$ and <0.05, respectively). The higher pH and alkalinity of trial 1 than trials 2 and 3 is reflective of the starting source water.

The alkalinities in the brackish water remained relatively stable throughout each trial, with average ±std. dev. of 2.37 ±0.05, 4.56 ±0.05 and 2.65 ±0.12 mM for trials 1, 2 and 3 respectively. The brackish water trials showed significant differences exists between the trials, ANOVA, [F (2, 25) =1696.2, $p<0.05$]. A Tukey post-hoc analysis shows all three trials are significantly different from one another, (Trial 1 and 2, $p<0.05$, Trial 1 and 3, $p<0.05$, Trial 2 and 3, $p<0.05$). The initial source water at the start of the trials is responsible for the variation between trials.

Trials 1, 2 & 3 had changes between minimum and maximum values of 0.20, 0.26 and 0.35 mM alkalinity as CO_3^{2-}, representing 7%, 9% and 11% increases from starting values, respectively. Significant differences occur in alkalinities between marine water trials, ANOVA [F (2, 25) =15.159, $p<0.05$]. Tukey test shows no significant alkalinity differences between trials 1 and 2, ($p= 0.99$). Differences are apparent between trials 1 and 3 ($p<0.05$) and trials 2 and 3, ($p<0.05$).

2.4.1.4 Total Ammonium Nitrogen (TAN)

The freshwater treatment values for Total Ammonium Nitrogen (TAN), measured as ammonium, ranged from <0.55 to 4.99 µM among the three trials with an average of 1.30 ±1.31 µM, (Figure 2.9A). No significant differences in TAN concentrations between trials exist, ANOVA [F (2, 25) =2.426, $p=0.11$].

Brackish water TAN values ranged from <0.55 to 17.19 µM with an average ±standard deviation of 3.17 ±5.28 µM. Trials 1 and 2 showed a sharp decrease over the first 7 days of the experiment (Figure 2.9B). Then, remain below detection limit (<0.55) for the remainder of the trials. Trial 3 shows an increase on day 2 followed by a rapid decrease. ANOVA [F (2, 25) =0.4126, p=0.66] results indicate there is no significant difference between TAN concentration occurring with all three brackish water trials.

Marine water TAN concentrations ranged from <0.55 to 10.53 µM with an average ±standard deviation of 1.48 ±2.21 µM. Levels decreased with trials 1 and 2 over 8 days to below detection limits (Figure 2.9C). Trial 3 started below the detection limit over this same period, and show three elevated points during the latter half of the trial. There is no clear explanation as to why three TAN spikes would occur after 10 days into the experiment. No significant differences exist with TAN concentrations between saltwater trials, ANOVA [F (2, 25) =0.4514, p=0.64].

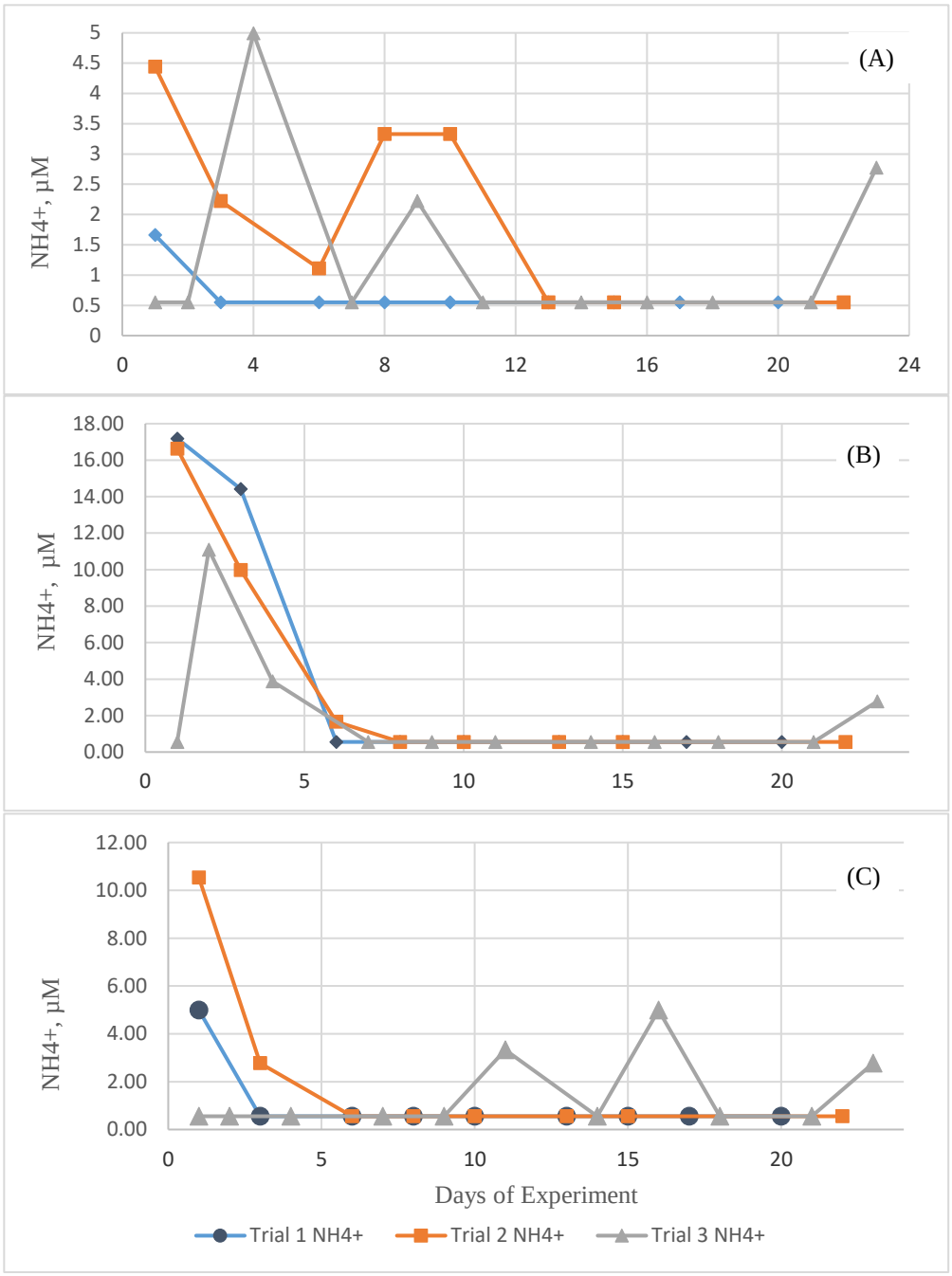


Figure 2.9 Concentration of TAN during trials 1, 2 and 3 for the canister filter treatments filled with oolitic aragonite sand. (A) Freshwater, (B) brackish and (C) marine waters. Note that the scales on the y-axis differ.

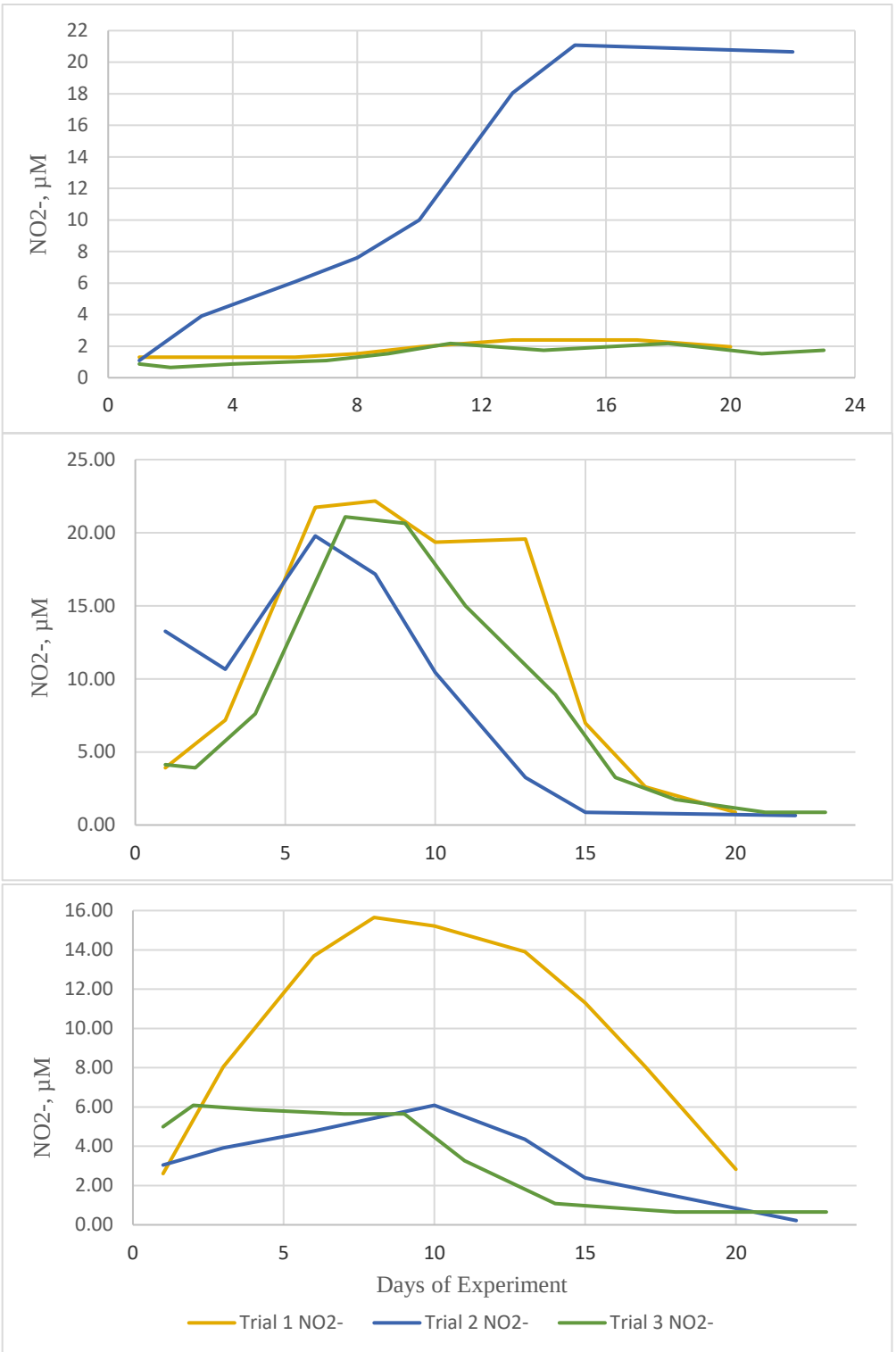


Figure 2.10 Concentration of NO_2 during trials 1, 2 and 3 for the canister filter treatments filled with oolitic aragonite sand. Note, scales differ with the y-axis in the three graphs. (A) Freshwater. (B) Brackish. (C) Marine.

2.4.1.5 Nitrite

Nitrite concentrations in the three freshwater trials averaged 4.33 ±5.91 µM and a median value of 1.96 µM. The first and third trials remain constant throughout duration of experiment with average ± std. dev. of 1.83 ±0.49 µM and 1.48 ±0.54 µM, respectively, (Figure 2.10A). The second trial was significantly different in that the nitrite concentration increased consistently during the trial, rising from 1.09 µM to a maximum of 21.08 µM before leveling off for the remainder of the trial. The second trial average was 11.06-±7.83 µM. A smaller peak in NH_4 occurred with trial 3, but did not result in any increase in NO_2, as with trial 2.

The nitrite levels in the brackish water treatment peaked on days 6 to 9, followed by a steady decrease (Figure 2.10B). The NO_2 peaks occurred at the same time the NH_4 levels decreased to their lowest values, indicative of completion of nitrogen cycling. ANOVA [$F(2, 25) = 0.504$, $p = 0.61$] results indicate that there is no significant difference between NO_2 concentrations occurring with all three brackish water trials. Nitrite levels during the marine treatments ranged from 0.22 to 15.65 µM with an average ±std. dev. of 5.61 ±4.57 µM (Figure 2.10C). Significant differences exist with NO_2 concentrations between the saltwater trials, ANOVA [$F(2, 25) = 11.921$, $p<0.05$]. Tukey test shows significant NO_2 concentration differences between trial 1 and 2 ($p<0.05$) and trials 1 and 3 ($p<0.05$). No significant differences exist between trials 2 and 3 ($p=0.93$).

2.4.1.6 Nitrate

Nitrate sampling occurred only on trials two and three for each treatment. The nitrate levels remain relatively unchanged during both trials in all three salinities. All salinities showed NO_3 valves significantly different between trials 2 and 3. Significant differences exist between NO_3 levels in freshwater trials 2 and 3, ANOVA [$F(1, 5) = 2595.6$, $p<0.05$]. ANOVA [$F(1, 5) = 1245.9$, $p<0.05$] results indicate that there is significant differences between NO_3 concentration occurring with trials 2 and 3 in the brackish water trials. Significant differences also occur in NO_3 concentrations between marine trials 2 and 3, ANOVA [$F(2, 5) = 53.02$, $p<0.05$]. The differences in the source water at the start can explained the significant differences in nitrate levels during each trial. Table 2-3 shows that after the start of the trial, the nitrate concentrations only varied slightly over the course of the experiment.

Table 2-3 Average nitrate levels in canister filters for trials 2 and 3, mg/l ± standard deviation.

Salinity	Trial 2	Trial 3
Freshwater	3.17 ±0.04	1.89 ±0.01
Brackish water	7.29 ±0.12	10.41 ±0.10
Marine	2.05 ±0.05	2.26 ±0.02

2.4.1.7 Phosphate

The freshwater phosphate concentrations show differences between the three trials, ANOVA [$F(2, 15) = 243.84$, $p<0.05$]. A follow-up Tukey post-hoc test indicates all trials are significant different from one another with PO_4 concentrations, $p<0.05$,

respectively for all three trials. Initially, the phosphate concentrations decreased over the first three days of the trials, followed by a slower declining rate for the remaining of the experiments. The freshwater treatment trial 3 followed this same gradual, flat trajectory until the last sampling, in which it dropped 0.04 mM over two days. It is not clear how a rapid decrease could occur at the end of the experiment. A sampling or testing error may explain the deviation.

The brackish treatment show a similar trend in phosphate changes as the freshwater testing. ANOVA [$F(2, 25) = 276.31$, $p<0.05$] results indicate that there is significant differences between PO_4 concentrations occurring with all trials in the brackish water treatments. A Tukey post-hoc test illustrate that all trials were significantly different (trials 1 & 2, $p<0.05$, trials 1 & 3, $p=0$, trials 2 & 3, $p=0$).

In the marine treatment, the PO_4 levels are significantly difference between trials, ANOVA [$F(2, 25) = 343.86$, $p<0.05$]. Tukey analysis shows significant differences between all three trials ($p<0.05$ for all comparisons). The data of the three trials show the same initial decrease in concentration, followed by relatively flat, unchanging concentrations afterward.

Summary of Canister Study: One can conclude that the significant differences between all trials is the result of the starting phosphate concentrations. The initial drop in concentration followed by slight to no decline in phosphate concentration is possibly due to OAS reaching its capacity to remove phosphate from solution.

2.4.2 Fluidize Sand Filter

The three trials lasted for 32, 33, 26 days, respectively.

2.4.2.1 Flow Rates

Differences in flow rate between the CF and the FSF experiments lead to dissimilarities with velocities, hydraulic retentions times and hydraulic loading rates. Several of the FSF flows show a decline over the period of the experiments with some remaining little changed (Table 2-4). The water

Table 2-4 Percentage Decrease in Flow Rate in Fluidized Filter Trials. A negative value indicates an increase in the measured flow rate.

	Freshwater	Brackish water	Saltwater
Trial 1	9.62%	16.57%	1.98%
Trial 2	7.40%	-0.20%	-1.61%
Trial 3	9.65%	-1.18%	39.86%
Average	8.89%	5.07%	13.41%

velocity and hydraulic loading rate reflect the same pattern as well.

The CF and FSF were similar in their use of the same volume of OAS and operated on the same volume of water, they did have significant differences. The measured flow in the CF were 14.8 lpm versus the FSF at 1.9 lpm. The flow in the canister filters was 7.8 times that of the FSF. The CF produced average flow velocities of 4.02 cm/s, 5.7 times greater than the FSF at about 0.7 cm/s. All trials in both filter setups used a volume of 297 liters. Using the average flow rates, the CF have an average hydraulic retention time of 20.1 minutes, the FSF, 156.3 minutes. Water passed through the CF OAS at a significant more frequent rate than the FSF. The average flow rate through the FSF for all three trials were 1.86 ±0.01, 1.92 ±0.01 and

1.86 ±0.02 lpm for the freshwater, brackish and marine waters, respectively. There are no significant differences in the average flow rates, [ANOVA F (2, 30) =0.6129, p=0.548].

Overall, the percentage of sand bed expansion decreased over the course of the experiments. During all trials, velocity rates show no differences within salinities for freshwater, brackish water and saltwater ANOVA [F (2, 8) =1.86, p=0.22], [F (2, 8) =0.25, p=0.15] and [F (2, 8) =2.24, p=0.17], respectively. Comparisons of the average velocities between salinities show no difference, ANOVA [F (2, 30) =0.62, p=0.54].

The average hydraulic loading rates for the three trials is 431± 40, 444 ± 30 and 431 ± 56 lpm/m^2, for freshwater, brackish and saltwater, respectively. The hydraulic loading rate follows the same behavior as the velocity results. The values reveal no significant differences in the freshwater, brackish and saltwater ANOVA [F (2, 8) =1.91, p=0.21], [F (2, 8) =2.47, p=0.15] and [F (2, 8) =2.24, p=0.17], respectively. As with the velocity, the average hydraulic loading rates per salinity show no significant differences, ANOVA [F (2, 30) =0.63, p=0.54]. Sand loss over the course of the trials ranged from 15.71% to 23.25% with an overall average loss of 19.84%.

The rate of total and daily water losses in the FSF trials are similar between treatments. In order to limit evaporation, the tanks were covered. Even with this preventive measure, water was still lost.

2.4.2.2 Temperature

The average temperatures were 20.7 ±1.4°C, 19.3 ±1.3°C and 20.8 ±1.6°C for the freshwater, brackish and saltwater FSF, respectively. The brackish water temperatures are significantly difference from the freshwater and the saltwater temperatures ANOVA [$F(2,246) = 26.995$, $p<0.05$], Tukey test $p=0$, for both comparisons.

2.4.2.3 Water Clarity

Significant differences between the clarity of freshwater trials exist ANOVA [$F(2, 88) = 31.474$, $p<0.05$]. A follow up Tukey test shows no significant differences in the clarity of the water for freshwater trials 1 and 3, $p=0.656$. The brackish and marine

treatments showed no significant differences between the clarity of their respective trials ANOVA [F (2, 88) =0.322, p= 0.72], [F (2, 88) =1.348, p= 0.26], respectively.

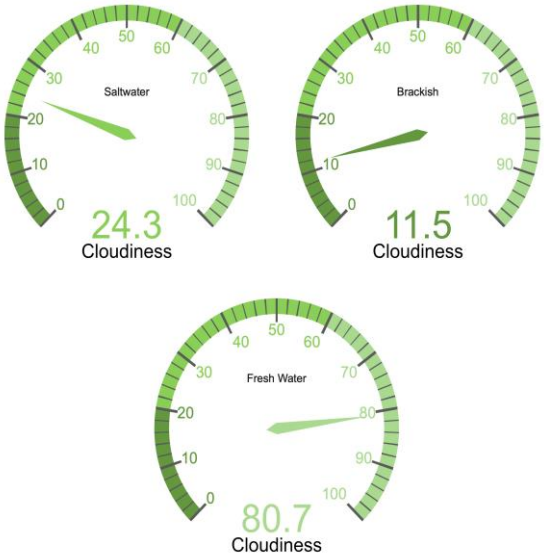


Figure 2.11 Treatment clarity during the three fluidized sand filter trials (T1, T2, T3) Scale: 0 meaning the water above the sand was clearly visible, 0.5 the water above the sand barely visible, and 1 water above the sand was not visible. Clarity Value for 20-day period. Dial read percentage of cloudiness.

Comparisons between the salinities show significant difference in the clarity data, ANOVA [F (2,270) =82.143, $p<0.05$], (Figure 2.11). A Tukey test shows no significant clarity differences occur between the brackish water and the marine trials, p=0.12. Differences in clarity do occur between freshwater and brackish water trials, as well as, freshwater and saltwater trials with $p<0.05$ for both comparisons. The freshwater treatments show a propensity to remain cloudy for extended periods compared to the brackish and saltwater treatments. The differences may stem from the low alkalinities in trials 1 and 3 which were below 1.92 mM $CaCO_3^{2-}$ for the durations of the trials. Trial 2 rose above the 1.92 mM $CaCO_3^{2-}$ threshold on day 13

and cleared on that same day. Alkalinities in trials 1 and 3 of the brackish water and all of the marine trials remained at or well above 2.08 mM $CaCO_3^{2-}$ during the treatment period. The second trial of the brackish water treatment did not increase above 1.77 mM $CaCO_3^{2-}$ at any time, though water clarity occurred on day 3.

The Langelier Saturation Index values were larger for the clear water conditions in all three salinities (Table 2-5). The freshwater Langelier Saturation Index values were 30% larger for the clear water conditions, though no significant difference occurred ANOVA [F93,56] = 2.41, p = 0.07]. The LSI values for the brackish clear water were 5.9 times greater than during the cloudy conditions. The saltwater LSI values are nearly identical, 1.01 and 1.05 (4.0% larger for clear) for cloudy and clear conditions, respectfully. Significant differences between cloudy and clear Langelier Saturation Index values occur between with the brackish water ($p<0.05$), but not the marine water ($p>0.05$).

Table 2-5 Langelier Saturation Index (LSI) values using data from the canister and fluidized sand filter experiments for clear and cloudy water conditions.

Average Langelier Saturation Index Values

	Cloudy		Clear	
	n =	LSI	n =	LSI
Freshwater	54	0.10	6	0.13
Brackish Water	9	0.09	51	0.53
Marine Water	16	1.01	44	1.05

Averaging of all of the LSI values for both cloudy and clear conditions show the brackish water average LSI values is 4 time that of the freshwater and the marine LSI is double that of the brackish water LSI values (0.11, 0.46 and 1.04 for freshwater, brackish and marine experiments).

2.4.2.4 Salinity

As with the CF salinities, variations in the FSF salinities stem from the starting source waters that can vary over the months between trials. The brackish and marine trials in the FSF show a gradual salinity increases for all trials likely due to season ambient temperature changes, evaporation, as well as placement of the setups.

The freshwater treatment showed an average salinity of 0.47 ±0.76 ppt. across the three trials. Salinity differences exist between freshwater trials, ANOVA [$F(2, 29) = 2907.2$, $p<0.05$]. A Tukey test shows no significant salinity difference between freshwater trials 1 and 3, $p=0.92$. The salinities in trial 2 are significantly differ with trials 1 and 3, (both with $p<0.05$). The salinity differences between trials results from the differences in the source aquaculture system. The salinities in all three trials remained stable throughout the treatment period. The change in salinity over the course of the trials ((Starting value –ending value)/ starting value) were 0%, 4.76% and 0% for trials 1, 2 and 3, respectively. The positive value of the percentage is indication of a decrease in salinity.

In the brackish water treatment, the salinity averaged 8.95 ±0.68 ppt. All the brackish water trials were significantly differ from one another, ANOVA [$F(2, 29) = 104.24$, $p<0.05$], Tukey test $p<0.05$ for all comparisons. Increases in salinity occurred with all brackish water trials with changes of 10.13%, 2.20% and 5.32%, for trials 1, 2 and 3, respectively.

With the marine treatment, the salinity averaged 34.05 ±1.92 ppt. Salinity differences occurred between all the marine trials, ANOVA [$F(2, 29) = 22.196$, $p<0.05$], Tukey

test $p<0.05$ with each comparison. As with the brackish water trials, the marine treatment showed salinity increases is all trials, 18.54%, 3.89% and 4.43% for trials 1, 2 and 3, respectively.

Conclusively, the starting salinities of the source waters dictated the overall differences between salinities in the different trials.

Salinities in the freshwater trials remained stable over the trial time period, with the brackish water and saltwater treatments showing a gradual increase. Trial one in both the brackish and marine treatment show the strongest increases with changes of 1.0 ppt. and 6.1 ppt., respectively. The reason for the >10% salinity increases with these two trials is not clear, as the position and setup of the experiments were the same for all trials. The change in salinity during the trials is likely a result of the evaporation concentrating the salts.

2.4.2.5 Water Quality pH

The freshwater pH ranged from a low of 7.72 to a high of 8.18. The pH has averaged 8.09 ±0.09. The freshwater trials show no significant pH differences between trials, ANOVA [$F(2, 29) = 0.236$, $p = 0.791$]. Brackish water treatment pH arranged from 6.93 to 8.16 with an average of 7.94 ±0.26. There are pH differences between brackish water trials, ANOVA [$F(2, 29) = 8.664$, $p<0.05$]. Tukey test shows that the brackish water pH values for trials 1 and 3 are similar, $p=0.95$. Significant difference exist between trials 1 and 2, and 2 and 3, $p<0.05$ for both comparisons. The saltwater treatment shows the pH ranged from 8.02 to 8.23 with an average of 8.09 ±0.05.

2.4.2.6 Water Quality Alkalinities

The freshwater alkalinities ranged from 1.09 mM CO_3^{2-} to 2.15 mm CO_3^{2-}, with an average of 1.55 ±0.24 mM CO_3^{2}-. All three trials show an increasing trend. Significant alkalinity differences exist between freshwater trials, ANOVA [F (2, 29) =12.758, $p<0.05$]. A Tukey analysis shows no significant alkalinity differences between freshwater trials 1 and 3, $p=0.98$. Significant differences do occur between trials 1 and 2 and 2 and 3, ($p<0.05$ for both).

The brackish water alkalinities ranged from 1.15 mM CO_3^{2-} to 2.72 mM CO_3^{2-}, with an average of 2.33 ±0.50 mM CO_3^{2-}. The alkalinities all show a positive trend. Significant alkalinity differences exist between the three brackish water trials ANOVA [F (2, 29) =169.12, $p<0.05$]. A Tukey test shows that the alkalinities in all three of the brackish water trials are significantly different from one another, all with p values <0.05. The starting alkalinities were 1.95 mM CO_3^{2-}, 1.15 mM CO_3^{2-} and 2.40 mM CO_3^{2-}, for trials 1, 2 and 3, respectively. The brackish trials in order increased in alkalinity by 11.5%, 28.3% and 8.14%. One could postulate the differences between trial alkalinities stems from the starting alkalinities of the source water. The saltwater alkalinities ranged from 2.39 to 3.22 mM CO_3^{2-}, with an average of 2.93 ±0.19 mM CO_3^{2-}. No significant alkalinity differences occur between saltwater trials, ANOVA [F (2, 29) =1.412, $p=0.26$].

2.4.2.7 Water Quality Ammonia (TAN)

The TAN concentrations in the freshwater treatment ranged from <0.55 to 11.09 µM, with an average value of 2.13 ±2.64 µM. The brackish water treatment ammonium

levels ranged from <0.55 to 32.15 µM, with an average of 6.05 ±7.13 µM. The saltwater treatment showed a range from <0.55 to 15.52 µM, with an average of 2.22 ±3.53 µM. Trials 2 and 3 of all the salinities general showed a decrease from the starting concentration, due to the source water, and remained at or below 0.55 µM. The second trial of the marine treatment showed three increases on days 6, 11 and 15, followed by a decrease on day 18, where it remained until the end of the trial. The fluctuation in the second trial produced two peaks greater than the starting concentration. The ammonium concentrations of trial 1 in all three salinity treatments show an increase starting on day 15 of the experiment and continuing to the end of the trial (Figure 2.12). The peculiar observation that the trial 1 concentrations increased on the same day of all three treatments leads one to suspect that an anomaly may have occurred, which continued for the remainder of the trial. Even though, sufficient nitrification bacteria appear present to lower TAN concentrations starting as high as 32.15 µM. No significant differences in ammonium data were found between trials for each salinity treatment as test by ANOVA [F (2, 29) =1.968, p=0.158], [F (2, 29) =0.334, p=0.719] and F (2, 29) =0.089, p=0.915] for freshwater, brackish water and saltwater treatments, respectively.

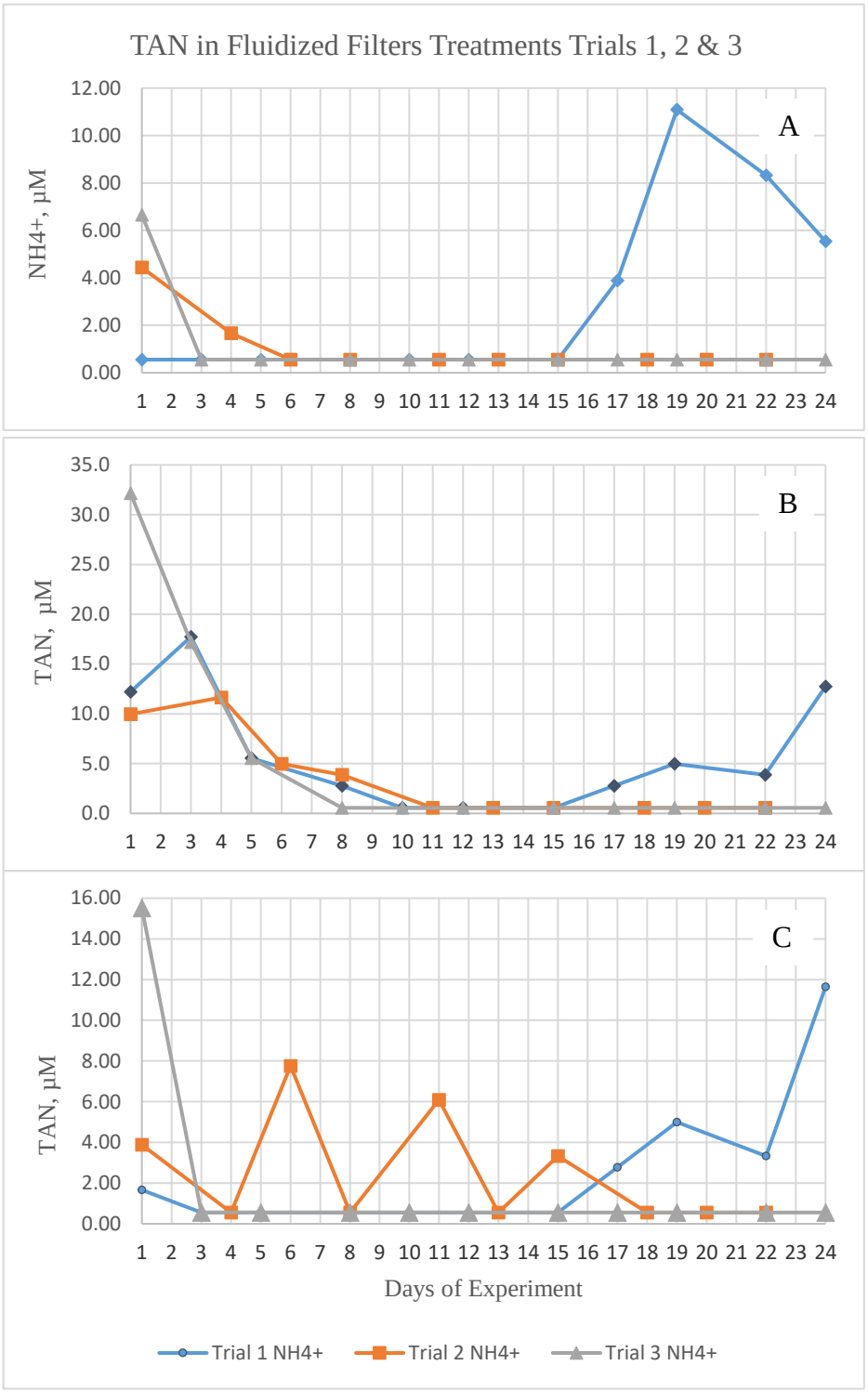


Figure 2.12 Total ammonium nitrogen (TAN) in fluidized sand filter treatments for three trials. A. freshwater, B. brackish, C. marine water. Note y-axis scale vary.

2.4.2.8 Water Quality Nitrite

The Table 2-6 illustrates the minimum, maximum and average NO_2 concentrations that occurred with all treatments and trials. Significant NO_2 differences occur between freshwater trials, ANOVA [$F(2, 29) = 6.383$, $p<0.05$]. A Tukey test

Table 2-6 The minimum, maximum and average ± standard deviation of nitrite in fluidized sand filter treatments, µM.

	Freshwater	Brackish water	Saltwater
Min Value =	0.43	0.22	0.22
Max Value =	11.30	35.43	15.65
Average ±std dev =	3.22 ±2.38	13.32 ±9.53	5.5 ±3.27

shows no significant NO_2 concentration differences between freshwater trials 1 and 2, and 2 and 3, ($p=0.28$, 0.15, respectively). Significant differences do occur between freshwater trials 1 and 3, ($p<0.05$). Trials 1 and 2 do not show a typical peaking of NO_2 values, followed by a gradual decrease as typically seen in cycling of a biofilter, (Figure 2.13A). The data in these trials show an increasing trend. Trial 3 does show the typical rise and fall of NO_2 concentrations commonly observed in the establishment of a biofilter, reaching its high value on day 17. All the brackish water trials show the standard rise and fall of nitrite concentrations of a developing biofiltration (Figure 2.13B). The levels peaked on days 12, 11 and 10 for trials 1, 2 and 3 respectively. The trial 1 NO_2 reading on day 10 appears to be in error with its decrease of 21.74 µM from day 8 to day 10, following by an increase of 22.61 µM on day 12. No NO_2 concentration differences exist between trials in the brackish water treatment, ANOVA [$F(2, 29) = 0.219$, $p=0.805$].

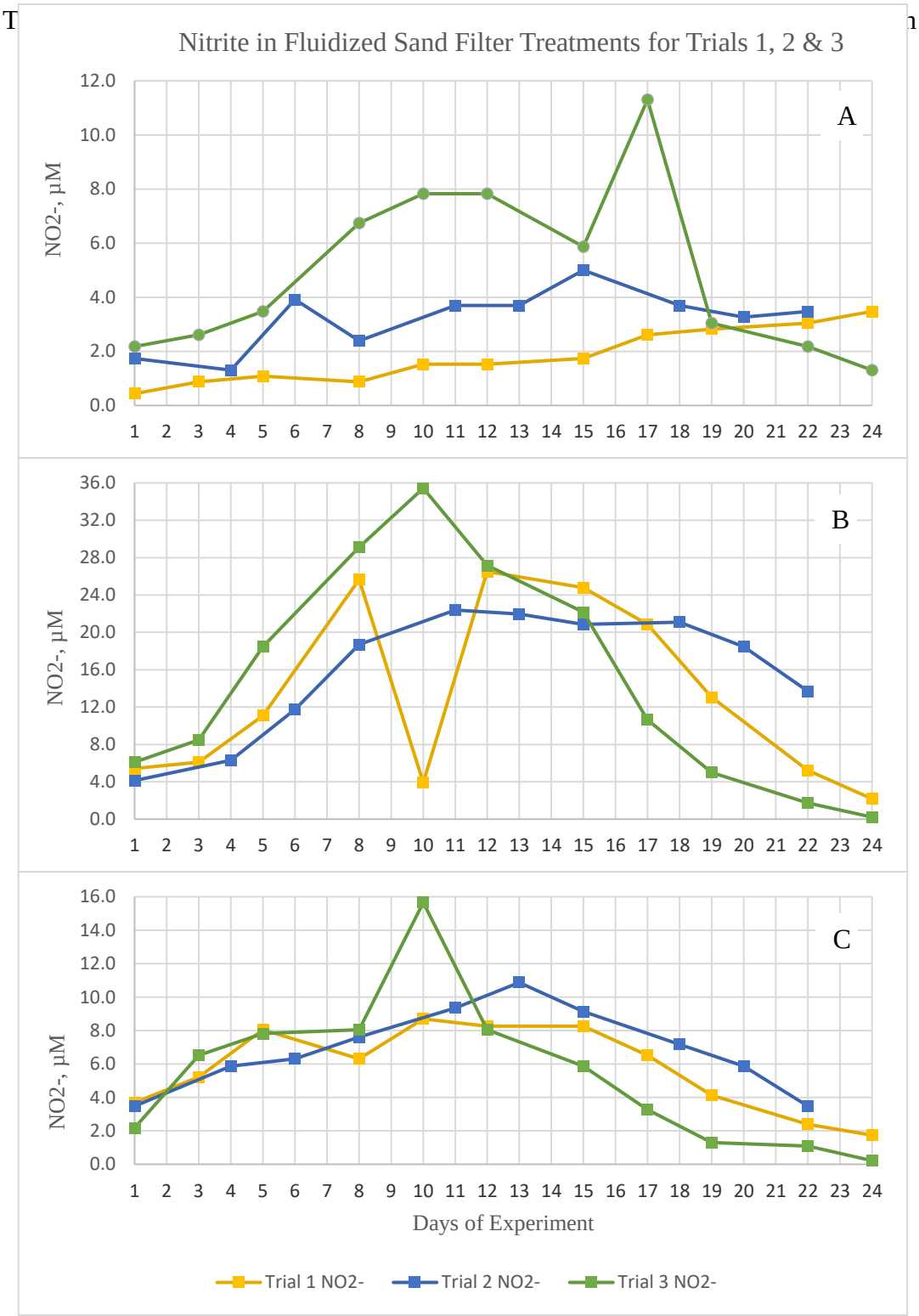


Figure 2.13 Nitrite in fluidized sand filter treatments for three trials. A. freshwater, B. brackish, C. marine water. Note y-axis scale vary.

The marine treatment also show the same trend as the brackish water treatment with peak values occurring on days 11, 13 and 10 for trials 1, 2 and 3, respectively (Figure 2.13C). No significant differences occur in the nitrite values between trials, ANOVA [F (2, 29) =0.559, p=0.578].

2.4.2.9 Water Quality Nitrate

Table 2-7 tabulates the average nitrate concentrations with trials per salinity. Examination of Table 2-7 shows the nitrate levels remained consistent through the trials by the relatively small standard deviation per trial and flat trajectory of data points.

Table 2-7 The average ± standard deviation of nitrate in fluidized sand filter treatments, mM.

Trials	Freshwater	Brackish water	Saltwater
1	1.39 ±0.03	9.16 ±0.18	2.15 ±0.04
2	2.01 ±0.01	12.19 ±0.08	1.93 ±0.03
3	2.21 ±0.06	10.75 ±0.14	1.96 ±0.04

Analysis shows significant nitrate difference between freshwater trials ANOVA [F (2, 7) =243.3, $p<0.05$]. A Tukey test shows significant nitrate difference between all three freshwater trials with $p<0.01$ for all comparison. There are significant nitrate differences between brackish water trials, ANOVA [F (2, 29) =492.95, $p<0.05$]. A Tukey test shows nitrate concentrations significantly difference between all brackish water trials, $p<0.01$ for all. Variations between marine trials exist with NO_3 values, ANOVA [F (2, 29) =33.12, $p<0.05$]. The Tukey test shows no significant nitrate differences between marine trials 2 and 3, p=0.41. However, significant nitrate differences do occur between trials 1 and 2, and 1 and 3, $p<0.05$, <0.05, respectively. The nitrate concentrations variations between trials is due to starting levels.

2.4.2.10 Water Quality Phosphate

During the freshwater trials, the phosphate values ranged from 0.012 to 0.059 mM PO_4^{3-}, with an average of 0.035 ±0.011 mM. The decreasing phosphate trend occurred in all three trials. The continuing decrease in PO_4 level over the course of the trials is an indication the aragonite sand in the FSF had remaining capacity to remove additional PO_4. Significant differences exist between PO_4 valves in the freshwater trials, ANOVA [$F(2, 29) = 13.418$, $p<0.05$]. A Tukey test shows phosphate similarities between freshwater trials 1 and 3, $p=0.16$. Significant phosphate differences occur between trials 1 and 2, and 2 and 3 ($p<0.05$ for both). The percentage decrease between phosphate measurements (starting and ending) ranged from 23.05% to 67.1%, overall average decrease of 44.0%. Most changes between measurements occurred in the range of -28.51% to 14.24%, though there was strong variation with trial 1 on days 17 and 19 and a lesser variability on trial 3 for day 17. A clear explanation of these uncharacteristic measurement changes is not forthcoming. The data shows a stead removal of phosphate with an overall removal of 0.030, 0.014 and 0.022 mM for trials 1, 2 and 3, respectively. The brackish water starting phosphate concentrations all showed markedly different concentrations of 0.48, 0.63 and 0.56 mM PO_4^{3-} for trials 1, 2 and 3, respectively. The measurements ranged from 0.45 to 0.66 mM, with an average value of 0.52 ±0.06 mM. Significant differences occurred between phosphate values in brackish water trials, ANOVA [$F(2, 29) = 143.37$, $p<0.05$]. The data remained relatively stable in trials 1 and 3. Trial2 does show a concentration drop over the first 6 days, then remaining stable

afterwards. The changes between phosphate measurements ranged from -14.0% to 12.2%, with an average of -0.028 ±4.73%. The median value for the percent phosphate changes was 0.071%. The accumulative phosphate changed in the brackish water treatment overall shows mixed results. The first trial shows two decreases in the data, but three positive changes as well, resulting in an overall loss of 0.011 mM representing only a phosphate decrease of 2.4%. The second trials shows a strong increase on day four followed by a strong decline on day six resulting 0.039 mM phosphate loss representing a 6.1% loss. The third trial shows an initial phosphate drop on day 3 then increasing until day 22. The last day of the trial shows a strong drop resulting in an overall phosphate loss of 0.029 mM representing, 5.1%. The marine phosphate concentrations ranged from 0.045 to 0.065 mM, with an average of 0.058 ±0.005 mM. The three trials all showed declines in the phosphate concentrations over the first 8, 11 and 19 days for trials 1,2 and 3, respectively. Following the decline, the values remained stable with an increase in the last sampling for all three trials. Analysis shows significant phosphate differences between marine trials ANOVA [$F(2, 29) = 70.815$, $p<0.05$]. A follow up Tukey test shows significant phosphate differences occur between all trial comparisons with $p<0.01$. The variation of phosphate concentrations between trials stems from the different starting concentrations received from the source waters. The percentage phosphate changes between measurements show a strong initial loss of phosphate before day 8, followed by a series of positive and negative change ending in strong

increases in the data. The trials show accumulative losses of 1.3%, 12.1% and 9.8% for trials 1, 2 and 3, respectively.

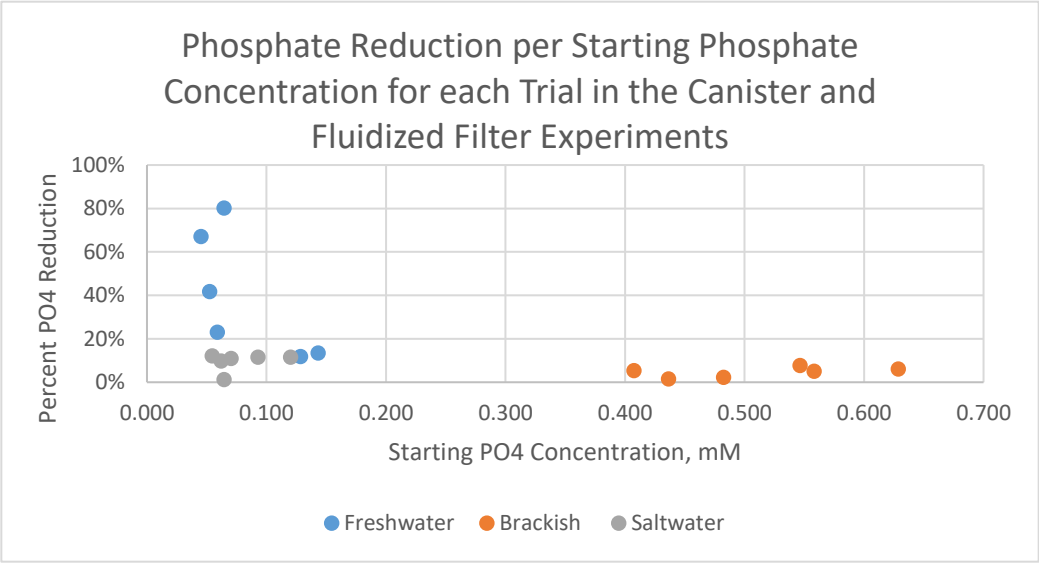


Figure 2.14 The percentage of phosphate removed per starting phosphate concentration of phosphate in the canister and fluidized sand filter trials for freshwater, brackish water and saltwater treatments.

Reviewing the results of the CF and the FSF treatments, one can see similarities with respect to phosphate removal. Both treatment setups used the same amount of oolitic aragonite sand (1000 g) and experimental arrangement only differencing in the filter holding the sand. Therefore, combining the CF and FSF data to see differences between salinity treatments for phosphate removal is prudent. The plot of percent phosphate removal per starting concentration clearly show a relationship with the clustering of data points according to salinity (Figure 2.14). The same grouping occurs with the ratio of PO_4 removed per weight of sand vs. starting concentrations (Figure 2.15).

The clustering of the data indicates that phosphate removal is independent of the contact method (i.e. Canister filter, fluidized sand filter) assuming the water is able to

make sufficient contact to the sand. The ability to remove phosphate from a water source is dependent on the starting concentration and salinity. The treatments of freshwater and saltwater had starting phosphate concentration of ≤0.143 mM. Within

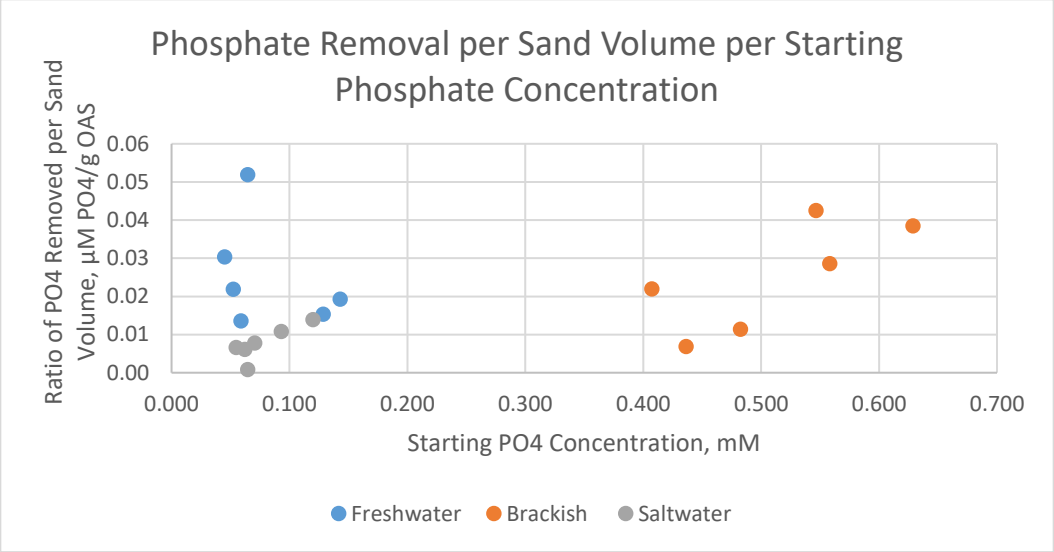


Figure 2.15 The ratio of phosphate removed per gram of oolitic aragonite sand used versus the starting concentration of phosphate in the canister and fluidized sand filter trials for freshwater, brackish water and saltwater treatments.

this concentration threshold, the phosphate removal rate ranged from 1.30% to 80.29% for both treatments. The freshwater and saltwater treatments do differ in the percent of phosphate removed (averaged for freshwater 39.6%, saltwater 9.58%). The current arrangement of experiments did not explore the capacity to remove phosphate at higher starting phosphate concentrations and at concentrations comparable to those of the brackish water in these experiments.

The saltwater and brackish water treatments both had percentage removals of ≤11.65% (average for saltwater 9.58%, brackish water 4.73%), though their starting average phosphate concentrations were 0.078 mM and 0.510 mM, respectively. Comparison of these two treatments indicates that the OAS in saltwater has over two

times the capacity to remove phosphate than does brackish waters on a percent phosphate reduction perspective. In an opposing observation, treatments below 9.0 ppt. appear to be able to remove more phosphate on an mM basis than the saltwater is capable of performing (Table 2-8). In determining the amount of phosphate removed per treatment in relation to the weight of aragonite sand used, the complied data used the following calculation:

> Total Average PO_4 Removed per salinity treatment (mg/kg) = (Average PO_4 Removed of 6 Trials per treatment (mM) x Starting volume of system (l))/Weight of oolitic aragonite sand used per trial (kg)

Table 2-8 Average phosphate removed and salinity using combined data from canister and fluidized sand filter trials for freshwater, brackish water and marine treatments.

Treatment	Average Salinity, ppt.	Average PO4 Removed, mM
Freshwater	0.63 ±0.61	0.0254
Brackish	8.79 ±0.74	0.0250
Marine	34.3 ±1.76	0.0077

Results using this calculation reveals that 716, 705 and 217 mg/kg total PO_4 (234, 230 and 70.1 mg P/kg OAS) are removed from the freshwater, brackish water and marine water treatments, respectively. The freshwater and brackish water treatment therefore are capable of removing 3.3 and 3.2 times more PO_4 per kg aragonite sand than marine water, respectively.

2.4.3 Citric Acid Recovery of Phosphate from Oolitic Aragonite Sand

Initial investigations of the ability of citric acid solutions to remove phosphate from oolitic aragonite sand started with marine exposed sand using a concentration of 0.2%

citric acid. Because of that testing, the effects of higher concentrations were needed to be used starting with a 2% citric acid solution followed by a 1% solution being tested.

Exposure of unused oolitic aragonite sand to 0.2%, 1.0% and 2.0% citric acid solutions occurred to access the amount of phosphate naturally present. The three washes at the 0.2% citric acid concentration shows little various with an average of 0.314 ±0.120 μM PO_4^{3-}. The observation is an indication that this level of citric acid is not effective in releasing phosphate from the OAS. The washes at 0.2% concentration show a declining phosphate removal rate at each successive wash, 0.390 ±0.119, 0.311 ±0.186 and 0.242 ±0.045 μM PO_4^{3-} for washes 1, 2 and 3, respectively. This declining phosphate removal rate is also present at the 1.0%

Table 2-9 Comparison of PO_4 and P removed from unused OAS when treated with 0.2%, 1.0% and 2.0% citric acid solution.

Citric Acid Concentration:	Average mg PO_4/g OAS ± Standard Deviation =	Average mg P/g OAS ± Standard Deviation=
0.20%	0.00044 ±0.00016	0.00014 ±0.00005
1.00%	0.017 ±0.0008	0.0057 ±0.00026
2.00%	0.024 ±0.0018	0.0079 ±0.00059

concentration, though the amount of phosphate removal is significantly greater ($p<0.05$). At the 2.0% citric acid concentration, the values for first and second washes overlap, 19.3 ±1.04 and 22.1 ±2.98 μM PO_4^{3-}. A Tukey analysis shows no significant difference between the set of washes at the 1.0% and 2.0% citric acid concentrations, ($p=0.13$). Results of the exposure of the unused oolitic aragonite samples at differ concentrations of citric acid was used to determine the appropriate

strength of citric acid to free phosphate from the OAS. Seeing no significant differences between the 1% and 2% citric acid treatments, use of a 1% citric acid solution would be sufficient.

Table 2-9 compares the amount of PO_4 and P recovered from three citric acid concentrations per gram of OAS used. Each increase in citric acid concentration yielded a greater amount of phosphate and phosphorus per gram OAS.

Exposure of the OAS remaining from the marine CF trials to citric acid concentrations of 0.2%, 1.0% and 2.0% occurred for washes 1, 2 and 3. The first wash remove the largest concentrations of phosphate followed by the second and third washes. The first and second washes at the 0.2% citric acid solution concentration showed no significant differences, 0.237 ±0.037 µM and 0.242 ±0.030 µM PO_4^{3-}, respectively.

As seen in the Table 2-10, the OAS weight decreased in all three citric acid concentrations and treatments. The reaction of the calcium carbonate based OAS with the citric acid appears to dissolve the sand, as witness by its weight loss. During the varying citric acid exposures, bubble formation occurred on the surface of the sands. Though not confirmed, the acidic reaction and resulting gas release is likely

Table 2-10 Percentage of sand loss per treatment and citric acid concentrations

Average Percentage Loss of Sand due to Citric Acid Treatment

	Citric Acid Concentrations		
Sand Exposure	0.2%	1.0%	2.0%
Unused sand	6.0%	16.5%	20.0%
Freshwater		16.0%	
Brackish water		17.0%	
Saltwater	5.0%	16.0%	22.0%
Overall Average per Acid Concentration	5.5%	16.4%	21.0%

carbon dioxide. The overall weight losses per citric acid concentrations were 5.5%, 16.4% and 21.0% for 0.2%, 1.0% and 2.0% acid concentrations, respectively (Table 2-10). Following the citric acid results of the unused sand and the marine-used sand exposed to the three concentrations, selection of a 1.0% citric acid concentration occurred to treat sand from the freshwater and the brackish water CF trials due to its ability to remove phosphate and its limited effect on sand loss. The data shows a decreasing concentration of removed phosphate in each subsequent wash, as occurred in the unused sand and the marine-used sand citric acid tests at the 0.2%, 1.0% and the 2.0% levels.

The concentrations of phosphate removed by each wash does not reveal the total quantity of phosphate captured by the oolitic aragonite sand, nor the portion removed by the sand with the citric acid treatment. In order to accomplish that endeavor, the calculation of the total amount of phosphate removed from the experimental water using the following formula:

> Total Weight of Phosphate Removed per experiment, (mg) = (Starting PO_4 concentration (mg/l) x System Volume (l)) – (Ending PO_4 concentration (mg/l) x system volume (l))

The percentage of phosphate recovered from the OAS by the citric acid treatment was determined by taking into account the total phosphate removed from the water and the amount naturally occurring on the sand with respect to the amount released from the OAS using the following formulation:

> Percentage Phosphate Recovered from Oolitic Aragonite Sand from citric acid treatment = [Average Phosphate Removed from OAS per gram of Used OAS (mg PO_4/g) / (Total Weight of Phosphate Removed per experiment (mg) +

Average Phosphate Removed from OAS per gram Un-Used OAS (mg PO_4/g)) x 100

The percentage of phosphate recovered from the OAS varies with citric acid concentration and water salinity (Table 2-11). Comparison of the saltwater exposed

Table 2-11 Percentage of phosphate recovered from used and un-used oolitic aragonite sand in 0.2%, 1.0% and 2.0% citric acid washes.

Percentage of Phosphate Recovered from Citric Acid Treated Oolitic Aragonite Sand from Third Canster Filter Trial						
	Total Weight of Phosphate Removed per experiment (mg)	Average Phosphate Removed from acid washed used-sand, mg PO4/g	Average Phosphate Removed from acid washed used-sand, mg PO4/kg	Average Phosphate Removed from acid washed unused-sand, mg PO4/g	Average Phosphate Removed from acid washed unused-sand, mg PO4/kg	% Phosphate Recovered from sand
		0.2% Citric Acid Concentration				
Saltwater	219.78	0.0003253	0.3253	0.0004402	0.44	0.15%
		1.0% Citric Acid Concentration				
Freshwater	1464.21	0.2568	256.84	0.0174	17.38	17.34%
Brackish water	1199.88	0.5326	532.61	0.0174	17.38	43.75%
Saltwater	219.78	0.1821	182.10	0.0174	17.38	76.78%
		2.0% Citric Acid Concentration				
Saltwater	219.78	0.2281	228.06	0.0242	24.18	93.48%

OAS shows a direct correlation between citric acid concentration and percentage recovery of phosphate with recovery rates of 0.15%, 76.78% and 93.48% for 0.2%, 1.0% and 2.0% concentrations, respectively. Scrutiny of the results of the 1% citric acid treatment show an inverse relationship with

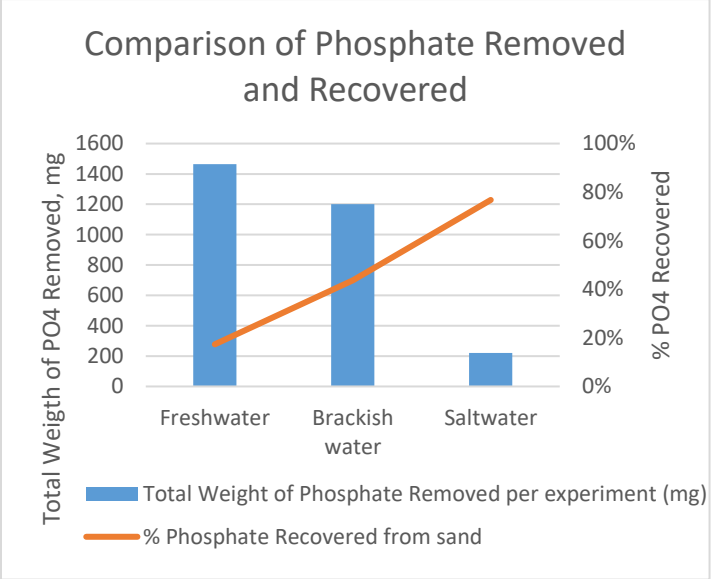


Figure 2.16 Comparison of the total phosphate removed from the canister filter study by OAS per treatment with the percentage of phosphate recovered with a 1% citric acid solution.

salinity for total phosphate removed and the percent phosphate recovered, (Table 2-11, Figure 2.16). As salinities decrease, the OAS appears to have a greater capacity to capture phosphate from an aqueous solution, but shows a reduction of its ability to released phosphate by citric acid occurs. Increased salinities show a lower ability to bind phosphate, but release of bound phosphate is easier by citric acid. In lower salinity waters, aragonite removes more phosphate than higher salinities. In contrast, aragonite in higher salinity waters is able to release more of the capture phosphate.

2.4.4 George Barley Water Prize (GBWP)

The 14-day GBWP experiment occurred twice, with a citric acid wash of OAS treatment occurring between trials and at completion. The first trial ran from July 25, 2017 to August 8, 2017. Circulation of the deionized water filled tank started on July 25, with the phosphorus stock solution added on July 26. The second trial occurred from August 14, 2017 to August 28, 2017. Sampling occurred as outlined in GBWP Stage 2 Guide Entry Guide (barleyprize.org, Table 2-1).

2.4.4.1 Water Conditions Flow Rates

Before the start of the first trial, the systems measured flow rates were 4.44 ± 0.06 and 4.58 ± 0.08 lpm, respectively. The flow increased by 2.9 times from the first to the third flow measurement on days 12. The change in flow during operation was unexpected. The FSF that held the OAS contained gravel on the bottom to disperse the incoming water evenly over the cross sectional area of the filter, as is typical of this type of unit. One can surmise that the flow may of been partial blocked by fine

partials in the OAS which later dissolved and/or were washed out the filter. However, the water's clarity did not change as would be expected if fine particles washed out of the filter. The flows of 9.52 ± 0.10 lpm and 10.13 ± 0.06 lpm occurred 5 days before the start and on 2 day for trial two. They show a more stable rate than that of trial one. The measured water flows on trial two are representative of the last measurement from trial one. This observation would lend support to the idea of a change in the internal resistance of the OAS sand bed within the FSF. A possible explanation could be that the OAS bed developed channeling within it. Channeling occurs when the sand bed is not fully fluidized allowing sand to settle at the bottom of the filter and a channel to form. The resulting increase in flow rate in trial one likely occurred early in the trial and appears to have reoccurred during the second trial. Between the trial one and two, the OAS was acid washed with a 2% citric acid solution according to the method detailed in the 2.6 Material and Methods section. This action would likely remove smaller sand particles by dissolving them into solution and changing the porosity and structure of the sand particles. Such changes may lend the OAS particles to be more uniform reducing the chance of channeling or compaction of the media.

The GBWP fluidized filter cross sectional area is 0.0410m^2 (0.442 ft2), as calculated by direct measurements of the filter. The resulting hydraulic loading rate of the fluidized sand filter in both trials is representative of the measured flow rates. The loading rate ranged from a low of 108 ± 1.6 to 316.8 ± 8.0 lpm/m2. The flow rates of both GBWP trials averaged 8.3 lpm, falling between the CF trials (14.8 lpm) and

the FSF trials (1.9 lpm) previously conducted. The hydraulic loading rates of three OAS experiments: CF, FSF and GBWP fluidized filter are notably different from one another with averaged rates of 2410 ± 36, 435 ± 42 and 203.1 ± 3.1 lpm/m^2, respectively. In the GBWP trials, the averaged system turnover rate was 82.1 ± 1.2 minutes with 21.2 ± 0.3 turnovers per day.

2.4.4.2 Sand Losses

The weight of the OAS at the end of the GBWP trials decreased from 20.000 kg to 16.048 kg (19.76% reduction). The assumption that this loss of sand is from the two citric acid treatments. The 19.76% sand loss is slightly lower than that expected from the 2% citric acid treatments of OAS conducted previously. Those treatments saw a 21% sand loss due to a single citric acid treatment. One can estimate using a 21% loss of sand per citric acid treatment, the calculated weight of the OAS after following the GBWP trial would be 12.482 kg, representing a loss of 37.59%. The discrepancy between the estimated and the measured weights may be due to the exposure of the OAS to the citric acid solution and the agitation strength. In initial citric acid concentration determination experiments, immersion of the OAS samples occurred in the citric acid solution with a greater ratio of citric acid solution to volume of OAS than in GBWP work. The agitation by the shaker was likely stronger than that produced by the flow through the FSF. In the GBWP experiment, the citric acid solution passed through the sand in the FSF, which may have not mixed the OAS as well as on a shaker. The velocity of the water flowing through the FSF likely caused less turbulence than that of the shaker. The combination of a possible

decreased exposure to the citric acid solution and reduced movement of sand particles may have lowered the weight of sand dissolved by the acid treatment. These actions may explain the differences in sand loss from that of the predicted. The GBWP trials occurred at ambient temperatures, without any direct measurement from the experimental system.

2.4.4.3 Water Quality

During the GBWP trials, the water laboratory analyzed samples only for phosphorus and no other water quality parameters. The experiment achieved the phosphorus target concentration for the first trial for week 1 with an average of 0.075 ±0.0002 mg/l and a median value of 0.0748 mg/l (Figure 2.17). The second week average was 0.218 ±0.001 mg/l phosphorus, 0.9% below the target of 0.220 mg/l phosphorus (Figure 2.17). The median value (0.217 mg/l) for the second week was 1.4% below the phosphorus target concentration. The OAS removed an average of 0.065 ± 0.007 mg/l and 0.147 ±0.032 of phosphorus per day, representing a removal rate of 85.3% and 65.1% for week 1 and 2, respectively. The amount of daily removal of phosphorus by the OAS decrease gradual over the two-week trial. Fitting of a linear regression line to the outflow concentration data occurred (in mg/l). The regression

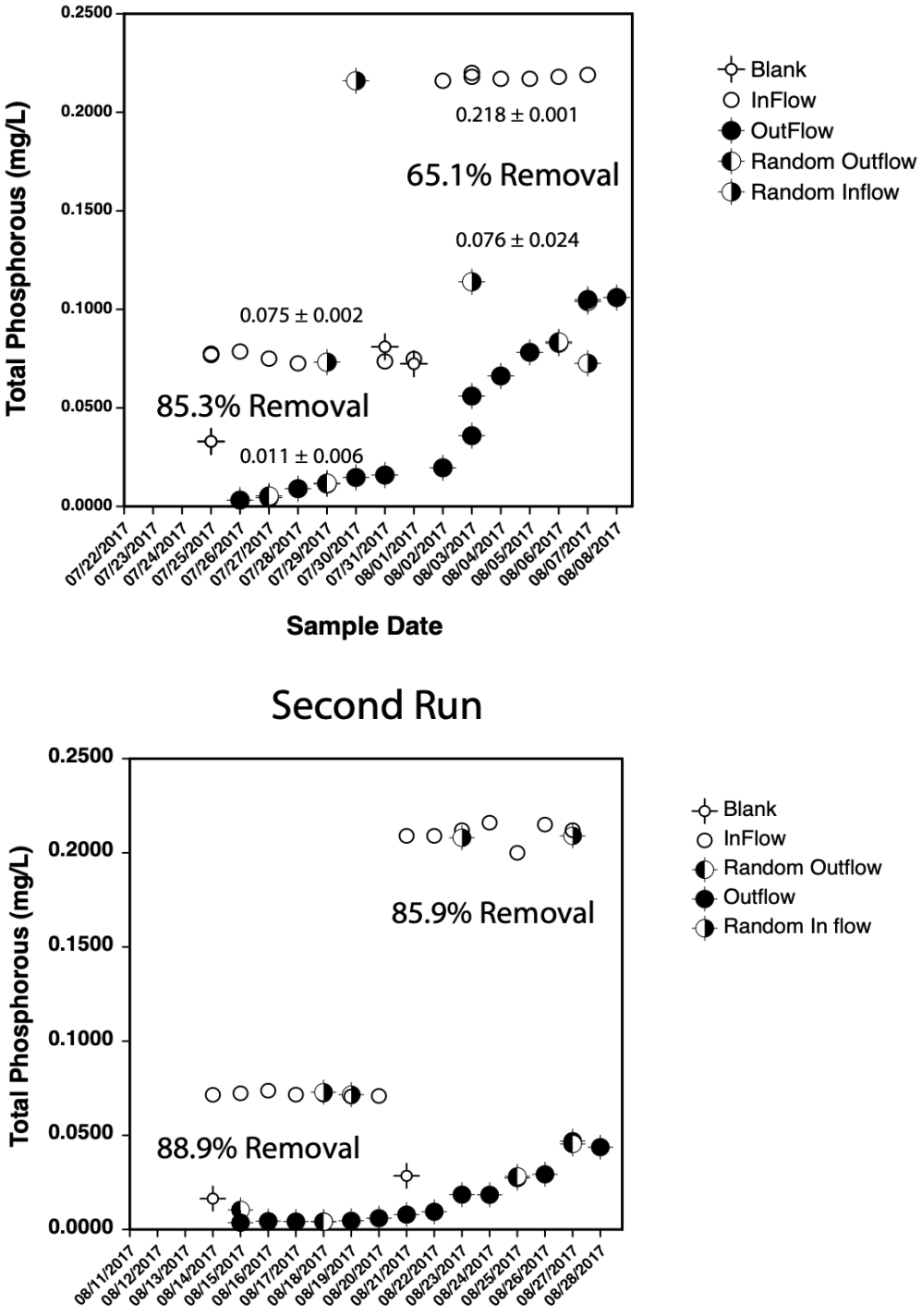


Figure 2.17 Phosphorus concentrations in the inflow and out flow of the George Barley Water Prize study for the first and second runs.

line equation is $y=0.0088x - 0.0314$ with a fit of $R^2 = 0.932$. In order to estimate the

capacity of the OAS for phosphorus removal, the regression equation was set to the average phosphorus concentration during week two, 0.210 mg/l phosphorus, in order to calculate the number of days theoretically remained with the sand if the daily phosphorus additions continued. The regression equation projected the point of OAS phosphorus saturation at day 27.4, or an additional 12.4 days.

Significant differences exist between with phosphorus levels between week 1 of both trials and the target phosphorus level for week 1, ANOVA [$F(2, 18) = 10.329$, $p<0.05$]. A Tukey test shows significant differences in phosphorus concentration between week 1 in trials 1 and 2 ($p<0.05$). The target phosphorus level was met with trial 1 week1 ($p=0.98$), but not with the second trial, ($p<0.05$). No significant differences occur between the second week phosphorus levels for trial 1, trial 2 and target phosphorus concentration level, ANOVA [$F(2, 18) = 1.789$, $p=0.20$].

The averaged inflow concentration during the first week of the second trial was 0.0719 ±0.00095 mg/l phosphorus and a median value of 0.0716 mg/l phosphorus. The OAS removed 0.0669 ± 0.0017 mg/l P the first week for an 88.9% removal rate. During the second week of the second trial, the averaged inflow concentration was 0.210 ±0.005 mg/l P with a median value of 0.210 mg/l P. The OAS removed on average 0.188 ±0.0143 mg/l P per day during the second week with a removal rate of 85.9%. The phosphorus removal rate stayed more stable than that of trial one. The phosphorus removal rate averaged 91.02% ±4.98 % per day over both weeks. The percentage of phosphorus removal during trial 2 ranged from 78.08% on day 14 to

96.22% of day 8. A polynomial equation fit to the daily phosphorus removal percentages: $y = -0.0025x2 + 0.0324x + 0.8497$ occurred with a fit of $R^2 = 0.863$. The phosphorus removal rates for week 1 and week 2 increased in the second trial substantial over the first trial. In week 1 of the second trial, the percentage removal rate increased by 3.6%. The rate in the second week was 24.8% greater. The execution of the experiments were identical in both trials. After the first trial, treatment of the OAS with a 2% citric acid wash occurred as descripted in the Material and Methods 2.3.6. The action the citric acid treatment therefore likely increased the effectiveness of the OAS to removal phosphorus from solution. Returning to the plot using percentage of phosphate removed per starting phosphate concentration (Figure 2.14) from the earlier CF and FSF work, the calculation of the phosphorus concentrations from the phosphate values occurred and plotted along with the GBWP phosphorus removal rates (Figure 2.18). The GBWP phosphorus starting values are the smallest in comparison with the other values, but show the largest rate of removal. The position of the GBWP data on the graph would seem to indicate that at lower concentrations of phosphorus the OAS has better ability to remove phosphorus. In other words, the OAS has a limit to the amount of phosphorus it is able to hold. There is a saturation limitation for the OAS. In Figure 2.18, the freshwater, brackish water, saltwater and GBWP phosphorus removal experiments were group and a trend line fit to the data. The best mathematical description of the data is by an Excel fit of the power equation, $y = 0.0299x^{-0.61}$, to the data in the graph, with an R^2 fit of 0.5993.

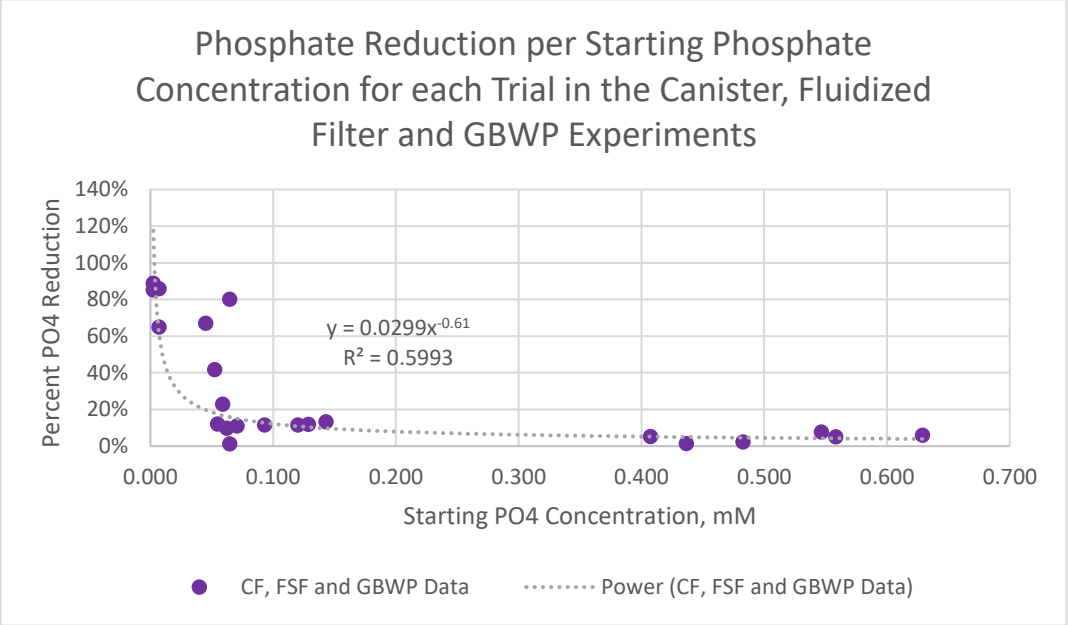


Figure 2.18 The relationship comparison of the percentage of phosphorus removed per starting concentration of phosphorus in the canister filter, fluidized sand filter and George Barley Water Prize trials for freshwater, brackish water and saltwater treatments.

As commented on before, the data sets lack appropriate phosphate/phosphorus concentrations for the brackish water and saltwater experiments. Table 2-12 displays the average starting phosphorus concentrations between these pooled groupings. The saltwater and freshwater average starting concentrations are two magnitudes greater than the GBWP concentrations. The brackish water levels were double those of the saltwater and freshwater starting phosphorus concentrations. Without data sets within

Table 2-12 Average starting salinities per experiments. GBWP include trials one and two. The salinity groupings of freshwater, brackish and saltwater each include all trials from both the canister filter and the fluidized filter work.

Experiment	Average Starting Concentrations of phosphorus, µM
GBWP	4.78
Saltwater	160
Freshwater	178
Brackish	405

each salinity with similar starting concentrations, one could be lead to a possible misinterpretation of the data. The display of the existing data in Figure 2.18 shows an inverse relationship, decreasing percentage of phosphorus removal with increasing starting concentration. This relationship is similar to the behavior of a saturated

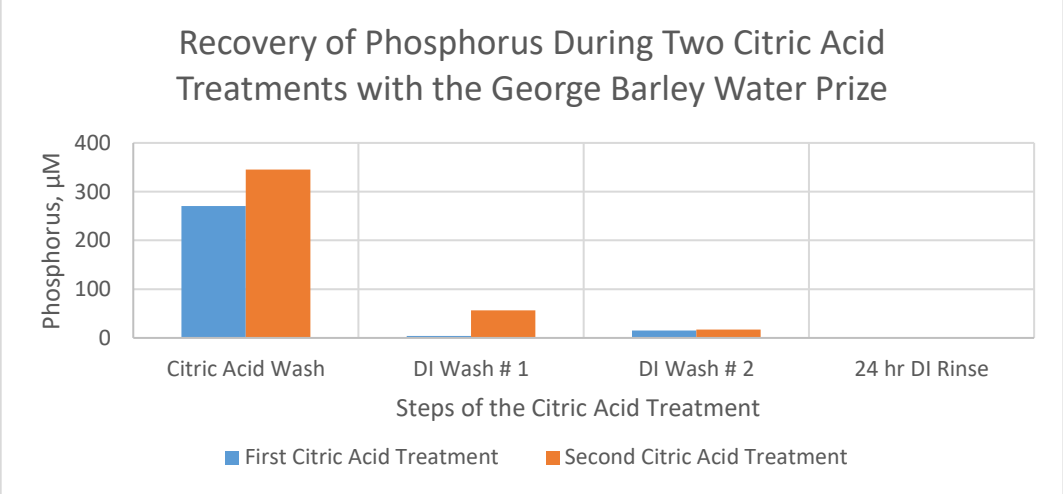


Figure 2.19 Phosphorus concentrations in the citric acid wash, the first deionized water wash, the second deionized water wash and the final 24-hour deionized water rinse for the first and second trials of the GBWP.

substrate.

In order to remove captured phosphorus on the OAS in the GBWP study after both the first and second trials, the application of the citric acid treatment occurred according to the protocol. Phosphorus measurements occurred on the citric acid solution, deionized water wash #1, deionized water wash #2 and a final 24-hour DI rinse from each treatment (Figure 2.19).

Generally, the measured concentration of phosphorus decreased in both trials at each progressive step. The exception is with the deionized water wash #1 in the first trial with a concentration of 4.10 µM phosphorus, compared to the 56.8 µM in the second

trial. The reason for the magnitude of the difference between the two samples is not clear, but may be a sampling error.

The 2% citric acid wash of the OAS in the GBWP first trial removed 524.93 mg of total phosphorus representing 39.4% of the phosphorus added during weeks one and two, along with the estimated amount of phosphorus calculated to be natural occurring on the OAS (Table 2-13). The calculation of the natural occurring

Table 2-13 The percentage of phosphorus removed from OAS after first and second trials of GBWP by 2% citric acid treatment. Total phosphorus per trial is the summations of naturally occurring on OAS and daily phosphorus additions each week.

GBWP Trial 1

	Week One	Week Two	Total, mg	% Phosphorus Recovered
Estimated phosphorus natural occurring on OAS, based on 2% citric acid experiment (0.0079 mg P per g) and 20 kg of OAS			158	
Total Phosphorus Additions, mg	298.2	874.72	1172.92	
Total Phosphorus Accumulated on OAS, mg			1330.92	
Total Phosphorus Removed by Citric Acid Treatment, mg			524.93	39.44%
Phosphorus Remaining on OAS			805.99	

GBWP Trial 2

Phosphorus Remaining on OAS			805.99	
Total Phosphorus Additions, mg	298.2	874.72	1172.92	
Total Phosphorus Accumulated on OAS, mg			1978.91	
Total Phosphorus Removed by Citric Acid Treatment, mg			791.62	40.00%
Phosphorus Remaining on OAS			1187.29	

phosphorus using the experimental data from the 2% citric acid concentration experiment with unused OAS occurred. That work determined that naive OAS contained 0.0079 mg P per gram of OAS. Using this value, extrapolation of the

amount of phosphorus in the 20.000kg of OAS used at the start of the GBWP occurred (Table 2-13).

The acid washed OAS was not removed from the FSF after trial one, but immediately reused for trial two. The OAS in trial two contained the remaining phosphorus from trial one and the additions of phosphorus during trial two. The amount of phosphorus calculated to be on the OAS in trial two increased by 48.69% from trial one. Even with this increase in phosphorus, the citric acid wash at the completion of trial two removed 40.00% of the phosphorus, very similar to that of the first trial.

With nearly a 50% increase in phosphorus, the 2% citric acid treatment was still able to remove the phosphorus at the same rate as trial one. A plausible explanation may be that the citric acid washing of the OAS changed the surface of the sand, increasing the surface area that phosphorus could bind too. The sand is calcareous in composition and reactive to acids, which could affect the surface area. The investigation of the idea of an increased surface area occurred in sections 2.4.5 and 2.4.6 of the GBWP trials.

2.4.5 Surface Area Measurement of Oolitic Aragonite Sand

Quantachrome® ASiQwinTM- Automated Gas Sorption Data analyzed the surface area of the OAS before and after 2% citric acid treatments. The OAS from the GBWP was treated with citric acid twice, once after trial one and again after trial two. The analytical service company LabQMC preformed two independent surface determinations for each sample. The unused OAS has a surface area measurement of

2.805 m^2/g and 2.769 m^2/g. The double acid washed OAS surface area increased to 4.769 m^2/g and 4.488 m^2/g. The surface area increased by 1.66 times in the citric acid wash OAS.

During trial one of the GBWP, the phosphorus removal percentage rate decreased to a low of 52%. In trial 2, the phosphorus removal rate did not decrease any lower than 78%. The 26% increase in percentage phosphorus removal in trial two is likely due to the increased surface on the OAS, offering a great ability to bind phosphorus and pull it out of solution.

In order to place this measured surface area into perspective, a comparison of the surface area of standard sand, used in concrete production, and OAS occurred. Summerfelt (2006) notes that the surface area of regular sand ranges from 4,000 to 20,000 m^2/m^3 fluidized sand biofilters. Using this range and the standard sand densities of 1520 -1680 kg/m^3 (Nemati, 2005), the calculated maximum surface area of regular sand ranged from 0.0119 to 0.0132 m^2/g. Using the average m^2/g values for the regular sand, naïve OAS and citric acid treated OAS, the naïve OAS surface area is 22 times that of regular sand. The citric acid washed sand surface area is 369 times greater than regular sand. Overall, OAS in both naïve and acid washed forms contain surface areas significantly greater than that of regularly used sand in fluidized sand filters.

2.4.6 SEM of Oolitic Aragonite Sand Before and After Citrate Washing

Scanning EM images of the naïve, untreated OAS and the twice-treated citric acid washed OAS are shown in Figure 2.20. The naïve OAS (Figure 2.20A) shows the surface of the aragonite with a surface coating layer. The surface appears not to be smooth, but shows a roughness with high and low areas. The twice-washed citric acid treated OAS (Figure 2.20B) reveals a reduced surface coating than that seen in the naïve OAS. The surface roughness appears greater than that of the naïve sand as the dark areas seem to be void openings. The citric acid treated image reveals the needle-like structure of the aragonite particle. The surface of the citric acid wash sand shows increased complexity as needle-like structures and apparent voids indicating a greater surface area than the naïve sand. The two scanning electron microscopic images support the increased measured surface area reported in the Quantachrome® ASiQwinTM- Automated Gas Sorption analysis.

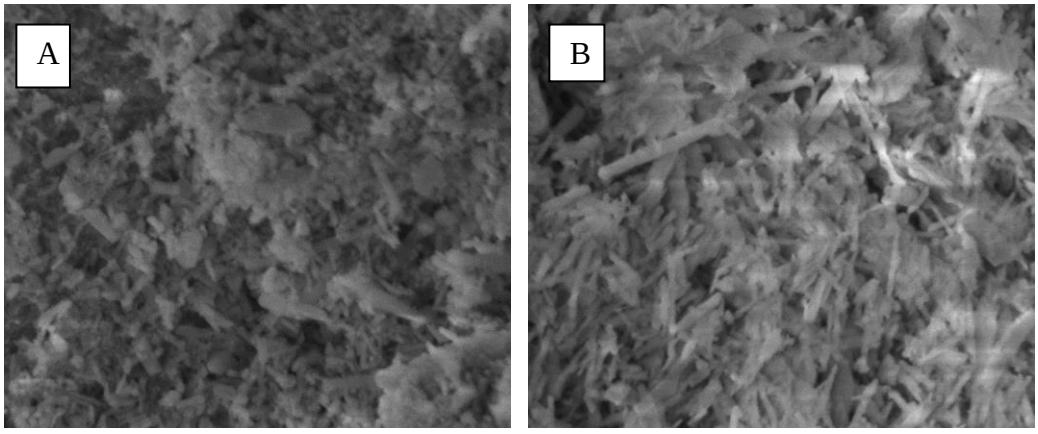


Figure 2.20 Scanning electron microscopic images of the naïve OAS and that of the GBWP OAS treated twice with a 2% citric acid treatment. Image A – naïve OAS. Image B – Twice-treated citric acid exposed OAS.

2.4.7 Microbiome Analysis

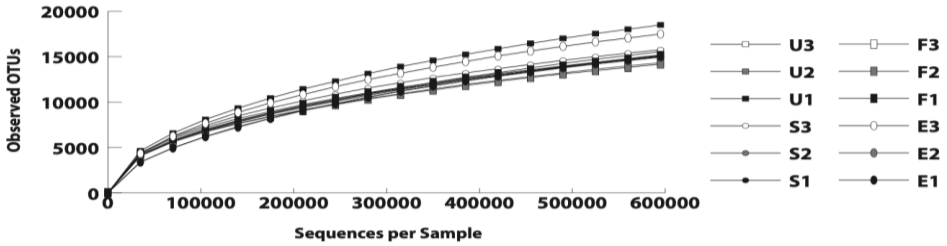
Examination of the bacterial colonization of the OAS show surprising similarities with the unused and used sand, though differences do occur. The rarefaction plot overall shows similar OTU diversity between the naïve sand and those used in freshwater, brackish and marine conditions (Figure 2.21A). The number of new OTU does not increase substantially in subsequent sequence reads. One sample from the naïve sand (U1) and brackish water exposed sands (E3), show closer diversity similarities between each other, than the other samples due to an increase in OTU. The freshwater and marine samples, along with the remaining brackish water and naïve sand samples show similar diversities as their curves group together with increasing reads.

Seven named bacterial phyla occurred with the four samples and an unassigned grouping (naïve, freshwater, brackish water and marine water exposed sands) with Proteobacteria having a relative abundance of ≥40% of the detected phyla (Figure 2.21B). All the sampled contained varying abundances of the detected bacteria phylum.

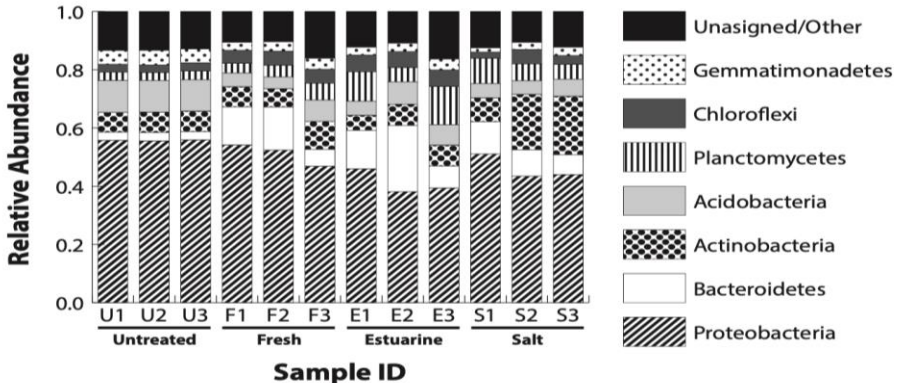
The principal coordinates analysis (Figure 2.21C) show little variation in the PC1 and PC2 axis, indicating less differences between the naïve sand and treatments. Each treatment, as well as, the naïve sand show a strong similarity within each treatments. The marine and estuarine group near to one another, as would be expected, assuming they would harbor bacteria tolerant of varying saline conditions. The untreated sand plot a closer distance with the estuarine and marine treatments than the freshwater

treatment, is plausible being from a marine source. Positioning of the freshwater treatment group further away from the other three groupings signifies less similarity with those groupings.

A



B



C

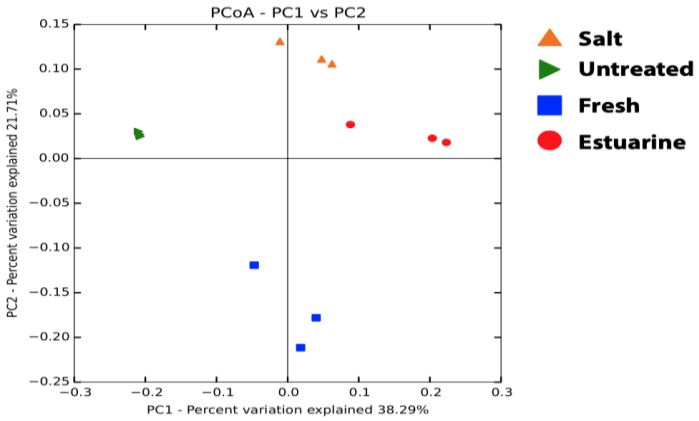


Figure 2.21 **A**. rarefaction plot. **U** –naïve sand, **F**-Freshwater exposed sand, **E**-Brackish water exposed sand and **S**- marine water exposed sand. The plot shows that the number of new OTUs (bacteria phyla) identified is not increasing substantially as sampling occurs with new sequences. B. Relative abundance of bacteria Phyla observed in the data for the three treatments. C. Principal coordinates analysis for the three treatments and naïve sand.

2.5 Discussion

In review of research on whitings in the Bahamas and the Dead Sea, Friedman (1993) concluded that different triggers are involved in initiating the events. Morse et al. (2003) presented reaction kinetics of calcite showing whitings caused by carbonates adhering to resuspensions of sediments rather than direct precipitation. Purkis et al. (2017) suggest the trigger for whitings occurs when the Florida Current brings $CaCO_3$ supersaturated water over the Great Bahama Bank.

Larson & Mylroie (2014) suggest there exist two different types of whiting events: large light colored whitings in deep bank water lasting 2 days and a smaller type, found in shallow water, darker in appearance and with a duration of one day. Bahamian anglers report of smaller, near shore whiting formations attributed to disturbances of bottom sediment by fish as causing the whitings. In consideration of large whiting events, Shinn et al. (1989) used multiple methods to determine if there were fish presence within whiting events. They were not able to locate fish schools and concluded that resuspension of bottom sediments by activity of fish schools do not account for most of the whiting events in the Bahamas. Dierssen et al. (2009) points out that whitings reportedly remain visible up to 45 hours and any fish activity resuspensions likely dissipate within 6 hours, meaning they could not be the sole initiation action to maintain these conditions. Robbins et al. (1997) observed some whiting events on photographs that remained visible up to 7 and even 11 days after first sighted, showing a significantly longer period than previously reported.

Dierssen et al. (2009) noticed evidence of phytoplankton association with whitings from measurements of chlorophyll concentrations. Dierssen et al. (2009) provides evidence of Langmuir currents as being a possible means to suspend sediments into the water column due their reach from surface waters to bottom sediments and current velocities.

Using NASA photographs of whiting events, the authors show that April and October months had the highest frequency of occurrence, pointing to spring and fall as the seasonal periods of whitings, though they do occur year round (Robbins et al., 1997). Purkis et al. (2017) supports a strong seasonality to whiting events with 70% occurring during the winter months between November and the end of April, contrary to the accepted idea that the summer provides the optimal conditions.

The authors first termed the aragonite grains as ooid or oolite structures consisting of a nucleus and up to a few layers of aragonite crystals (Newell et al., 1960). Newell et al. (1960) noted that the oolitic aragonite contained organic matter and hypothesized the sand stemmed from a recrystallization or precipitation process. The physical structure of oolitic aragonite has a greater porosity than calcite or dolomite due its crystal structure (Cherkas et al., 2018). Shearman et al. (1970) report the observation of concentric layer formation on aragonite oolites from the Persian Gulf. Margolis & Rex (1971) using light and scanning electron microscopy reveal the porous structure of oolitic aragonite grains formed by both boring algae and the needle-like crystals of the aragonite. Mitterer (1972) eludes to the possibility that oolitic aragonite particles precipitated due to the organic material embedded in the $CaCO_3$ assemblage.

Edgcomb et al. (2013) in studying the oolitic sands of Highborne Cay in the Bahamas report observation of endolithic borings in the grains, which are habitats for various bacteria.

As a measure of the productivity of the whitings, Shinn et al. (1989) conducted cruises through ongoing whiting events showing carbonate sediment concentrations averages of 10 mg/l within the whitings, compared to 1.5 mg/l outside of the event. Shinn et al. (1989) measured settling rates of particles within the whitings at a rate as high as 34 $g/m^2/hr.$, theorizing that whitings are actively producing sediments continuously. The measured time to settle particles from whitings or other suspended bottom sediments take about six hours. With this short length of time to settle, the Shinn et al. (1989) would expect the events would be short and not lasting for days as observed.

The Everglades Foundation (evergladesfoundation.org) is a Florida based organization dedicated in restoring the American Everglades through education, science and support for cleanup and protection. In 2016, they started a competition, George Barley Water Prize (GBWP) to find new technologies able to remove phosphorus from freshwater to very low levels. The competition had four stages: stage 1, stage 2, pilot study and grand challenge stage. The reward of a prize purse for passing each stage occurred. This work represents the second stage of the competition.

In the current studies, the CF flow rate all remained consistent with an average of 14.8 lpm. The FSF study also shows average measured flow rates per treatment to be

consistent. The average flow rate through the FSF for all three trials were 1.86 ±0.0077, 1.92 ±0.0141 and 1.86 ±0.0243 lpm for the freshwater, brackish and saltwater, respectively. There are no significant differences in the average flow rates, ANOVA [F (2, 30) =0.613, p=0.55]. The CF flows were 7.9 times the FSF, signifying a significant difference in turnover rate and hydraulic retention time.

The differences between the CF and the FSF flow rates stems from the filter type. The CF included a built-in pump. The FSF required a separate pump. A flow control value was not a component of the CF nor was one installed. The CF experiments used the full flow of the filter. The outflow of the pumps used for the FSF required a control valve to adjust the flow, so that the sand bed would expansion and fluidize but with a velocity to keep the sand from being pumped from the filter. The flow limitation of the FSF resulted in the differences. An FSF of increased volume would have allowed similar flow rated between the CF and FSF experiments.

Overall, in the FSF the percentage of sand bed expansion decreased over the course of the experiments. The brackish water trial 2 showed a slight increase on the final measurement, but below the starting percentage expansion. The saltwater trial 1 shows a strong increase in sand bed expansion on day 19 of the experiment, followed by a gradual decline after that point. The ending value remained 6% above the starting measurement.

The expectation of gradual declines in sand bed expansion from decreases in flow rates, as with the freshwater treatment is expected. As the experiments proceeded, the sand began to separate into layers, presumably consisting of particles of differing

densities in some of the filters. The upper most layer of sand usually appeared as a cloudy, moving mass with its surface changing shape and elevation, making for an accurate measurement imprecise. The measurements likely reflect some of this imprecision. Even though the oolitic aragonite sand used for the experiments came from the same collection, there may have been areas with the sample sand that were not uniform in consistency. Since the cloudy sand area in the FSF did not exist at the beginning of the experiment, it is a reasonable conjecture that some of the components of the sand may have been broken down to smaller particles due to the mixing and friction between sand grains. The smaller particles, being of a lighter density, presumably could have formed the cloudy layers.

Particle dissolution into the experimental water and breakage of sand grains to small, lower density particles likely contributed to losses, allowing flow water to transport them to the receiving tank. The differences between minimum and maximum percentages show a decreasing spread in the range, 7.56%, 4.42% and 2.31% for trials 1, 2 and 3, respectively. An explanation for this observation is not readily forthcoming. One could speculate that the narrowing of the range may be due to refinement of flow adjustment and setup of the circulation pumps in each treatment. The trials and salinities do show some variation in the weight of sand lost during experiments, but no significant differences exist, ANOVA [$F(2, 6) = 1.33$, $p = 0.33$]. The CF water clarity shows a strong delay in clearing in the freshwater treatment as opposed to both the brackish and marine treatments. In contemplating a mechanism for these differences, the interaction of the salts with the particles of the cloudy water

may have decreased the clarity. If this were true, a higher concentration of salts in the marine treatment would likely clear the water sooner than the brackish water treatments. Observations do not support this idea, as the brackish water tank cleared sooner (1 day) than the marine treatment (5 days). The FSF trials show a similar clarity clearing as in the CF trials. The FSF freshwater trials remained cloudy for the majority of the experiment. The brackish water trials cleared sooner than the marine trials, as seen in the CF trials. The FSF brackish and marine treatments took a longer period to clear than the CF, which could in part be from the slower HRT in the FSF than the CF. The assumption is the fine partial components of the OAS cause the cloudiness in the water, since rinsing did not occur prior to use. If two assumptions are considered, the OAS added to each filter was uniform and therefore likely to contain a similar amount of fine particles; and the flows within each filter group (CF and FSF) are similar, then the cloudiness/particles in the water should settle out of suspension at similar rates. The lack of this occurrence indicates there is an intrinsic interaction between the cloudiness with the different salinity waters resulting in dissimilar water clarities.

Another possible consideration for the cause of the long duration of cloudiness in the freshwater and to a lesser degree in the brackish and marine experiments, may stem from an imbalance in the concentration of calcium carbonate saturation in the experimental waters of the canister and fluidized filter experiments. The Langelier Saturation Index (LSI) is a calculation, which incorporates water temperature, pH, total dissolved solids, calcium concentration and alkalinity using the formula. The

LSI calculation generates values indicating if the water is under saturated (negative values, corrosive) or over saturated (positive values, scale forming). The LSI values during the clear water conditions remained higher than when cloudy conditions existed in all salinities, with significant differences only occurring in the brackish water treatments. Seeing that the brackish and marine waters experiments cleared at a significantly faster rate than that of the freshwater treatments, differences in the Langelier Saturation Index between clear and cloudy conditions does not appear to explain the differences. The overall average Langelier Saturation Index values show progressively increasing values as the salinity increases, which is understandable since total dissolved solids and the calcium concentrations are increasing with salinity as well. However, the brackish water treatments cleared faster than the marine treatments, the LSI again is not likely the main driver in this observation. The low average LSI value for the freshwater treatments (4 and 9 times lower than the brackish and marine treatments) may be involved in the extended cloudy conditions. The complete understanding of the mechanism for the observed cloudiness between salinity treatments is not clear.

The maintenance of proper pH and alkalinities within RAS systems is essential for production of finfish (Wills et al., 2016). Wills et al. (2016) demonstrated the use of a fluidized sand filter using aragonite sand to control pH and alkalinities in RAS systems. The aragonite filled reactor reached water condition stasis of 7.0 and 135 mg/l $CaCO_3$ for pH and alkalinity, respectively, within 4 days (Wills et al., 2016). In

addition, the reported influent and effluent phosphorus measurement of their small-scale aragonite reactor show a 16.9% decrease in phosphorus (Wills et al., 2016). The current work shows how oolitic aragonite sand influences the water properties in freshwater, brackish and marine waters. These affects include changes in the physical water conditions and nutrient load within the water it contacts.

The pH and alkalinities play a critical role within aquaculture systems to minimize stress on inhabitants, as well as their influence on the state of nitrogen compounds in the system. Use of OAS in the CF and FSF trials demonstrated pH and alkalinity changes beneficial to a RAS to counter the effects of nitrification. The CF pH's in all trials and salinities show increases from starting values. The CF alkalinities showed increases over the starting values for the freshwater and the marine treatments. The brackish water treatment shows no robust alkalinity response from the OAS in the CF. The FSF pH and alkalinity concentrations show increasing values, as with the CF trials. Only the FSF marine trial 1 showed a decline in the pH value occurring, in spite of an increasing alkalinity value. The general increases in pH and alkalinity in both CF and FSF trials points to the ability of the OAS to effect positive and balancing influence over these two parameters in aquaculture systems. The use of a FSF (CycloBio®) to improve pH, alkalinity and remove dissolved CO_2 in a freshwater spring with limestone sands proved effective in increased pH and alkalinity, at the same time reducing CO_2 concentrations (Watten et al., 2017). The authors did not apply the process to saline waters. The starting pH (5.27) and alkalinity level (about 4 mg/l) was significantly below those of this work (Watten et

al., 2017). Even with considering these differences, the one can see that a $CaCO_3$ substrate used within a FSF can be a practical method to modify pH and alkalinity conditions in aquaculture waters. In line with the current work, Wills et al. (2016) operated a large-scale pilot project on a RAS stocked with Florida Pompano (*Trachinotus carolinus*) with water conditions of 26 ppt. salinity and 28.3°C. The authors utilized a CycloBio® FSF with aragonite sand to understand how alkalinity, pH and CO_2 levels are affected at high and low starting alkalinity concentrations (>200 mg/l and ~70 mg/l, respectively) (Wills et al., 2016). The two alkalinity starting levels both reached an equilibrium of about 130 mg/l and a pH of 7.0 over 4 days during the trial (Wills et al., 2016). The work clearly shows the ability of aragonite to control pH and alkalinities in a RAS operation stocked at a moderate density (56.6 kg/m3) (Wills et al., 2016).

The form of nitrogen that exists in RAS is contingent on feeding rates, pH, alkalinity, temperature and the presence of an active nitrifying bacteria population (Timmons & Ebeling, 2010, pg. 293-299). In the CF, the starting TAN concentrations per trial varied according to that of the source water for each treatment. In the brackish and marine treatments, the TAN values fell over the first 7 days of the trial. In the freshwater treatment, the TAN level dropped in the first trial, but reached similar levels over 15 days. The TAN levels at the start of the FSF trial were, as in the CF trial, a result of the source water supplies. In addition, the TAN levels show similar decreases as in the CF trials with some variation among trials and treatments in the FSF work. Contrary to the CF work, the FSF freshwater trial did not show a delay in

the start of nitrification, but it did show similarity to the brackish and marine treatments. Besides drying of the OAS sand, the product did not receive any additional processing. The sand, being unsterilized, likely harbors marine sourced bacteria capable of nitrification. The observation of endolithic borings in OAS (Edgcomb et al., 2013) and the large surface area measurement per volume and scanning electron micrographs in the current study reveal voids and space on the aragonite particle suited for harboring bacteria. Assuming this is the case, the brackish and marine waters would be a more suitable water source than the freshwater. Therefore, the nitrification process would start sooner in saline waters over freshwater.

The nitrite values at the start of the CF trials also stem of the source waters, as does the TAN concentrations. Once the nitrification process starts, nitrite concentrations will rise as is typical of cycling of biofilters (Timmons & Ebeling, 2010, pg. 293-299). The nitrite levels in the CF trials show these initial increases followed by decreases in the brackish and marine treatment though the patterns appear differ between the two treatments. The freshwater treatment shows no changes for trials one and three. Trial two did show an increase in concentration, but without dropping for the rest of the experiment. These results are indicative of an active nitrification bacteria existing within the experimental setup.

The source of the starting water came from systems housing fish with active biofilters. Water from such live systems would contain a variety of live bacteria. If the source waters seeded the experimental setup with nitrifying bacteria, as opposed

to the OAS, one would expect the TAN and nitrite concentrations to behave similarly in the three salinity treatments because the seeded bacteria are from the source water. Seeing a delay in the peak nitrite value by approximately 7 days in the CF trial two and no change with the other two trials in the freshwater treatment compared to the brackish and marine treatments, could indicate the nitrification bacteria poorly establishment to freshwater conditions. Therefore, marine sourced nitrifying bacteria likely reside with the OAS starting the nitrification process in saline waters readily; however, a freshwater environment would likely hinder marine sourced bacteria.

All three treatment in the FSF trials show comparable nitrite graphs showing nitrite levels peaking mid-way through trials, afterwards declining in concentration. The scenario explaining the source of nitrifying bacteria in the CF does not hold for the FSF TAN and nitrite results. The rapidity of the beginning nitrification activity in both CF and FSF trials appears to be occurring rather rapidly. The initial ammonium peaking and declines occurred on day 1 to 4 with the CF study and 8 to 13 days with the nitrite, though one freshwater trial was delayed until day 15. The FSF study had TAN peak periods on days 1 to 4 and nitrite peaks on days 10 to 15.

Timmons & Ebeling (2010) indicate typical biofilter establishment takes 14 days for the ammonium level to peak and 28 days for the nitrite, acceleration occurs with additions of ammonium and nitrite at the start. A case study of biofilter performance on low (8% of maximum density) and high (100% maximum density) with post-smolt Atlantic salmon (*Salmo salar*) showed ammonium and nitrite peaking on days 7 and 16, and days 10 and 12, respectively (Roalkvam et al., 2021). Referencing the

Roalkvam et al. (2021) work, the CF and FSF studies TAN levels peaked appropriately in half the time, though the temperature range was lower at 9.3 to 17.7°C. The peak nitrite concentrations of the CF and the FSF are in agreement with Roalkvam et al. (2021).

The ending product of nitrification, nitrate, shows a different trend than the TAN and nitrite compounds. The measured CF nitrate concentrations in all treatment changed little over the course of the experiments for trials 2 and 3, as nitrate levels in trial one were not examined. The marine nitrate concentrations did show a slight increase in both trials indicating nitrification is proceeding toward nitrate production. One can draw from the findings that the starting nitrate concentrations are due to the levels in the source water and nitrification lagging in the freshwater and brackish waters to form nitrate by the end of the trials. The nitrate concentrations in the FSF also show the starting values stemming from the source waters and remain little changed over the course of the trials. The same limitations in nitrate increases in the CF likely apply to the FSF trials as well.

The quantities of nitrogen present in the source waters are responsible for the nitrogen levels in the experimental setups for CF and FSF trials. During the trials, no introduction of additional nitrogen occurred therefore; the nitrogen levels in the experiment systems were finite. The starting TAN and nitrite concentrations in all trials were below 55.4 µM. Considering the NH_4+ to NO_3- reaction is a one to one conversion, the expected nitrate increase in each trial would only be expected to change by maximum of <0.0323 mM (Timmons & Ebeling, 2010, pg. 293-299). The

starting nitrate concentrations in the trials were generally above 1.61 mM, with only one trial being between 1.29-1.45 mM. Assuming a change of 0.0323 mM nitrate due to existing TAN and nitrite concentrations, the expectation is the trial nitrate concentration to change by ≤ 2% change, a level that may not be observable in the nitrate concentration due to the high starting concentrations in most of the trials.

As with the salinity, TAN, nitrite and nitrate concentrations, the phosphate values at the beginning of the CF trials between all treatments is a result of the source waters. The starting values vary greatly between treatments, but all showed an initial decline usually in the first 3 days of the trials followed by a stable concentration or slight declining readings until the conclusion of the experiment. The accumulative phosphate changes do vary; they show sharp initial declines on some trials with others as gradual continuing declines. With all starting phosphate readings about 0.0632 mM, a level considered high in other operations (True et al., 2004b, Buhmann & Papenbrock, 2013, Davidson et al., 2016).

The FSF phosphate concentrations followed suit as with the CF trials with initial declines and subsequent declines or stable concentrations. Millero et al. (2001) reported of a two-stage adsorption process onto aragonite and calcite, marked by rapid adsorption of phosphate quickly (30 minute) followed by an longer slow adsorption period (> 1 week). The daily fluctuations of phosphate changes in FSF trials show periods of stability, as well as relatively large changes both positive and negative. The plot of the percent of phosphate removed per starting phosphate concentrations graph (Figure 2.14), which includes both the CF and FSF data, give

the impression that freshwater exposed OAS at low phosphate concentrations may be better capable of removing phosphate from solution than when in marine conditions. The brackish water treatments with starting phosphate concentrations between 0.400-0.632 mM showed removal of 0.007 – 0.043 mM phosphate. In freshwater treatments at lower starting phosphate concentrations, show similar removal amounts. The design of the experiments did not include phosphate concentrations in the water sources at low, moderate and high levels for all three salinity treatments, as that method would clarify the effectives of salinity and phosphate concentration would have on phosphate capture. In comparisons of phosphate adsorption on aragonite, the process decreases as the salinity increases, though the presence of Ca^{2+} and Mg^{2+} ions in seawater can increase adsorption (Millero et al., 2001).

Reviewing the results of the canister filter and the fluidized sand filter treatments, one can see similarities with respect to phosphate removal. Both treatment setups used the same amount of oolitic aragonite sand (1000 g) and experimental arrangement only differencing in the filter holding the sand. Therefore, compilation of phosphate removal data is appropriate to see differences between salinity treatments. The plots of percent phosphate removal per starting concentration and using the ratio of PO_4 removed per weight of sand vs. starting concentrations clearly show a relationship with the clustering of data points according to salinity (Figure 2.14, Figure 2.15). The clustering of the data indicates that phosphate removal is independent of the contact method (i.e. Canister filter, fluidized sand filter) assuming the water is able to make sufficient contact to the sand. The ability to remove phosphate from a water

source is dependent on the starting concentration and salinity. The treatments of freshwater and saltwater had starting phosphate concentration of ≤0.143 mM. Within this concentration threshold, the removal rate of phosphate ranged from 1.30% to 80.29% for both treatments. The freshwater and saltwater treatments do differ in the percent of phosphate reduction (averaged for freshwater 39.61%, saltwater 9.58%) (Figure 2.14). The current arrangement of experiments did not explore the capacity to remove phosphate at higher starting phosphate concentrations and at concentrations comparable to the brackish water in these experiments.

The saltwater and brackish water treatments both had percentage removals of ≤11.65% (average for saltwater 9.58%, brackish water 4.73%), though their starting average phosphate concentrations were 0.078 mM and 0.510 mM, respectively (Figure 2.14). Comparison of these two treatments indicates that the OAS in saltwater has over two times the capacity to remove phosphate than does brackish waters on a percent phosphate reduction perspective. In an opposing observation, calculated average salinities and average phosphate removed in CF and FSF (freshwater 0.63ppt and 0.0254 mM, brackish 8.79 ppt and 0.0250 mM and marine 34.3 ppt and 0.0077 mM), treatments below 9.0 ppt. appear to be able to remove more phosphate on an mM basis than the saltwater is capable of performing. In order to determine the amount of phosphate removed per treatment in relation to the weight of aragonite sand used, the complied data used the following calculation:

> Total Average PO_4 Removed per salinity treatment (mg/kg) = (Average PO_4 Removed of 6 Trials per treatment (mg/l) x Starting volume of system (l))/Weight of oolitic aragonite sand used per trial (kg)

Results using this calculation reveal that 716, 705 and 217 mg/kg total PO_4 are removed from the freshwater, brackish water and marine water treatments, respectively. The freshwater and brackish water treatment therefore are capable of removing 3.3 and 3.2 times more PO_4 per kg aragonite sand than marine water, respectively.

2.5.1.1 Determination of Citric Acid Concentrations for Phosphate removal

Exposure of OAS to varying citric acid concentrations proved effective in determining that the 1% and 2% citric acid concentrations for OAS used in freshwater, brackish and marine water applications effectively removed phosphate from the sand. The percentage of phosphate recovered from marine exposure OAS show rates of 0.15%, 76.8% and 93.5% for citric acid concentrations of 0.2%, 1% and 2%, respectively (Table 2-11). The freshwater and brackish water exposure OAS showed lower percent recoveries, but they only received testing at the 1.0% citric acid concentration. Even though the 2.0% citric acid concentration showed increased phosphate removal over the 1.0% solution, the loss of OAS sand was greater (21.0% at 2.0% vs 16.4% at 1.0%, Table 2-10). In selection of a citric acid concentration for phosphate removal, the benefit of increased removal rate needs balanced with the rate of sand loss. As seen in the GBWP, the sand losses following two citric acid treatments were significantly less than predicted (19.8% actual, 37.6% predict). The discrepancies are likely to due to the exposure of the OAS to the citric acid solution and the agitation strength.

In the initial development of the citric acid phosphate removal methodology, the consideration of finding of phosphate on the unused OAS did not occur. Although the amount of naturally occurring phosphate adhered to the OAS is an order of a magnitude lower than the initial 2% citric acid wash of the marine exposed OAS, one needs to take into account existing phosphate on naïve OAS when used as a phosphate removal agent. The use of the citric acid as a release agent for OAS bound phosphate is effective; the concentration used needs balanced against its dissolution of the OAS. In testing 0.2%, 1% and 2% citric acid solutions on used marine exposed OAS, the high concentration was able to remove 28.1% more phosphate than the 1% solution. The 2% solution showed 25% more sand loss by weight than with the 1% citric acid solution. An increase in phosphate removal due to higher citric acid concentrations means a concurrent and increased loss of OAS.

In the current work, the distilled water washes was limited to two. The last distilled water washes results in concentrations of phosphate of 0.0485 mM and 0.0582 mM for 1% and 2% citric acid treatments, respectively. These concentrations in the last washes represent 18% and 17% of the concentration in the first citric acid wash for the 1% and 2% concentrations, respective. The relatively high amount of phosphate remaining in the last distilled water washes would suggest that perhaps additional washes could further reduce the phosphate from the OAS or a longer washing duration. If used in a nutrient reduction application, the number and length of washes would need to be determine based on recovery, time and cost.

Examining the percentage of phosphate the citric acid treatment is able to recover by the three tested concentrations reveals the effectiveness of the treatment. Using the marine exposed OAS at the 0.2%, 1% and 2% concentrations, the method was able to recover 0.15%, 76.78% and 93.48% of the phosphate the OAS captured and naturally occurred for the three concentrations, respectively. Treating OAS used for phosphate removal with a 2% citric acid solution shows a capability of removing nearly 94% of the phosphate removed by the OAS.

The GBWP competition provided an opportunity to show the effectiveness of OAS as a phosphate/phosphorus removal method that is applicable, rechargeable and simple to apply. Over the two weeks of the first GBWP trial, the OAS was able to remove an average of 75.2% of the phosphorus added to the system. The second week of the first trial was able to remove an average of 65.1% of the added phosphorus. Between the first GBWP trial and the second, the OAS underwent a 2% citric acid treatment to remove the adhered phosphorus and recharge the OAS for continued phosphorus removal for the second GBWP trial.

The OAS showed an increased ability to remove phosphorus following the citric acid treatment. The first week of the second trial showed 88.9% phosphorus removal rate. The second week showed 85.9%. The OAS improved its phosphorus removal by 3.6 percentage points over the first week of the first trial and 20.8 percentage points over week 2 of the first trial. The action of the citric acid treatment on the OAS particle unlocks additional surface area in the interior of the aragonite structure enhancing the

available surface area. This increased surface area likely provides the additional capacity in the OAS for more phosphorus attachments to the OAS.

The OAS logically has a limitation to the amount of phosphorus it is able to remove from a body of water. In examining the current work, the equations fitted to the outflow data and for the percent of phosphorus removed estimated when the OAS would be exhausted in both trials. The equations between the trials are different, but the condition of the sand is also different between the trials. The OAS used in trial one was unused, naive OAS, but the OAS in trial two had been citric acid washed once before the start of trial two. By setting, the point where the outflow equation meets the inflow concentration will determine the number days until the OAS reaches capacity.

In GBWP trial one, the actual measured outflow results are used for the first 14 days for the calculations. After that point, the equation ($y=0.0088x-0.0314$) is used for calculating the expected outflow phosphorus concentration. Using this approach, there is a value for each day for the inflow (amount of phosphorus added per day) and an outflow value (either actual reading or a calculated value from equations. Subtracting the outflow value from the inflow value for each day into the future gives the amount of daily phosphorus removed. This difference is multiplied by tank volume (568 l) resulting in the milligrams of phosphorus removed by the OAS per day for the whole tank volume. Summation of the daily results show that trial one can theoretically remove 1383 mg of phosphorus from the water before day 28 when is becomes saturated. The assumed saturation point is that point where the calculated

phosphorus removal becomes greater than that added. The calculated capacity of the OAS is 69.2 mg P/kg OAS.

A similar approach is employed using the percent phosphorus removed per day equation (y=-0.0319x+1.0302) fitted to the data in GBWP trial 1. Using the equation, calculation of the daily percentage of phosphorus removed from the system water occurred for each day of the experiment. Multiplication of the added phosphorus concentration by the measured or calculated percent obtained the amount of phosphorus removed by the OAS. The daily amount removed multiplied by the volume of the system water generated the total amount of phosphorus removed per day. Summation of these daily removal amounts resulted in a calculation that 1502 mg removed over 32 days. Using this value, the OAS capacity to remove phosphorus is 75.1 mg P/ kg OAS.

Following the same methodology above but using the equation (y=0.0021e0.1975x) for the calculation of outflow phosphorus concentrations in GBWP trial 2, show that the OAS can remove 1670 mg of phosphorus over 23 days. The estimated capacity for the OAS to hold phosphorus is 83.5 mg P/kg OAS. Using the same method as in trial 1 with the equation (y=-0.0025x2 + 0.0324x + 0.8497) for the percentage of phosphorus removed during the GBWP trial two, the calculations show a total of 1669 mg phosphorus removed in 26 days. The resulting capacity phosphorus removal capacity calculated is 83.5 mg P/kg OAS.

Comparison of the calculated phosphorus removal capacity of the two GBWP trials show a significant improvement in the OAS to remove and hold phosphorus

following a single citric acid treatment. Averaging the two calculations for each trial, the first trial shows an average P removal capacity of 72.2 mg P/ kg OAS. The second trial shows an average of 83.5 mg P/kg OAS representing an increase of 15.6% in capacity over the capacity in trial one.

Comparison of these removal capacities of the GBWP OAS to the combined freshwater studies in the CF and FSF, show a capacity of 234 mg P/kg OAS. The CF and FSF phosphorus removal capacity of the OAS is 3.24 and 2.80 times greater than the GBWP trials 1 and 2, respectively. The average P exposure of the OAS in the CF and the FSF was 760 mg of total P. The GBWP OAS was exposure to 1670 mg of total P, 2.1 time more than the CF and FSF. The CF and FSF experiments had a longer duration running from 20 to 33 days, representing ≥ 43% longer period than the GBWP trials. The increased length of the experiment possibly allowed a longer amount of time for the OAS to extract the P from the water. The average flow between the CF and the FSF is 8.35 lpm and the GBWP is 9.82 lpm. The flows are similar, but the GBWP system volume is 1.9 times greater than the volume used in CF and the FSF studies. The CF and FSF systems turned over approximately twice the amount than the GBWP system providing additional contact time to the OAS. The additional turnover rate in the CF and FSF may have been sufficient to remove more P than the GBWP per kg of OAS.

The interaction of the starting phosphorus concentrations and the resulting percentage of removal from the water for the CF, FSF and GBWP work reveals a relationship showing greater phosphorus removal at lower starting concentrations (Figure 2.18).

Even though this relations seems plausible and the power equation matched to the data has a limited fit ($R^2 = 0.599$), further work with phosphorus concentrations in the low, moderate and high ranges for freshwater, brackish and marine waters should occur. A future project which incorporates these concentrations and salinities would provide a better understanding if salinity effects the ability of the OAS to removal phosphorus in aquaculture effluents.

With the initial citric acid testing to determine the most effective concentration in phosphorus removal from OAS and the actual use of that selection with the GBWP work, demonstrates the effectiveness of releasing phosphorus by citric acid treatment. One can view this in two ways. First, the proficiency in which a citric acid wash can release, recharge the substrate and actually improve the phosphorus capture capacity ensures that the OAS can be reused likely many times before needed supplemented with additional material. Secondly, phosphorus is a finite resource. As such, its usage should be conservative and in the proper proportions for the intended purpose. With this mindset, efforts to recover any phosphorus or phosphate in waste streams provide opportunities for reuse. The potential use of OAS followed by citric acid wash might be an effective approach for phosphorus removal in certain applications.

The OAS microbiome analysis shows the same phyla detected in the naïve sand also occurs within the OAS in the freshwater, brackish and saltwater used OAS. The three samples within each salinity group show some variation in relative abundance of the phyla. The similarities between the salinity groupings is surprisingly comparable in composition. One can conclude that the OAS can act as a microbial seeding source

for the different phyla observed on the naïve OAS. The families, genera and species these phyla represent may vary from the naïve, as determination at these taxonomic levels occur. In addition, the experimental setups where filled with water from actively operating RAS, which varied not only in salinity but also in fish species as well. The water sources and fish species likely provided their unique bacterial profile of organism differing than that of the naïve OAS.

Chapter 3: Anaerobic Ammonium Oxidation in Freshwater Mediated by Oolitic Aragonite Sand

3.1 Abstract

The release of nitrogen in effluent from recirculating aquaculture systems (RAS) have the potential to lead to eutrophication conditions in receiving waters and as such require treatment. Nitrogen removal can occur by denitrification and anammox bacteria under anoxic conditions. In order to limit nitrogen release into the environment and reduce nitrogen in aquaculture effluents, this work evaluated the use of oolitic aragonite sand (OAS) as a medium for denitrification and anammox

bacterial development in a fluidized sand filter (FSF). The system conditions were favorable for denitrification and anammox activity to occur. The study maintained oxygen levels below 15.6 µM over the duration of the study. The OAS controlled the water to an asymptotic pH of 7.55 and an alkalinity of 4.0 mM as CO_3^{2-}. The NH_4/NO_2 ratio varied throughout the study with ratios above 1.0 maintaining nitrates in the system below 0.093 mM. At a ratio below 1.0, nitrates began accumulating in the system and remained until the NH_4/NO_2 recovered to the 1.0 limit. The low ratio value was not due to nitrite limitation, but the total ammonium nitrogen appears as the limiting factor in spite of thirteen NH_4Cl additions. Microbiome analysis of OAS contained bacterial genera responsible for nitrification and denitrification including *Thermomonas*, *Clostridium sensu stricto*, *Opitutus*, *Nitrosomonas*, *Thermononas*, *Mesorhizobium*, *Hirschia* and *Parvibaculum*. The freshwater anaerobic study observed a decrease of 215.8 grams of nitrogen, representing a loss of 90.5% of the added nitrogen to the system. Without the detection of anammox associated bacterial genera on the OAS, the attribution of the lost nitrogen is likely denitrification instead of anammox, even though conditions were suitable, anammox cannot be completely ruled out as occurring.

3.2 Introduction

The aquaculture production in the United States is actively growing to meet the shortcomings of natural fishery supplies due to limited stocks (Yeo et al., 2004, Turcios & Papenbrock, 2014). The release of nitrogen in effluent flows from

recirculating aquaculture systems (RAS) have the potential to lead to eutrophication conditions in receiving waters (Chen et al., 2002, Vincenzo & Zonno, 2006). The US Environmental Protection Agency (EPA) regulates discharges from aquaculture operations based on production capacity of the facility, type of operation, receiving water characteristics and other factors in issuance of discharge permits in order to safe guard US waters (NRAC Publication N. 00.003 Mugg et al., n.d.). Turcios & Papenbrock (2014) notes that nitrogen effluent concentrations (~1.4 mg/l) can be low but still pose a risk to the environment. A European approach to curbing aquaculture effluent effects on the environment occurred with the AQUAETREAT project, which commissioned the creation and dissemination of methods, technology and knowledge to those in the aquaculture field (Vilella et al., n.d.). Information was disseminated on nutrient reduction methods such as sludge thickening, treatment lagoons, disc filtration, recycling of treated water, and applying sludge as agricultural fertilizers and the feasibility of the techniques (Vincenzo & Zonno, 2006).

In an effort to reduce nitrogen in effluent flows from aquaculture operations, different approached have been tested. Turcios & Papenbrock (2014) review approaches to reduce and mitigate nutrients into the environment by aquaculture operations. These included integrated multi-trophic aquaculture, which incorporate several species that reuse wastes from one another; RAS operations that reduce effluent to concentrated flows and aquaponics, which use plants to reduce nutrient loads on systems (Turcios & Papenbrock, 2014). Osti et al. (2020) utilized artificial float islands of water hyacinth (Eichhornia crassipes) as a nitrogen removal strategy

in tilapia production ponds, resulting in average total nitrogen (g/day) reductions of 64%. The method was effect but sizing of islands, hydraulic retention time (HRT), time to stabilize system and limits to certain species may constrain wide spread adoption (Osti et al., 2020). Artificial wetlands can provide total nitrogen removal of 95 - 98% with a low cost of construction, energy usage and maintenance (Turcios & Papenbrock, 2014). The approach does require sufficient space, which would likely be a limitation. In a small-scale experiment, Wahyuningsih et al. (2015) demonstrate a 49.7% nitrate removal rate in a tilapia/ romaine lettuce aquaponics setup showing the positive effect plants incorporated into finfish production unit can have on nitrogen levels.

Filtration in sewage treatment and aquaculture facilities rely on nitrification to convert ammonium ions to nitrite to nitrate in a two-step bacterial action (Fux, 2003, Ward, 2008, Timmons & Ebeling, 2010). The nitrate is a less toxic form of nitrogen. Though, nitrate is still a nutrient that if released in high quantities can cause environmental damage, therefore effluents need to meet discharge regulations (Fux, 2003, Shivaraman & Shivaraman, 2003). In some sewage treatment facilities, nitrate removal from the effluent stream by denitrification requires anaerobic conditions and a source of carbon to produce nitrogen gas (Fux, 2003).

The use of denitrification to reduce nitrates to N_2 gas by heterotrophic or autotrophic bacteria ensure the removal of nitrogen from RAS to the atmosphere (Timmons & Ebeling, 2010). The process needs to occur in anaerobic conditions with a carbon source for bacterial growth (Timmons & Ebeling, 2010). Using a upflow granule

packed vertical column reactor, Sauthier et al. (1998) cautions that denitrification reactors require stable total organic carbon/nitrogen ratio and a continuous carbon supply to maintain activity without producing undesirable conditions such as large negative swings in redox-potential and formation of sulfide to harmful levels for fish. Development of computer controlled denitrification systems, which monitor dissolved oxygen, oxidation reduction potential, pH and control carbon dosing and flow rates can produce nitrate levels <5 mg/l in a commercial scale RAS (Lee et al., 2000). Management of denitrification conditions prevent nitrite and hydrogen sulfide production (Lee et al., 2000). Nitrate removal rates by denitrification vary greatly (1-166 mg NO_3-N/l/h), likely due differences is denitrification reactor used, operating conditions, source water conditions, carbon sources, etc. (van Rijn et al., 2006). In a freshwater application, nitrogen removal rates of 670 – 680 g N/m^3/day were measured in an up flow denitrification reactor with floating plastic media showing no differences between carbon sources of methanol, acetic acid, molasses and Cerelose™ (hydrolyzed starch) (Hamlin et al., 2008). In order to reduce the chance of leaking residual organics back to culture water from liquid carbon sources in the denitrification reactors, Singer et al. (2008) employed cotton wool at a C/N ratio of 0.82 g cotton/g of nitrate N resulted in nitrate levels of < 10 mg/l.

Using endogenous carbon (i.e. fish feces) for denitrification, can accomplish nitrate removal rates of 26.9% using a fluidized sand with hydraulic retentions time of 15 minutes (Tsukuda et al., 2015). The authors postulated the nitrate removal rate would

increase by applying a longer retention time and ensuring enough carbon is present for denitrification activity to continue (Tsukuda et al., 2015).

Discovery of the bacteria group *Planctomycetales* in sewage treatment plants led to the process of anaerobic ammonium oxidation (Anammox) (Fux, 2003, Shivaraman & Shivaraman, 2003). The anammox process uses ammonium and nitrite ions to form dinitrogen gas directly under anoxic conditions with little biomass produced (Fux, 2003). However, anammox may occur from <0.04 to 8 mg O_2/l in different types of reactors with varying associated complications (Cho et al., 2020).

Most of the initial work into anammox active began with municipal sewage treatment facilities. In an application of domestic sewage treatment, Ma et al. (2015) allowed for short periods of elevated oxygen levels to control the nitrite-oxidizing bacteria so that anammox bacteria were better able to establish at a temperature of 20°C. Management of the dissolved oxygen levels in this fashion proved effective in decreasing total nitrogen levels in effluent to 6.6 ±2.7 mg/l, representing 89.9% decline in total nitrogen (Ma et al., 2015). Working with domestic sewage, Zhang et al. (2008) noted that startup times of anammox treatments are long, though the treatment is cost effective and can be improved with activate sludge inoculates. Cema et al. (2011) show that a limitation in anammox conditions in sewage treatment facilities is availability of nitrite in the source waters requiring low levels of oxygen to maintain limited nitrite generation. Control of dissolved oxygen levels at 3 mg/l, allow the achievement of a removal rate of 1.8 ±0.31g $N/m^2/d$ (Cema et al., 2011). Anammox activity in sewage treatment, once stabilized, appears to show a level of

recovery when temporarily exposed to dissolve oxygen levels around 5 mg/l, which minimal effect anammox if followed by decreased oxygen levels (Kimura et al., 2011).

One type of anammox treatment applied to an aquaculture operation is the SNAD (simultaneous partial nitrification, anammox and denitrification) (Lu et al., 2020). A SNAD bioreactor connected to a RAS system was able to maintain nitrate levels in the system at 0.75 mg/l over the 180-day trial representing an example of a nitrogen removal system exposed to low carbon and high oxygen influent waters (Lu et al., 2020).

Understanding the need to limit nitrogen built up in the environment and the requirement for aquaculture effluents to be low in nitrogen, this work seeks to evaluate the use of oolitic aragonite sand (OAS) as a medium in a fluidized sand filter (FSF) to establish conditions conducive for denitrification and anammox activities. The objectives are to 1. Demonstrate the establishment of suitable conditions for denitrification and anammox in freshwater using oolitic aragonite sand as a medium. 2. Describe how an oolitic aragonite sand filter on an anaerobic system handles repeated nitrogen additions. 3. Show removal of nitrogen from the freshwater setup, signifying occurrence of denitrification and/or anammox.

3.3 Methods

3.3.1 Experimental Design

The freshwater denitrification experiment was composed of a Solar Components Corporation (solar-components.com) tank of 700-liter volume (30" diameter x 5' high, item# 11012). The reservoir tank was raised approximately 9 cm above the floor with wood supports. Installation of a ¾" (19 mm) bulkhead fitting in the bottom center of the tank occurred with flexible tubing connecting it to a circulation pump intake (PAN World Magnetic Pump Model NH-50PX). The pump outlet was directed to a fluidized sand filter (MacClean Water Treatment System, Model#

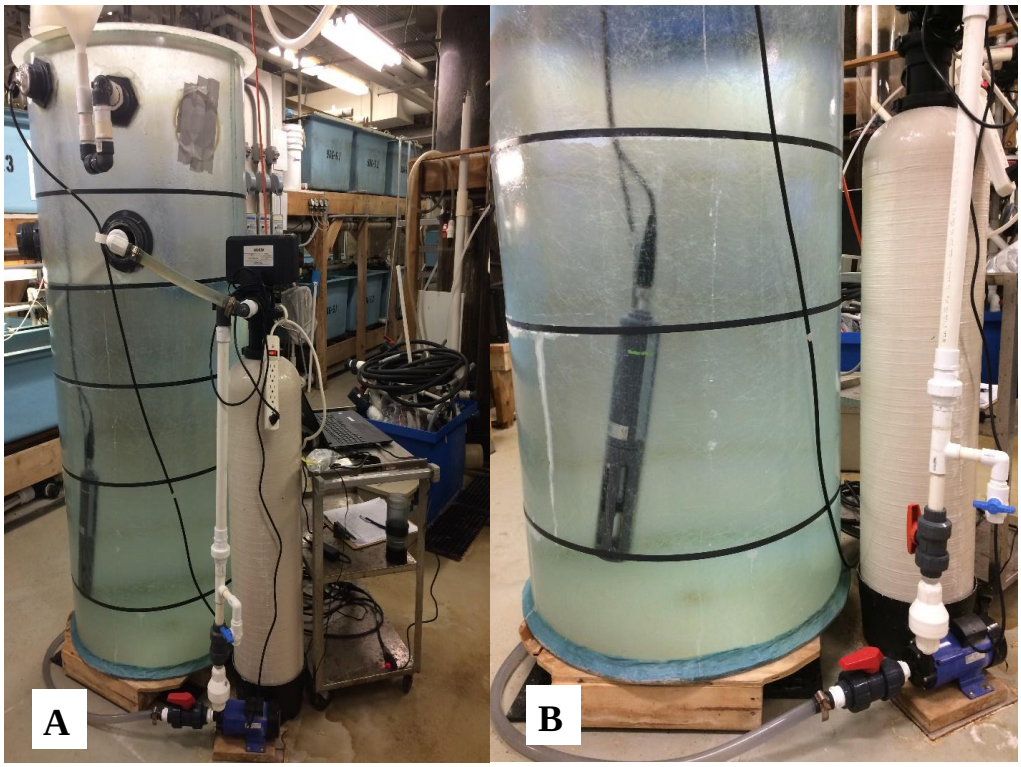


Figure 3.1 A. Arrangement of reservoir tank, circulation pump, fluidized sand filter, return to reservoir, addition port with funnel, monitoring probe in reservoir and computer recording data. B. Close-up of circulation pump, sampling port above pump and monitoring probe positioned in reservoir.

3P971, Rated Service Flow 5 gpm, Cuno Water Treatment Churubusco, IN, USA) (Figure 3.1A). Installation of a sampling port on the outlet of the pump acted as a sampling point (Figure 3.1B). In order to distribute in-flowing water, placement of inert gravel (9.3737 kg) on the bottom of the filter occurred. The used gravel was that which passed through a #8-mesh sieve (openings 2.38 mm, 0.0937 in.). Filling of the FSF with 15.0000 kg of OAS previously used in the second stage of the George barley Water Price (GBWP) competition occurred (Chapter 2, section 2.3.4). The OAS had been citric acid washed twice following the procedure employed in the GBWP work. The outlet of the FSF was direct back to the reservoir tank, so that the

placement of the returning pipe was below the water surface to avoid agitation. Deionized water (568 liters) filled the tank. Inoculation of bacteria into the system occurred with the addition of active seeded media and the oolitic aragonite sand in the fluidized filter. One liter of mature Kaldnes™ K1 filter media (Water Management Technologies Inc., www.w-m-t.com) from a biofilter on a recirculating system holding tilapia (*Oreochromis niloticus*) was hung in a mesh bag in the reservoir tank. Suspension of an YSI 6-Series multi-probe 6600 V2-4 Sonde in the reservoir with outputs connected to a laptop for recording conditions every 15 minutes. The Sonde included a dissolve oxygen, temperature, conductivity, pH and chlorophyll sensors. A computer running the software program Eco Watch Lite (https://www.ysi.com/customer-support/software-firmware-downloads/software) accessed and stored the data from the YSI monitor. Measurement and recording of dissolved oxygen occurred in the reservoir. Sealing of all penetrations through the reservoir tank prevented oxygen from entering the water. The inclusion of an additions port on the side of the reservoir tank using a water filled U-trap made from 1" (25.4 mm) PVC pipe and fittings provided an air-lock preventing oxygen penetration into the system (Figure 3.2). Additions of solutions of ammonium chloride and sodium

Figure 3.2 Additions port with funnel on side of reservoir tank. Piping filled with deionized water.

nitrite used the fill port and return of excess water during sampling from the sampling port.

An initial system test period conducted to confirm reliable operation of the system. Aeration of the reservoir tank occurred during this time with a diffuser positioned on the bottom. Expelling of oxygen from the reservoir tank by administering nitrogen metered in by a fine pore diffuser positioned on the tank bottom occurred to create an anaerobic environment. Aeration within the reservoir ended prior to the administration of the nitrogen gas. The diffuser was connected to a liquid nitrogen dewar by vinyl tubing, a control valve and regulator. Supply of a moderate flow of nitrogen maintained a gentle water circulation within the tank.

Spiking the system with 42.585 milliliters of a 1000ppb phosphorus stock solution from the GBWP experiment for a target reservoir concentration of 75ppb (2.42 µM) phosphorus marked the start of the study. Addition of ammonium chloride (117.902 g, purity 96.5%) and sodium nitrite (59.629 g, purity 97.0%) occurred to achieve a concentration of 70 mg/l for both ions.

At completion, collection of three samples of OAS occurred in sterile vials for bacterial analyze. Drying of OAS occurred by spreading the sand on trays with laminar airflow passing over the trays. Mixing of the sand occurred often during the drying period to allow complete evaporation of the water. The final weight of the dried OAS occurred by subtraction of weight of the sand from the original weight of the gravel. The calculated percent loss of sand used this formula: Percent Sand Loss = ((Original sand weight - used sand weight)/ Original sand weight) x 100.

3.3.2 Water Quality Testing

Water collections occur from the sampling port directly with purging approximately 2 liters of water from the sample port, one liter at a time. Return of unused water to the reservoir was by additions port. Collected water samples came from the second liter removed from the sample port. The Water Quality Laboratory of the National Aquarium Baltimore, MD (aqua.org) tested samples for pH, NH_4+, NO_2-, PO_4- and alkalinity once per week. The laboratory referenced the following methods from the manual Standard Methods for the Examination of Water and Wastewater, 21st Edition to analyze the parameters (2510, 4500- H+, 4500- NH_3, 4500- NO_3, 2320 and 4500- P, respectively). Samples for nitrate were taken at the sample time and analyzed by the Molecular Characterization and Analysis Complex laboratory at the University of Maryland Baltimore County using method 4500- NH_3/ 4110 from the manual, Standard Methods for the Examination of Water and Wastewater.

Over the course of the experiment, additional ammonium chloride (Sigma-Aldrich, ACS reagent, 96.5%) and sodium nitrite additions (Sigma-Aldrich, #237213-500G, ACS reagent, ≥ 97.0%) occurred based on water quality results. The additions occurred by way of the additions port on the side of the reservoir tank.

3.3.3 Microbiome Analysis

Weighing of triplicate oolitic sand samples occurred 100 mg aliquots and placed into the homogenization tubes from the Qiagen DNeasy powersoil kit (Germantown, MD). Performing homogenization of the twelve samples occurred for 30 seconds in

an MPbio (Santa Ana, CA) FastPrep-24 at a power setting of five. 450 µl of supernatant were removed from the homogenization tubes and placed into a Qiacube (Qiagen) carrier holder. Performing DNA extraction occurred using the protocol for the DNeasy powersoil kit on the Qiacube and the optional IRC step for PCR inhibitor removal. Removal of the remaining PCR inhibitors occurred by passing each sample through a Zymo Research Zymo-Spin IV-HRC column for 1 minute at 10,000-x g following removal of storage buffer. Amplifiability was tested for each sample using the bacterial 16S primers, 27F and 1492R (Suzuki 1995) and the 2x PCR mastermix from Promega (Madison, WI). Separation of PCR products occurred by electrophoresis on a 1% agarose TBE gel and imaged using ethidium bromide on a BioRad (Hercules, CA). Use of five ng DNA total occurred to generate the libraries as depicted in Figure 1. We sequenced V3-V4 region. It was a 2 X 300-cycle run. We10pM total pooled library to load the sequencer for sequencing. The gene-specific sequences used in this protocol target the 16S V3 and V4 region. Selection of sequences were from the Klindworth et al. publication (Klindworth A, Pruesse E, Schweer T, Peplles J, Quast C, et al. (2013) Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res 41(1).), as the most promising bacterial primer pair. Illumina adapter overhang nucleotide sequences are added to the gene-specific sequences. The full-length primer sequences, using standard IUPAC nucleotide nomenclature, to follow the protocol targeting this region are:

16S Amplicon PCR Forward Primer = 5'

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCA

G 16S Amplicon PCR Reverse Primer = 5'

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCT

AAT CC

Data Analysis Pipeline for Aragonite Sand Microbiome

1. Quality trimming

a. All read pairs quality trimmed using CLC Genomics Workbench (Qiagen, Hilden, Germany)

i. Trimmed using quality score (limit = 0.05)

ii. Trimmed ambiguous nucleotides (max. number = 2) iii. Minimum sequence length after trimming (n = 100 bp) iv. Discarding of orphan reads occurred

2. Merger of read pairs

a. All read pairs were merged using CLC Genomics Workbench i. Mismatch cost (n = 2)

ii. Gap cost (n = 3)

iii. Maximum unaligned end mismatches (n = 0) iv. Minimum score (n = 8)

Exclusion of unsuccessfully merged read pairs occurred from downstream analyses

3. Read normalization

a. Normalization of reads occurred across samples

i. ~697,000 merged reads/sample which is the lowest read per sample count. OTUs were picked using the open reference OTU method in QIIME

OTU picking method – uclust. Identity threshold (n = 0.97)

Reference database - Silva123_QIIME_release

Percent subsample (n = 0.001)

Prefilter percent identity (n = 0.0)

Minimum failure threshold (n = 100000)

vii.Minimum cluster size (n = 2)

Taxa summaries

Reporting of relative abundances of taxonomic groups occurred for all samples using QIIME. Reported taxonomies occurred for levels 2 through 6

Alpha diversity measures

a. Calculation of alpha diversity metrics occurred from constructed BIOM OTU table (output from open reference OTU picking). Alpha diversity metrics: Goods_coverage, chao1, observed_otus, simpson, simpson_e, PD_whole_tree, observed_species. Performance of multiple rarefaction occurred on OTU tables to generate average rarefaction plots

Calculation of Beta Diversity Beta diversity measures occurred from constructed BIOM OTU table (output from open reference OTU picking). Generation of weighted and unweighted 2-D and emperor PCoA plots occurred for all samples

3.3.4 Statistical Analysis

Calculated averages include ± standard deviation where appropriate. Calculated standard deviations used the STDEV.S formula in EXCEL.

3.4 Results

3.4.1 Water Quality

Starting on December 19, 2017, the experiment setup, water quality monitor, computer, FSF and circulation began for a trial period to test operation of components. Adjustment to the system occurred during this time. The freshwater anammox experiment started on February 7, 2018 with the addition of the phosphorus stock solution, and the ammonium and nitrite compounds. Sampling occurred before the additions. The water was circulated overnight, and a water sample taken. Stopping of the aeration, addition of the seeded media and start of nitrogen gas purging occurred at this time. The experiment ended after 97 days. At the completion of the experiment, the OAS in the FSF was samples for bacterial analysis. The temperature ranged from 20.55°C to 21.46°C, with an average of 20.91 ± 0.14°C. The median value was 20.92°C. The temperature during the experiment remained very stable throughout with a difference between the high and low values of 0.91°C (Figure 3.3).

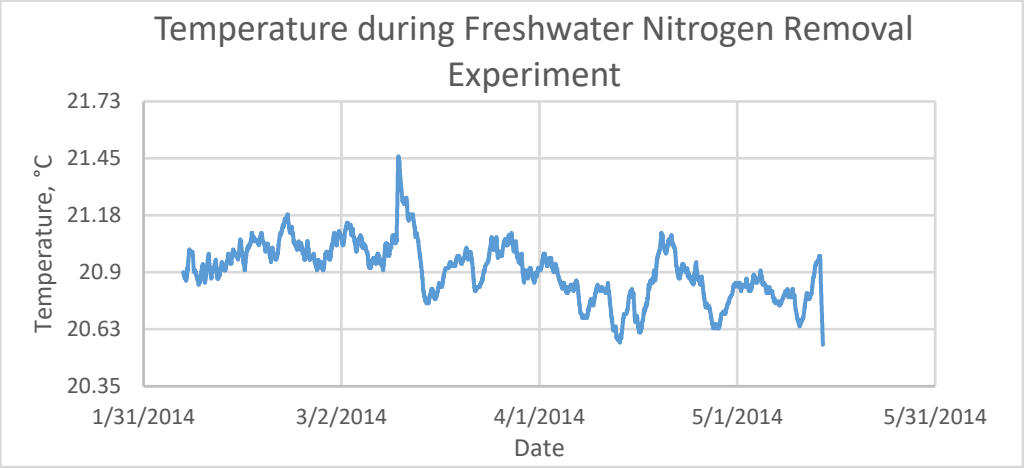


Figure 3.3 Temperature during the freshwater nitrogen removal study, recorded every 15 minutes.

The dissolved oxygen concentration on the first day of the experiment began at 292 µM and dropped to below 31.2 µM by day 3 at 8:00 AM. The dissolved oxygen averaged 10.0 ±3.12 µM from day 3 until the end of the experiment. During this time, the median value was 9.38 µM. Once the oxygen concentration reached below the 31.2 µM threshold, it remained stable for the completion of the project as evidenced by Figure 3.4 and the relative low standard deviation of the average over this period. The addition of nitrogen gas continued from the start until day 15, at which time the empty dewar was removed. By day 15, the dissolved oxygen concentration was at 8.13 µM. Since the oxygen concentration, reached levels well below 15.6 µM and appeared stable without the use of nitrogen; the decision was not to resume nitrogen additions.

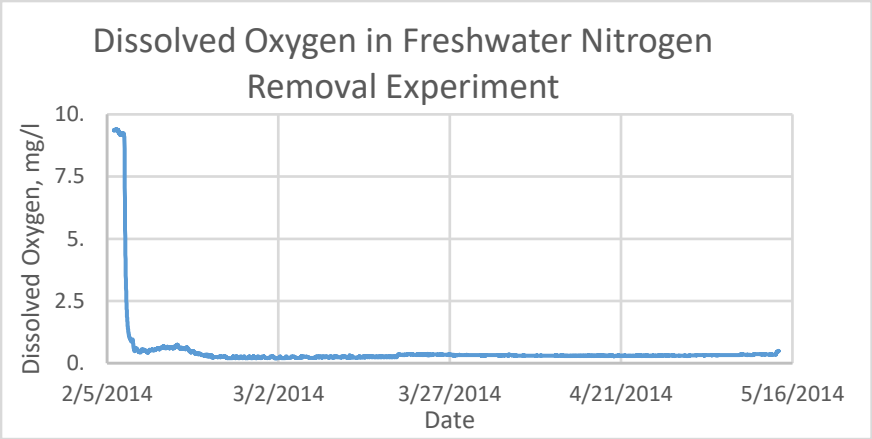


Figure 3.4 Freshwater nitrogen removal experiment dissolved oxygen data recorded from YSI Sonde unit from February 7, 2018 to May 14 2018, taken every 15 minutes.

The salinity increased from 0.1 ppt. to 2.5 ppt., though average 1.0 ±0.7 ppt. The pH trended downward with a high point of 8.44 pH units and a low of 7.51. The pH averaged 7.82 ±0.25, with a median value of 7.78 (Figure 3.5). The trend line fitted to the pH data is y= -0.19ln(x) + 8.4892, R^2 = 0.7538 (Figure 3.5). The alkalinity averaged 3.10 ±0.69 mM CO_3^{2-} and a median value of 3.32 mM CO_3^{2-}. The alkalinity reached a high of 4.11 mM as CO_3^{2-} and a low of 1.66 mM as CO_3^{2-} (Figure 3.5). The trend line fitted to the data is y=1.5763x0.1886, R^2 = 0.7526 (Figure 3.5).

The starting addition of 117.9 g of NH_4Cl resulted in a total ammonium nitrogen (TAN) concentration of 1.65 mM. The TAN concentration varied throughout the experiment with an average of 0.88 ±0.37 mM with a median value of 0.79 mM (Figure 3.6). The TAN ranged from 0.00055 to 1.66 mM over the course of the experiment. During the experiment, thirteen additions of NH_4Cl (59.9 g) were added on days 30, 37, 58, 66, 71, 73, 74, 75, 77, 79, 80, 81 and 82 (Figure 3.6). The nitrite starting addition of 59.629 g $NaNO_2$ resulted in a concentration of 1.07 mM on day

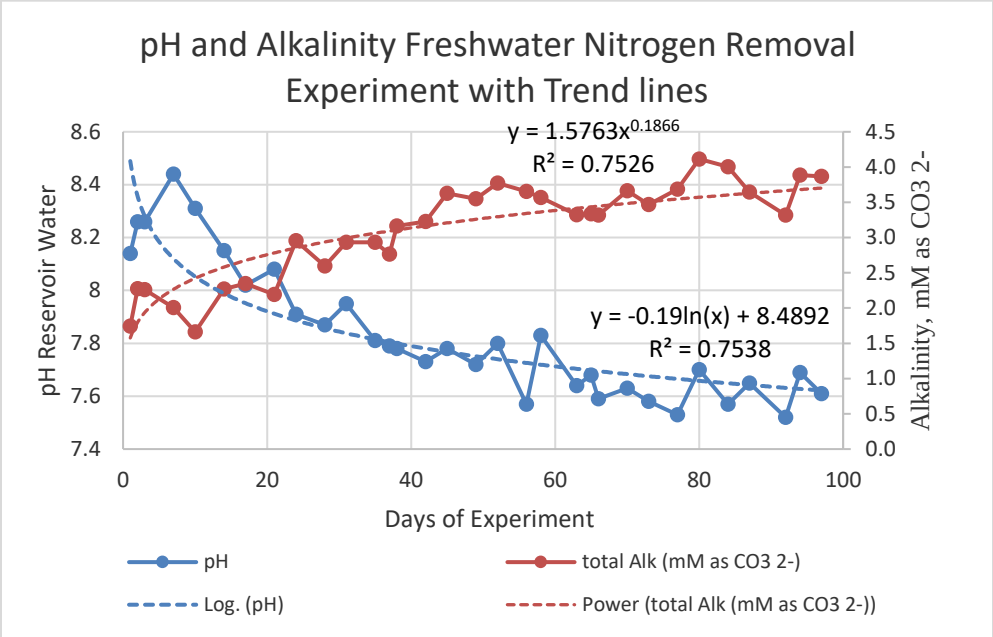


Figure 3.5 The pH and alkalinity during the freshwater nitrogen removal experiment. Excel fit a equations to the data, along with associated R^2 value.

two. The nitrite concentration varied as the ammonium concentrations. The nitrites ranged from 0.00022 to 1.25 mM with an average of 1.05 ±0.22 mM and a median value of 1.09 mM. With the additions of NH_4Cl, one would expect an increase in the TAN concentration. Following the first five additions, the resulting TAN concentration changed little or decreased. The next four additions show a strong decline in TAN concentration until day 77. The TAN concentration decrease from 0.729 to 0.169 mM, representing a 76.8% decrease. During this same period, days 73 to 77, showed a 29.1% increase in nitrite concentration. The TAN concentration decreased 0.301 mM from starting to end values, representing an 18.2% decline. The nitrite concentrations responded differently, with an increase of 0.167 mM or 15.6% increase from the starting value. The nitrate concentrations started at 0.015 mM at day 1 and ranged from 0.015 to 0.34 mM. They averaged 0.10 ±0.09 mM, with a

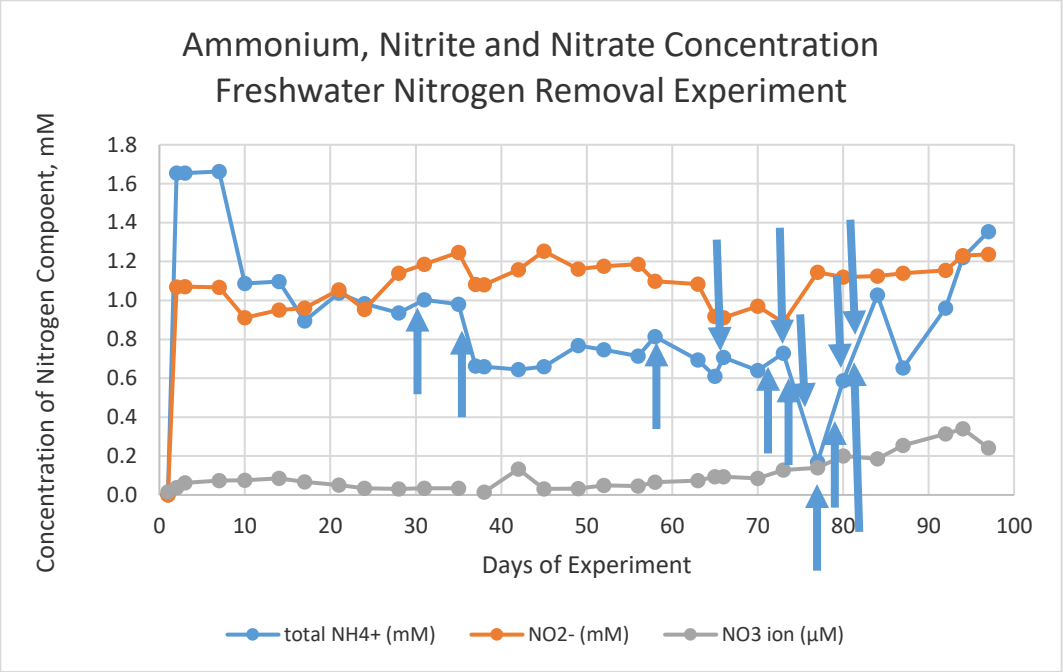


Figure 3.6 TAN, nitrite and nitrate concentrations during freshwater nitrogen removal experiment. Blue arrows indicate thirteen NH_4Cl additions (59.9 g each) on days 30, 37, 58, 66, 71, 73, 74, 75, 77, 79, 80, 81 and 82.

median value of 0.074 mM. Figure 3.6 shows a relatively stable level over the first 60 days, followed by an increasing nitrate from 0.074 mM on day 63 to 0.339 mM on day 94.

The TAN, nitrite and nitrate concentrations varied throughout the experiment. In order to access the ability of the OAS to mediate anammox activity, the researcher converted the measured nitrogen compounds in the system water and those of the additions to nitrogen to make comparisons. The nitrogen from the NH_4Cl and $NaNO_2$ additions show incremental increases (Figure 3.7). The nitrogen measured in system water samples show a stable, flat profile by contrast (Figure 3.7). The measured nitrogen shows an average of 16.04 ±3.85 g, with a median value of 15.68 g. The

nitrogen ranged from a low of 0.13 g to a high of 22.52 g. The repeated additions of nitrogen during the experiment did not affect the nitrogen concentration present in the water.

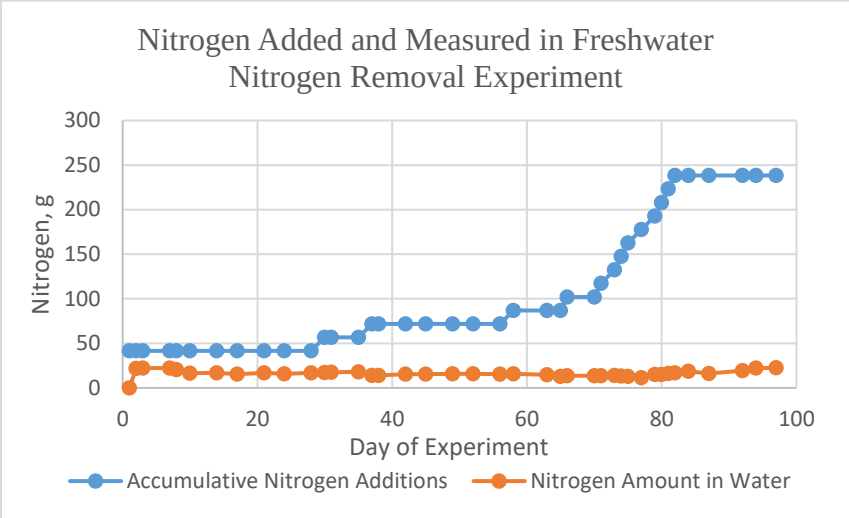


Figure 3.7 Comparison of accumulative nitrogen additions in the form of NH_4Cl and $NaNO_2$ with the measured nitrogen present in the water from TAN, nitrite and nitrate.

The difference between the amount of nitrogen added to the system and that measured in the water in the form of TAN, nitrite and nitrate reveal a loss of 215.8 gram of nitrogen (Figure 3.8). The loss of nitrogen amounts to 90.5% of the added nitrogen. Therefore, the additions of nitrogen reside in a sink within the system, since there is a lacking of a corresponding nitrogen increase within the water column with the stepwise nitrogen

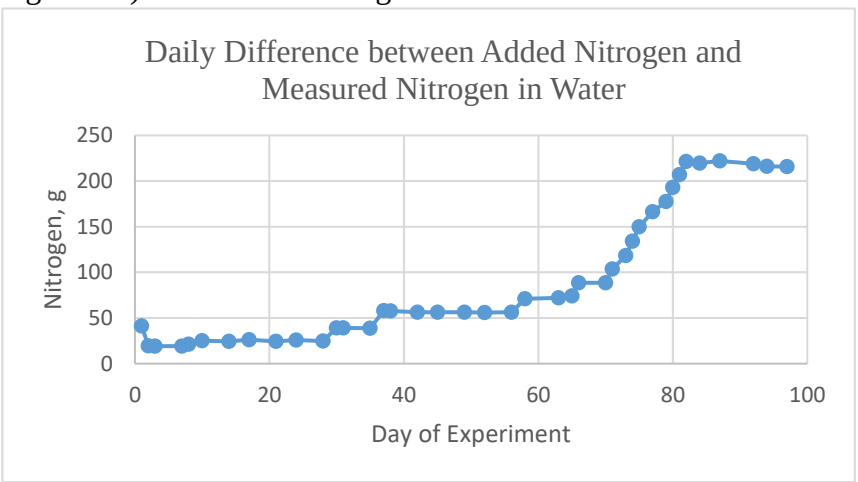


Figure 3.8 Daily difference between added nitrogen in the form NH4CL and NaNO2, minus the daily summation of nitrogen from TAN, nitrite and nitrate.

additions. One could speculate that an increase in bacterial biomass could be a reservoir for nitrogen. Another sink could be an increase in nitrogen in the air space above the water surface, indicating the presence of anammox, or denitrification, activity within the system. Sampling of the air space above the water surface was not undertaken. The plotting of the scale of the nitrogen discrepancy occurs in Figure 3.8. The profile of the graph closely matches that of the nitrogen additions in Figure 3.7. The balance of ammonium to nitrite is a decisive ratio for the anammox process to proceed forward to dinitrogen gas formation. Consequently, the ratio calculation used the measured ammonium concentration to measured nitrite concentration in the system (Figure 3.9). The ammonium/nitrite ratio ranged from 0.15 to 2.55. In Figure 3.9, the ammonium/nitrite ratio in comparison to the total nitrate levels shows a

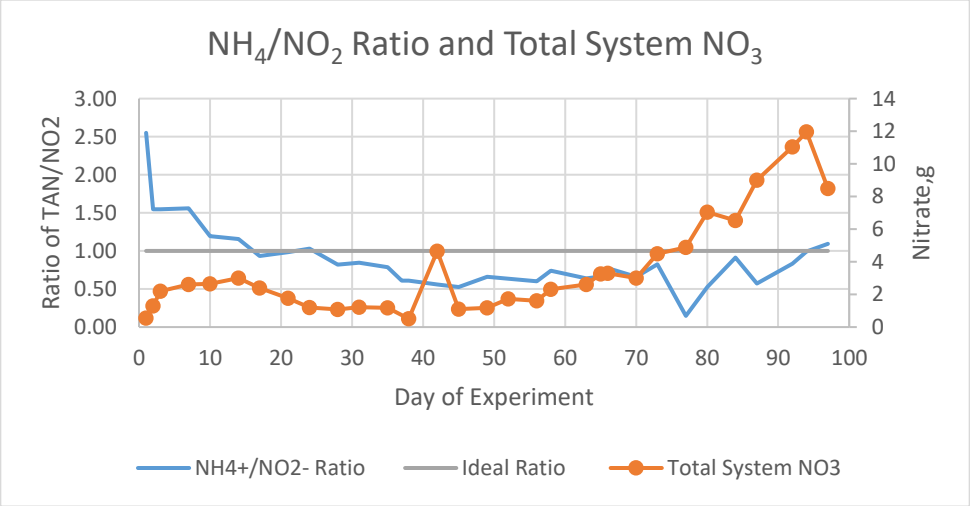


Figure 3.9 Ammonium/nitrite ratio in freshwater nitrogen removal trial using an oolitic aragonite sand filled fluidized sand filter. Calculation of the ratio used the measured amounts of ammonium and nitrite in system water. The ideal ratio line represents a value of one. Total system nitrate concentrations.

relationship inherent on the ratio of one. With the ratio >one, the nitrate

concentrations increased over day 1 to day 14. From day 14 until day 38, the nitrates continued a steady decline with the ratio decreasing from 1.14 to 0.61. The ratio stayed below 0.90 between days 38 to 80 resulting in gradual increases in nitrates. Once the ratio rose above 0.90 (day 84), the nitrate concentration shows a strong one day decrease. By day 94, the ratio rose to 0.99 showing a strong decrease in nitrates. These observations reveal a removal of nitrates occurring with an ammonium/nitrite ratio ranging from 0.90 to 1.15. When the ratio occurred above or below this range, nitrates accumulated in the system.

The phosphate concentrations behaved rather oddly considering that, a single addition occurred to produce a concentration of 2.42 µM P or a phosphate concentration of 7.42 µM (Figure 3.10). At no time during the experiment did phosphate concentrations approached this level; maximum concentration was 1.79 µM PO_4.

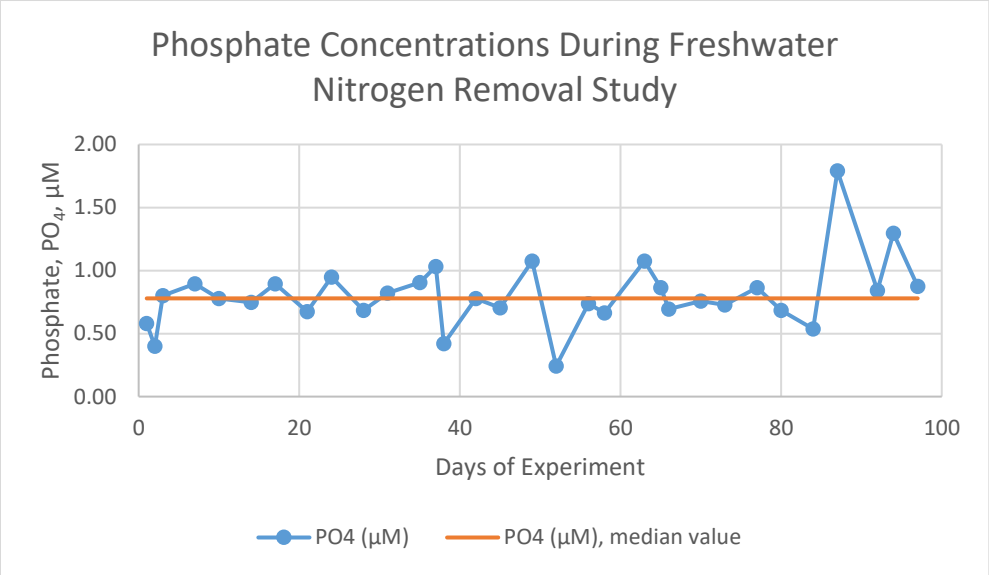


Figure 3.10 Phosphate concentration variations during the freshwater nitrogen removal experiment with median value line.

The average phosphate concentration was 0.806 ±0.28 µM PO_4, with a median value

of 0.779 µM PO4. The values ranged from 0.242 to 1.79 µM PO_4. As the Figure 3.10 shows and the relatively large standard deviation, supports the phosphate readings varied unexpectedly during the experiment. The phosphate concentration appears to cycle between being in solution and absorption onto the OAS.

3.4.2 Microbiome Analysis

The microbial community analysis was able to assign taxa to five phyla in the freshwater OAS in decreasing order of abundance: *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Verucomicrobia* and unassigned/other (Table 3-1). Assignment to a known phylum did not occur for less than 2 % of taxa. The identified phylum contain the genera *Clostridium sensu stricto 8, Opitutus, Nitrosomonas, Thermononas, Mesorhizobium*, *Hirschia, Fluviicol*a and *Parvibaculum*. Assignment of genera did not occur for members of the families *Chitinophagaceae*, *Caulobacteraceae*, *Rhodobacteraceae*, *Shingomonadaceae*, and *Comamonadaceae*. None of the described anammox genera from the *Planctomycetes* occurred in any replicate.

Table 3-1 Taxonomic groups identified in microbiome analysis of aragonite sand from the freshwater fluidized beds.

Percent Phylum	Phylum	Class	Order	Family	Genus
8.0%	Bacteroidetes	Flavobacteria	Flavobacteriales	Cryomorphaceae	Fluviicola
		Sphingobacteriia	Sphingobacteriales	Chitinophagaceae	
20.0%	Firmicutes	Clostridia	Clostridiales	Clostridiaceae 1	Clostridium sensu stricto 8
28.0%	Proteobacteria	Alphaproteobacteria	Caulobacterales	Caulobacteraceae	
				Hyphonmonadaceae	Hirschia
			Rhizobiales	Rhodobiaceae	Parvibaculum
				Phyllobacteriaceae	Mesorhizobium
			Rhodobacterales	Rhodobacteraceae	
			Sphingomonadales	Sphingomonadaceae	
		Betaproteobacteria	Burkholderiales	Comamonadaceae	
				Oxalobacteraceae	other
			Nitrosomonadales	Nitrosomonadaceae	Nitrosomonas
		Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	Thermomonas
5.0%	Verrucomicrobia	Opitutae	Opitutales	Opitutaceae	Opitutus
39.0%	Unassigned; Other				

3.5 Discussion

The nutrient conditions within the waterways of the United States have reached concerning levels as to impact on algae, invertebrate and fish life that reside in these waters (Manning et al., 2020). Using open-access data from United States Geological Survey North American Water Quality Assessment program, Manning et al. (2020) found nitrogen and phosphorus concentrations levels are at a point to negatively affect inhabitants of these ecosystems. The sources of nitrogen pollution in freshwater waterway stem from multiple sources such as residential fertilizers, pet waste, sewage effluent, storm water runoff and atmospheric sources (Hobbie et al., 2017, *Nitrogen and Water*, n.d.).

In order for anammox to occur in a system, meeting certain conditions are a necessity. These conditions include low dissolved oxygen, the presence of known anammox bacterial families, presence of NH_4+ and NO_2- ions and acceptable temperatures for anammox bacteria.

The study recorded temperatures of 20.91±0.14°C during the project. The temperature remained stable with only a 0.91°C difference in the temperature range. The observed temperatures were within limits recommended for anammox and denitrification (Timmons & Ebeling, 2010, Cema et al., 2011, Jin et al., 2012). Cema et al. (2011) report of temperate conditions of a pilot plant for anammox treatment at a wastewater treatment plant in Sweden using moving bed biofilm reactor filled with Kaldnes™ media. The operated reactor maintained an average temperature of 24±2.5°C. During the winter, with temperature control, the system was able obtain a

minimum temperature of 20°C (Cema et al., 2011). In a review of conditions inhibiting anammox, Jin et al. (2012)discuss the optimal temperature range as 30-40°C. Other authors report of anammox occurring at various temperatures between 18°C and 22°C, disclosing that anammox bacteria are capability of operating in at a lower temperature range, with varying degrees of activity (Jin et al., 2012). Temperatures for denitrification can occur over a broad range depending on the denitrifiers established in the filter (Timmons & Ebeling, 2010). The denitrification temperatures are reported to range from 14.6°C to 27.2°C (B. Zhou et al., 2019, Tsukuda et al., 2015, Singer et al., 2008, Lee et al., 2000). The current work is within this range.

The dissolved oxygen concentration within the test system decreased rapidly over the first three days to 31.2 µM, indicating the effectiveness of the nitrogen purging in the setup. Kimura et al. (2011) recommend sparging of nitrogen gas as a method to reduce dissolved oxygen concentration to acceptable levels in anaerobic applications. From that point forward, the dissolved oxygen averaged 10.0 ±3.12 µM. The low dissolved oxygen remained stable at this level for 94 days or 97% of the experiments duration. The ability of the system to maintain a low dissolved oxygen level throughout the remaining experiment without the continuation of nitrogen indicates how well the system is isolated from the ambient air. Tsukuda et al. (2015) showed denitrification can occur when the reactor dissolved oxygen concentration is <0.37 mg/l (<23 µM). Aerobic denitrification is reported at dissolved oxygen levels of 5.0 – 6.0 mg/l (0.31 – 0.38 mM), though denitrification is normally considered to occur in

hypoxic conditions with oxygen levels <0.2 mg/l (<12.5 µM) (Hong et al., 2019). Working with biodegradable plastic as an carbon source for denitrification, Zhang & Zhang (2018), observed nitrate removal rates of 30.6 g NO_3-N/m^3/d and 30.8 g NO_3-N/m^3/d at starting oxygen concentrations of <1.0 mg/l and 4.8 mg/l. The oxygen in the low oxygen treatment decreased to <1.0 mg/l during the trial, attributed to bacterial respiration.

Reports of oxygen concentrations for anammox to occur differ in ranges from a variety of anaerobic arrangements. In an experimental flow-through anammox system with wastewater, Kimura et al. (2011) detected no effect on anammox bacteria activity on a gel carrier when exposed to influent dissolved oxygen levels of ≤2.5 mg/l (≤78.1 µM). The use of dissolved oxygen concentrations of 0.5 mg/l (15.6 µM) and 3 mg/l (93.8 µM) in an ELAN® anammox reactor showed no differences in their ability to remove nitrogen in the domestic sewage pilot project (Morales Aqualia et al., 2014). The ELAN® process produces granules in the anammox process (Morales Aqualia et al., 2014). The granules formed in the higher dissolved oxygen concentrations (3 mg/l) tolerated higher levels of oxygen better than the lower oxygen (0.5 mg/l) granules due to the thickness of the biofilms being greater in the higher oxygen environment (Morales Aqualia et al., 2014). Yin et al. (2016) showed in a small scale sequencing batch reactor that dissolved oxygen levels of 0.5 mg/l (15.6 µM) produced the high specific nitrogen removal rates. Shivaraman & Shivaraman (2003) report maintenance of oxygen condition of <0.5% of air saturation for

anammox. In the current work, the oxygen levels occurred in a low enough range to allow anammox activity to occur.

The pH during the freshwater trial steadily declined throughout the experiment. However, the average pH was 7.82±0.25 (median 7.78) and a fitted logarithmic equation representing this trend becomes asymptotic to a pH of about 7.55.

The ideal pH for denitrification occurs between 7 and 8, with possible diminished activity outside this range (Timmons & Ebeling, 2010). Zhang & Zhang (2018) working with biodegradable plastics as a carbon source observed denitrification proceeding at pH values of 6.5 to 7.8. Xu et al. (2018) showed low pH valves (5.68) decreased nitrate removal by denitrification by 34% in work using PHBV/PLA polymer blends for denitrification. At high nitrate concentrations (2700 mg/l), denitrification is impeded at pH values of 6.5 and 7.0 (Glass & Silverstein, 1998). The authors found nitrite concentrations increasing with rising pH values of 7.5, 8.5 and 9.0 (Glass & Silverstein, 1998). Qian et al. (2019) working with synthetic wastewater at pH's of 5, 7 and 9 caused denitrification to produce more nitrite with increasing pH. By control of denitrification by higher pH and a low C/N ratio, partial-denitrification could be used to maintain a stable level of nitrite for anammox reactions (Qian et al., 2019). Published reports show the effective range for pH in anammox is 6.5-8.5 from work conducted on a sequence batch reactor (Yin et al., 2016). The observed pH in the present work is within the reported effective range for pH.

The declining trend of the pH over the 97 days of the experiment is unexpected with the large alkalinity increase, which occurred concurrently. Using the logarithmic equation fitted to the pH data, shows a decreasing rate of decline, become asymptotic to a pH of 7.55. A plausible explanation is the suggested nitrification occurring in the system. As mentioned earlier, ammonium present in the system and additional NH_4Cl added to the system over the course of the experiment resulted in rising nitrite levels indicating nitrification is occurring. The nitrification process forms H+ ion during the process leading to decreases in pH as the process proceed forward. The maintenance of a high, stable nitrite level and the occurrence of nitrite increases with NH_4Cl additions signifying the nitrification is taking place and the generation of hydrogen ions leading to a decreasing pH.

The biofilter media added to the system came from an established, long running freshwater system. Tal et al. (2004) by use of 16S-rRNA gene sequences detected the presence of anammox activity within moving mixed bed biofilters, with some being within the IMET-ARC facility. The presence of the anammox bacteria in RAS bacterial consortia provides strong support of these same bacteria being present in the seeding media (Tal et al., 2004). The media visually contained thick, dark biofilms with the internal surfaces of the media. Due to the age of the active biofilter media and the thickness of the observed biofilms (not measured), the deepest layers within the biofilms likely exceed the depth with which oxygen can penetrate creating an anaerobic habitat. The existence of a possible anaerobic zone within the biofilter media, lead to the assumption of the presence of anaerobic bacteria like anammox

type bacteria. The biofilm thicknesses on Kaldnes media used in anammox pilot treatment for wastewater ranged in thicknesses of 0.27 mm, 0.43 mm and 0.77 mm, at the start, 7 months and 12 month periods, respectively (Cema et al., 2011). Cema et al. (2011) reports that these biofilm thicknesses did not affect the nitrogen removal rate. The understanding is the anammox bacteria present within the biofilm prevent exposure to higher oxygen levels due to the need of diffusion and utilization by aerobic bacteria in aerobic portions of the biofilms (Cema et al., 2011).

In addition to the seeded media, earlier bacterial analysis on oolitic aragonite sand used in freshwater, brackish water, saltwater and unused aragonite showed the presence of the phyla *Planctomycetes* known to include functional anammox bacteria (worked recorded in this dissertation). Between these two inoculation sources, the assumption is denitrification and/or anammox bacteria were present in the experimental system. Confirmation of the presence of anammox and denitrification bacteria in the freshwater denitrification experiment can be confirm by bacterial analysis of the sand remaining from the experiment as well as determine

For the anammox activity to occur, the reaction relies upon the availability of both ammonium and nitrite ions in proper proportions (Fux, 2003). The ratio of ammonium to nitrite works best with a ratio close to one, though it continues with some variation (Fux, 2003). Nitrite can be the limiting factor in anammox activity with municipal wastewater (Cema et al., 2011).

The current NH_4/NO_2 ratio exposes conditions that may of lead to impaired anammox conditions and increased nitrate production during the end of the experiment.

Referring to Figure 3.9, one can see that the ratio only was at one at brief periods during the experiment. The ratio remained significantly below one with a median value of 0.80. Close observation of Figure 3.9 shows when the ratio declines below ratio = 1, the nitrate concentrations started to rise. In addition, during the last 20 days of the experiment, the figure shows, increases in the nitrate concentration occurred with a simultaneous increase in the ratio until it reached a value of 0.99. The speculation is there is a lag time between when the ratio increases into an acceptable range and when the nitrate level can respond with decreasing values. The nitrite concentration remained relatively stable during the experiment with an average of 1.05 ±0.22 mM. Reported low nitrite concentrations can be a limiting factor requiring the need for partial nitrification to generate sufficient nitrite to maintain a ratio closer to one for the anammox reaction to occur. In the current study, a NH_4/NO_2 ratio ranging from 0.90 to 1.15 appears sufficient for the anammox to occur as evidenced by a reduction in nitrate concentrations.

In this current application, nitrite does not seem to be limited. One can argue that ammonium is a limiting factor due to the NH_4/NO_2 ratio being much lower than one. The TAN concentration averaged 0.88 ±0.37 mM, compared to 1.05 ±0.22 mM NO_2. A higher ammonium (TAN) concentration would increase the NH_4/NO_2 ratio closer to one. Even before the first NH_4Cl addition to the system (days 7-28, Figure 3.6), the NH_4 concentration decreased, while the NO_2 concentrations showed a slight increase. This may be an indication of nitrification is progressing forward. When NH_4Cl additions began (day 30), this same observation occurred over days 24-35 and

38-45 (Figure 3.6). Considering the system is closed, this observation is likely due to nitrification activity occurring. Introduction of nitrification bacteria occurred with the seeded Kaldnes™ media and can assumed to be present. The dissolved oxygen levels in the water were <15.6 µM, assumed to be inappropriate for nitrification to occur. The position of the probe recording the dissolve oxygen level was near the bottom of the tank. Though circulation remained continuous, oxygen measurements in the upper water column and in the headspace between the water and the tanks cap did not occur. The suspension of a mesh bag of the seeded media just below the water surface acted as a source of bacteria. If oxygen did occur in sufficient quantities in the headspace, the position of the seeded media at the water surface would be nearer to where diffused oxygen may be present. Taking into account all of these circumstances, the possibility exists that oxygen may have been sufficient in the portion of the upper water column at a concentration sufficient to allow nitrification at a rate to consume part of the NH_4 present and maintain the nitrite concentrations measured. If this scenario existed, not nitrite, but ammonium would become a limiting component. The NH_4Cl additions certainly aided in improving the ratio, but fell short of the optimal ratio. Increased scrutiny of the NH_4 to NO_2 ratio during the experiment, would have allowed a greater addition of NH_4Cl to maintain the ratio closer to one.

The freshwater denitrification/anammox test setup began with a fixed starting concentration of ammonium and nitrite. As the experiment continued, introductions of nine NH_4Cl additions started occurring on day 30 to day 77. The effect of the

NH_4Cl additions depended on the NH_4/NO_2 ratio at the time of the addition. When the NH_4/NO_2 ratio was ≤ 0.90, the NH_4Cl addition resulted in increased NO_2 and NO_3 concentrations due to nitrification and presumably inhibition of anammox activity. If the NH_4/NO_2 ratio was above 1.15, nitrite concentrations tended to increase. The nitrate concentrations generally increased with additions with a ratio outside the range of 0.90 to 1.15. The anammox activity requires the presence of similar concentrations of NH_4 and NO_2 ions (NH_4/NO_2 ratio ~ 1) in order for anammox to proceed. In this work, when the ratio trends low the ammonium ion becomes a limiting factor in the anammox reactions.

The understanding of the changing concentrations of phosphate and phosphorus in the system potentially could be a result of the existence of varying oxygen concentration zones within the experimental setup. In cyclic exposure to aerobic and anaerobic conditions, wastewater from domestic sewage can free phosphorus from bacteria, followed by bacterial up taken up in aerobic conditions as polyphosphate (Timmons & Ebeling, 2010). Timmons & Ebeling (2010) describe phosphorus removal by heterotrophic denitrification bacterial under aerobic or anoxic conditions without the need of alternating between oxygen levels. This phosphorus removal pathway occurs in both freshwater and saltwater RAS (Timmons & Ebeling, 2010).

Zeng et al. (2003) using a simultaneous nitrification, denitrification and phosphorous removal (SNDPR) in a sequencing batch reactor under aerobic and anaerobic conditions demonstrated phosphorus release in anaerobic conditions followed by uptake of phosphorus through the aerobic phase. The variability of the phosphorous

and phosphate concentrations during the experiment could be the result of this occurring, assuming that aerobic and anaerobic areas existed spatially in the experimental setup. The loss of denitrification during SNDPR occurred not by the formation of N_2 gas, but by N_2O (Zeng et al., 2003). The occurrence of SNDPR in the current work may explain the variability of the phosphorus and phosphate concentrations and reveal another avenue for nitrogen loss from the system. Recently, Wang & He (2020) isolated a *Thauers* sp. strain SND5 from domestic wastewater showing it is capable of simultaneous nitrification, denitrification and phosphate removal using an internal carbon source of polyhydroxybutyrate (PHB), thus reducing the need for carbon in the denitrification process. Bacterial pathways exist for sequestering phosphate in anaerobic conditions suggesting a possible explanation for changing phosphate concentrations.

The current work shows a steady increase in alkalinity over the course of the experiment. The utilization of alkalinity during nitrification process of converting NH_4 to NO_3 decreases alkalinity. On the other hand, the denitrification process typical generates alkalinity, but anammox does not consume alkalinity in the process. The existence of a rising alkalinity may be indicative of denitrification or buffering of the water from the OAS within the FSF. In all likelihood, both sources of alkalinity increases probably contribute to the elevated concentrations seen. Previous work in this dissertation with OAS in CF and FSF show alkalinity increases due to the sand. The CF and FSF work showed alkalinity increases of about 0.767 mM over a 23-day period, maximum. The anammox alkalinity change is appropriately 0.767 mM over a

97-day period. With a simple extrapolation of the CF and FSF results, the OAS could conceivably account for most if not all of the alkalinity increases. The dissolve oxygen levels within the system were at levels conducive to denitrification to occur. The presence of alkalinity would provide a carbon source, though total nitrate in the system remained <12 g in total (with a maximum concentration of 0.339 mM). If alkalinity provided the carbon for denitrification to occur, the expectation would be a declining alkalinity. The current study shows a steady increase in alkalinity reaching an asymptotic concentration of 4.0 mM as CO_3^{2-}. One cannot dismiss the existence of denitrification. The speculation is that the alkalinity formation would be small in relation to the action of the OAS.

A visual observation that the anammox or denitrification processes are occurring is the presence of N_2 gas bubbles. No observation of N_2 gas bubbles in the reservoir tank occurred, nor witness of them in the water flow returning to the tank. The lack of nitrogen gas bubble does not preclude the processes occurring. Possibly, due to their size or rapid movement into the headspace above the water, missing of their detection is reasonable.

The interpretation of the data leaves an interesting question. Which media is responsible for mediating the denitrification and/or anammox reduction of nitrogen in the system? The added Kaldnes™ media or the OAS sand filled FSF. The reason for the addition of the Kaldnes™ media was to ensure anaerobic bacteria assumed to be residing in the biofilms attached to the inter surface of the media was introduced to the anammox system. The Kaldnes™ K1 media contains a surface to volume ratio of

500m^2 per m^3 (evolutionaqua.com/k1-media). One liter of seeded Kaldnes™ media added therefore contains 0.5 m^2 of surface area. The previously used OAS in the freshwater anammox FSF was citric acid washed twice. The surface area calculation completed by LabQMC (results shown in chapter I) for the OAS following two citric treatments resulted in two measurements of the surface area, 4.769 m^2/g and 4.488 m^2/g. To determine the surface area of the OAS in the FSF, the two measurements were averaged and the surface area calculated for 15 kg of OAS (4.629 m^2/g x 1000 g/kg x 15 kg) resulting in a surface area of 69, 435 m^2. The OAS provided a surface area five magnitudes greater than the Kaldnes™ media. The reported slow growth rate for anammox bacteria affects the establishment of anammox filtration (Fux, 2003, Kuenen, 2008). The large, available surface area of the OAS would provide a prime habitat for anammox bacteria to colonize. In addition, the flow within the FSF and constant mixing of the OAS would provide greater contact between the water and the OAS established anammox bacteria. The Kaldnes™ media did not receive direct flow from the water returning from the FSF; therefore, possible boundary layers could impede sufficient contact of NH_4 and NO_2 ions to anammox bacteria.

The observed genera from the freshwater OAS contained groups associated with nitrogen fixation, nitrification and anaerobic conditions. The detected genus *Nitrosomonas* (*Proteobacteria*) is a known ammonia-oxidizing bacterium commonly found in commercial RAS with published examples for hybrid grouper, as well as for Atlantic salmon (Huang et al., 2018, Roalkvam et al., 2021). *Nitrosomonas* represented 5% of the observed taxa. Observation of *Nitrosomonas* along with

several archaeal species occurred in a biofilter study on a commercial RAS operation for freshwater Yellow Perch (*Perca flavescens*) (Bartelme et al., 2017). Known nitrogen-fixing species in soils occur in the *Mesorhizobium* genus, though represented only 3% of the taxa in the study (Helene et al., 2019). The phyla *Firmicutes* and *Verrucomicrobia* contained the detected genera *Clostridium sensu stricto* and *Opitutus* that are generally associated with anaerobic environments including soils and digestive tracts (Alou et al., 2018, Chin et al., 2001). These phyla represent 20% and 5% of the detected freshwater taxa, respectfully. The phylum *Proteobacteria* contains the genus *Thermomonas* (5% of observed taxa), which include six valid species as of 2019, know from biofilms, hot springs and soils (Ju et al., 2019). Work on *Thermomonas aquatic sp. nov.* screened from activated sludge discharge is able to grow in anaerobic conditions and breakdown a range of nutrients associated with sewage sludge (Ju et al., 2019). The phyla *Proteobacteria* and *Bacteroidetes* do contain denitrification bacteria genera though known genera were not revealed (Deng et al., 2020).

Bacteria of the genus *Fluviicola* of the *Cryomorphaceae* family accounted for 7% of the found taxa in the study, though the genus is not well understood (Newton & McLellan, 2015). O'Sullivan et al. (2005) first proposed the genus in 2005 point to their distinctive traits of orange colored colonies and their ability to produce flexirubin pigments. A study of the bacterial community diversity between Lake Michigan and urban waterways of Milwaukee, Newton & McLellan (2015) report the genus *Fluviicola* included numerous oligotypes, classified as either generalist or

specialist in these areas. The genus includes some novel bacterium. An isolated bacterium, *Fluviicola taffensis gen. nov., sp. nov.*, from a United Kingdom river shows it to be a strict aerobic freshwater species, intolerant of Na and lacking abilities to reduce nitrate (O'Sullivan et al., 2005). Dahal & Kim (2018) isolated *Fluviicola kyonggii sp. nov.* from forest soil in the Republic of Korea showing it to be strictly aerobic and able to grow with up to 2.0% NaCl. The species lacked capability to reduce nitrate to nitrite.

Information on the ecological function of the genera *Hirschia* is minimal. The *Hirschia baltica* species prefers an aerobic brackish water habitat with salinities between 0.5 – 8.6 %NaCl considered a chemoheterotroph utilizing amino acids, organic acids and sugars as carbon sources (Chertkov et al., 2011). Members of the *Hirschia* genera reportedly utility nitrate, though these are marine adapted species (Park & Yoon, 2013). The novel genera *Parvibaculum* is unique in its ability to degrade a number of compounds as linear alkylbenzenesulfonate (a laundry surfactant), polycyclic aromatic hydrocarbons, alkanes, hydrocarbons, denitrification of linear-nonylphenol, linear alkyl benzenes, polychlorinated biphenyls and in nitrate reductions (Schleheck et al., 2011). *Paribaculum* inhabit seawater, marine rocks, soils, river sediments, groundwater, biofilms, deep-sea sediments and geothermal springs environments contaminated with these organic compounds (Schleheck et al., 2011).

The OAS contained bacterial genera responsible for nitrification in the breakdown of sewage nutrients and those adapted to anaerobic conditions like denitrification. Thus,

a microbial driven nitrification process was likely occurring as TAN concentrations decreased and nitrite levels increased (Figure 3.6). The presence of nitrifying bacteria that can metabolize nitrogen of various oxidation states support this idea. The presence of the anaerobic genera *Clostridium sensu stricto, Opitutus* and *Thermomonas* suggest the possibility that one or more of the species within these genera possess denitrification capabilities, though not confirmed. The omission of the phylum *Planctomycetes* is surprising as they are commonly associated with anammox activity (Fux, 2003). Previous work with the OAS in CF and FSF showed the presence of the phylum *Planctomycetes* occurred on the OAS used in the freshwater, brackish and saltwater application, as well as, the unused naïve sand (Figure 2.21B). Presence of this anammox containing phyla in all four of these sand samples, supports the possibility that the phyla would be present in the current work, though confirmation of the anammox genera would need to confirmation. The total lack of the phyla from the anaerobic FSF is contrary to the expectations. Although nitrogen removal occurred, the absence of any known anammox genera in the OAS lessens the likelihood that anammox occurred in the OAS. The mature Kaldnes™ K1 filter media added at the start of the study may have harbored anammox bacteria, though the media did not undergo microbiome analysis to verify. Verification of the presence of *Planctomycetes* in the system the Kaldnes™ K1 media came from did not occur. The present study cannot conclusively confirm anammox activity occurring in the OAS fluidized filter. A portion of the added nitrogen (NH_4Cl and $NaNO_2$ additions) to the system did undergo nitrification and remain in the system as TAN,

nitrite and nitrate, although the majority of the nitrogen (>90%) was lost from the system, likely due to either anammox and/or the denitrification process.

The bacterial analysis showed a large portion (39%) of unassigned bacterial units. Lacking the functionality of these bacterial groups, one could speculate these groupings conceivably contain bacteria with the ability to conduct denitrification or anammox activities.

In conclusion, the current work showed that the conditions for denitrification and anammox activity were present in the system setup. Those being low dissolved oxygen, proper temperature, pH, availability of NH_4 and NO_2 and the presence of anaerobic seeded media. The addition of supplemental NH_4Cl resulted in nitrate increases when the NH_4/NO_2 ratio fell below 0.90 due to the occurrence of nitrification. If the NH_4/NO_2 ratio was between 0.90 and 1.15, the nitrate levels decreased. The anaerobic system was able to remove 238 grams of added nitrogen, representing a nitrogen loss of 90.5%.

Chapter 4: Nitrogen Removal in Saltwater Under Anoxic Conditions Mediated by Oolitic Aragonite Sand

4.1 Abstract

The reduction of nitrogen releases into the environment can diminish the likelihood of developing eutrophic condition, which result in deterioration of ecosystems. However, the development of environmentally friendly recirculating aquaculture systems (RAS) control all aspects of their water quality, their reduced effluents still require close oversight to limit nitrogen releases to the environment. The feed used in RAS produce ammonium, nitrite and nitrate by the action of nitrification bacteria. The resulting form of nitrogen, nitrate, requires management to limit release. Reduction of nitrate to nitrogen gas occurs through denitrification and anammox processes causing lower nitrogen in discharges. The objectives of this study encompassed the establishment of denitrification and anammox conditions in marine water using oolitic aragonite sand as a substrate, show the response of repeated nitrogen additions and show nitrogen removal capacity from the system. Temperature, salinity and dissolve oxygen remaining in acceptable ranges for denitrification and anammox activity to occur. The pH and alkalinity values fluctuated in opposition to each other of during the first 210 days, then remained stable to day 270. Phosphorus concentrations decreased from 2.48 µM to 0.158 µM in the first three weeks, followed by variations in concentrations until day 203, which it remained below 0.196 µM. The ammonium and nitrite concentrations showed alternate profiles up to day 182. Nitrate levels remained below 0.20 mM for 217

days. Afterwards, climbing sharply to 1.80 mM on the final day. Additions of ammonium and nitrite resulted in ammonium declines and nitrite increases between days 77 to 175. Variation in pH, alkalinity, phosphorus and phosphate occurred during same period. The difference between added nitrogen and that measured in the water was 253.04 g, representing an 87.6% loss. The ratio of NH_4/NO_2 fluctuated from 0.06 to 1.86, outside the ideal value of one for anammox activity. The ratio remained below one from day 77 to day 182. Microbiome analysis of the aragonite sand identified the phylum *Actinobacteria*, *Proteobacteria*, *Bacteroidetes* and unassigned with percentages of 28.0%, 22.0%, 3.3% and 46.7%, respectively. Classes and families within these phylum do contain bacteria know to function as denitrifiers. No members of the *Planctomycetes* phylum, which contain known anammox bacteria, occurred. The conditions for denitrification and anammox bacteria to function existed within the setup, though determination of their presence did not occur. The loss of 87.6% of the added nitrogen likely occurred due to denitrification activity within the system.

4.2 Introduction

The impairment of the coastal water by nitrogen influxes have created eutrophic conditions such as low oxygen zones, harmful algae blooms, changing habitats, sea grass beds decreases, altered biodiversity, etc. (Driscoll et al., 2003, Howarth, 2008). Watersheds transport released nitrogen to coastal areas where it can accumulate to sufficient levels leading to eutrophic conditions (Driscoll et al., 2003). In work

commissioned by the Hubbard Brook Research Foundation, Driscoll et al. (2003) determined anthropogenic nitrogen inputs into the environment for the Northeast United States include atmospheric deposition from emissions, wastewater treatment and fertilizer in the form of chemical and animal manures. Nitrogen pollution of estuaries in the Northeast United States stem from multiple sources and vary according to watershed. Therefore, mitigating actions must vary for the watersheds (Driscoll et al., 2003). Using the Watershed Assessment Tool for Evaluating Reduction Strategies for Nitrogen (WATERSN), Whitall et al. (2004) revealed that reducing nitrogen in wastewater treatment plants in urban watersheds produced 32 – 57% reductions in nitrogen. In less developed areas, the primary nitrogen sources were from agricultural operations (Whitall et al., 2004). Denmark enacted multiple nitrogen reduction legislation starting in the 1980's, which lowered nitrogen loading from land sources by 50% over a 25-year period (Windolf et al., 2012). The study noted different levels of nitrogen reduction per studied watershed (Windolf et al., 2012). A reason for these difference is likely, nitrogen enriched ground water supplies imparting a delay in watershed improvement with nitrogen reductions actions taken (Windolf et al., 2012).

In developing marine aquaculture production, one needs to address management of effluents in order to lessen the impact to receiving waters (Guerdat, 2012). Increased nitrogen discharges in natural waters can generate accelerated primary production, hindering ecological systems producing eutrophication in the worst-case scenarios (Chen et al., 2002). RAS systems possess the capability of storing and concentrating

effluent flows, minimizing environment effects (Zohar et al., 2005, Guerdat, 2012). Using this inherent benefit of RAS, improvements to the effluent treatment can lead to lower nutrient releases curbing potential eutrophication of environmental conditions (Guerdat, 2012).

Normal biology filtration in sewage treatment and aquaculture facilities use the nitrification process of converting ammonium ions to nitrite to nitrate in a two-step bacterial action (Fux, 2003, Ward, 2008, Timmons & Ebeling, 2010). The nitrate ion is a less toxic form of nitrogen. Nitrate is still a nutrient that if released in high quantities can cause environmental damage; therefore, effluents need to meet discharge regulations (Fux, 2003, Shivaraman & Shivaraman, 2003). In some sewage treatment facilities, nitrate removal from the effluent stream by denitrification requires anaerobic conditions and a source of carbon to produce nitrogen gas (Fux, 2003).

The use of denitrification to reduce nitrates to N_2 gas by heterotrophic or autotrophic bacteria ensure the removal of nitrogen from RAS to the atmosphere (Timmons & Ebeling, 2010). The process needs to occur in anaerobic conditions with a carbon source for bacterial growth (Timmons & Ebeling, 2010). Using a up flow granule pack vertical column reactor, Sauthier et al. (1998) cautions that denitrification reactors requires stable total organic carbon/nitrogen ratio and a continuous carbon supply to maintain activity without producing undesirable conditions such as large negative swings in redox-potential and formation of sulfide to harmful levels for fish. Development of computer controlled denitrification systems, which monitor

dissolved oxygen, oxidation reduction potential, pH and control carbon dosing and flow rates can produce nitrate levels <5 mg/l in a commercial scale RAS (Lee et al., 2000). Management of denitrification conditions prevent nitrite and hydrogen sulfide production (Lee et al., 2000). A literature search revealed nitrate removal rates by denitrification vary greatly (1-166 mgNO_3-N/l/h), likely due differences is denitrification reactor used, operating conditions, source water conditions, carbon sources, etc. (van Rijn et al., 2006). In a freshwater application, nitrogen removal rates of 670 – 680 g N/m^3/day were measured in an up flow denitrification reactor with floating plastic media showing no differences between carbon sources of methanol, acetic acid, molasses and Cerelose™ (hydrolyzed starch) (Hamlin et al., 2008). In order to reduce the chance of leaking residual organics back to culture water from liquid carbon sources in the denitrification reactors, Singer et al. (2008) employed cotton wool at a C/N ratio of 0.82 g cotton/ g of nitrate N resulted in nitrate levels of < 10 mg/l.

Using endogenous carbon (i.e. fish feces) for denitrification, can accomplish nitrate removal rates of 26.9% using a fluidized sand with hydraulic retentions time of 15 minutes (Tsukuda et al., 2015). The authors postulated the nitrate removal rate would increase by applying a longer retention time and ensuring enough carbon is present for denitrification activity to continue (Tsukuda et al., 2015).

Discovery of the bacteria group *Planctomycetales* in sewage treatment plants lead to the process of anaerobic ammonium oxidation (Anammox) (Fux, 2003, Shivaraman & Shivaraman, 2003). The anammox process use ammonium and nitrite ions to form

dinitrogen gas directly under anaerobic conditions with little biomass produced (Fux, 2003).

Most of the initial work into anammox active began with municipal sewage treatment facilities. In an application of domestic sewage treatment, Ma et al. (2015) allowed for short periods of elevated oxygen levels to control the nitrite-oxidizing bacteria so that anammox bacteria were better able to establish at a temperature 20°C. Management of the dissolved oxygen levels in this fashion proved effective in decreasing total nitrogen levels in effluent to 6.6 ±2.7 mg/l, representing 89.9% decline in total nitrogen (Ma et al., 2015). Working with domestic sewage, Zhang et al. (2008) noted that the startup time of anammox treatments are long, though the treatment is cost effective and can be improved with activate sludge inoculates. Cema et al. (2011) showed that anammox conditions in sewage treatment facilities limited by nitrite concentrations in the source waters require low levels of oxygen in treatment to generate limited amounts of nitrite for the reaction. By controlling dissolved oxygen levels at 3 mg/l, a removal rate of 1.8 ±0.31g $N/m^2/d$ were achieved (Cema et al., 2011). Anammox activate in sewage treatment, once stabilize, can show a level of recovery when temporarily exposed to dissolve oxygen levels around 5 mg/l, which minimal effect anammox if followed by decreased oxygen levels (Kimura et al., 2011).

Freshwater RAS operations have advantages over marine systems. Discharging of freshwater effluent to wastewater treatment facilities or use as an agriculture fertilizers and soil amendment can occur (Van Rijn, 2013). Marine aquaculture does

not have this benefit, hence the need for appropriate treatment of discharge to meet environmental regulations and transparency of the operation to governmental agencies and the public (Van Rijn, 2013). One of the first reported detections of an anammox bacteria group, *Planctomycetales*, in an RAS system was by Tal et al. (2006) and Zohar et al. (2005). The slow growth rate of anammox bacteria and potential competition from denitrifying bacteria along with a proper supply of ammonia and nitrite could limit development of an active filter unit for a RAS system (Yossi Tal et al., 2006). A thorough understanding of the bacterial consortium, their niche and stability is essential in development of nitrogen removal technology for RAS to gain an increased presence in aquaculture (Schreier et al., 2010). Kessel et al 2011 report of the detection of anammox bacteria in the fish feces and the intestinal tract of finfish could be seeding material for inoculation of an anammox filtration. In support of this, Lahav et al. (2009) detected anammox bacteria in the intestinal tracts of sea bream and in their feces, proposing a similar inoculation theory. The authors also note that oxygen depleted areas can exist within biofilms in nitrification biofilters presenting a location for anammox establishment alongside nitrify bacteria ((Van Kessel et al., 2011).

Understanding the need to limit nitrogen built up in the environment and the requirement for aquaculture effluents to be low in nitrogen, this work seeks to evaluate the use of oolitic aragonite sand as a medium in a fluidized sand filter (FSF) to establish conditions conducive for denitrification and anammox activity. The objectives are to 1. Demonstrate establishment of denitrification and anammox

conditions in marine waters using oolitic aragonite sand as a medium. 2. Describe how an oolitic aragonite sand filter handles repeated nitrogen additions. 3. Show removal of nitrogen from the marine setup.

4.3 Methods

4.3.1 Experimental Design

The layout for the saltwater denitrification and anammox experiment was composed of a Solar Components Corporation (solar-components.com) tank of 700-liter volume (30" diameter x 5' high, item# 11012). The reservoir tank was raised approximately 9 cm above the floor with wood supports. Installation of a ¾" (19 mm) bulkhead fitting occurred in the bottom center of the tank with flexible tubing connecting it to a circulation pump intake (PAN World Magnetic Pump Model NH-50PX). The pump outlet was directed to a FSF (MacClean Water Treatment System, Model# 3P971, Rated Service Flow 5 gpm, Cuno Water Treatment Churubusco, IN, USA) (Figure 4.1A). Installation of a sampling port occurred on the outlet of the pump, between the pump and FSF (Figure 4.1B). Placement of inert gravel (9.3737 kg) occurred on the bottom of the filter to distribute in-flowing water. The used gravel was that which passed through a #8-mesh sieve (openings 2.38 mm, 0.0937 in.). The reservoir tank, piping, pump and FSF were the same arrangement as used in the George Barley Water Price GBWP competition (Chapter 1, section 2.4.4). The researcher filled the FSF with 15.000 kg of OAS previously used in the GBWP work and citric acid washed twice according to the procedure from used in the GBWP. The outlet of the

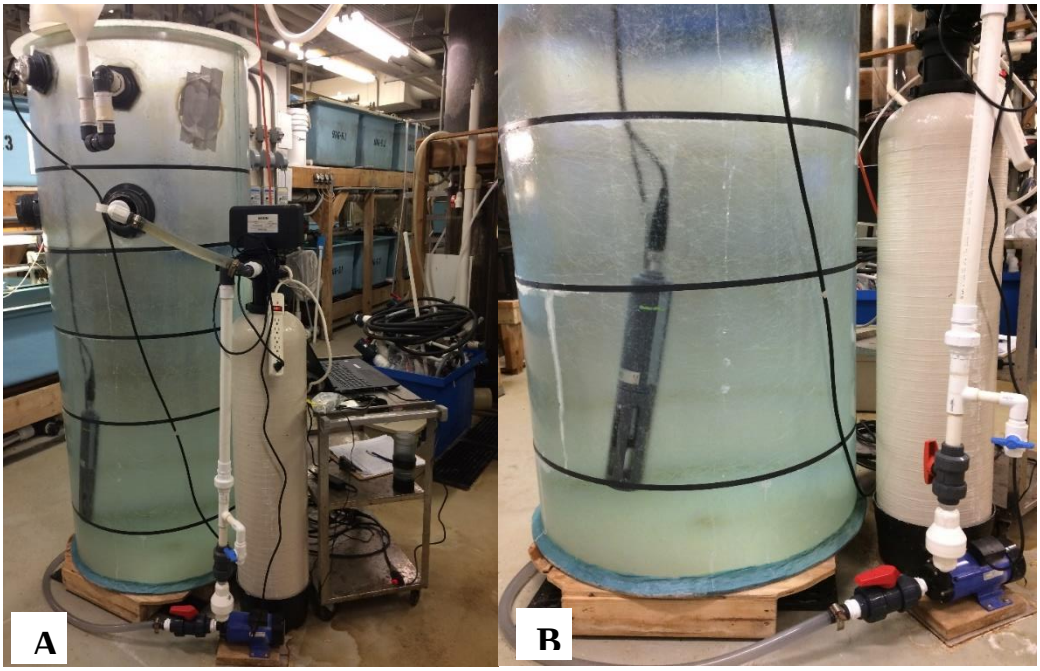


Figure 4.1 A. Arrangement of reservoir tank, circulation pump, fluidized sand filter, return to reservoir, addition port with funnel, monitoring probe in reservoir and computer recording data. B. Close-up of circulation pump, sampling port above pump and monitoring probe positioned in reservoir.

FSF was direct back to the reservoir tank, with the position of the returning pipe below the water surface to avoid agitation. Filling of the tank with 568 liters of deionized water and Crystal Sea® Marinemix from Marine Enterprises International, LLC occurred. Adding of salts occurred in four additions over 25 days to raise the salinity to 35 ppt. During the salt's additions, strong aeration in the reservoir from blower supplied air mixed the water to dissolve the salts and providing a uniform salinity profile. Inoculation of bacteria into the system occurred with the addition of active seeded media and the oolitic aragonite sand in the fluidized filter. One liter of mature Kaldnes™ K1 filter media (Water Management Technoloies Inc., www.w-m-

t.com) from a biofilter on a recirculating system holding nurse sharks (*Ginglymostoma cirratum)* was hung in a mesh bag in the reservoir tank.

At the end of the experiment, the flow rate into the reservoir tank was calculated. Flow rates measurements occurred by filling a beaker with water from the filter outlet and timing the period with a stopwatch. The flow rates in liters per minute were calculated using the formula: Flow Rate (lpm) = (timed volume, l/ time, s) x 60. The measurements occurred five times and the results averaged for the measured flow rate.

Figure 4.2 Additions port with funnel on side of reservoir tank. Piping filled with deionized water.

The suspension of an YSI 6-Series multi-probe 6600 V2-4 Sonde occurred in the reservoir with outputs connected to a laptop for recording conditions every 15 minutes. The Sonde included dissolve oxygen, temperature, conductivity, pH and chlorophyll sensors. A computer running the software program EcoWatch Lite (www.ysi.com) accessed and stored the data from the YSI monitor. Measurement of dissolved oxygen occurred in the reservoir.

A Solar Components friction fit cap blocked oxygen addition to tank from the room air. The installation an additions port on the side of the reservoir tank using a water filled trap made from 1" PVC pipe and fittings occurred (Figure 4.2). Additions of ammonium chloride, sodium nitrite and return of excess water during sampling from

the sampling port occurred at the additions port. The trap maintained a watertight seal that limited oxygen, assisting in the development of anaerobic conditions.

An initial system test period conducted to confirm reliable operation of the system. Aeration of the reservoir tank occurred during this time with a diffuser positioned on the bottom. In order create an anaerobic environment; administration of nitrogen metered by a fine pore diffuser positioned on the tank bottom occurred to expel oxygen from the reservoir tank. Aeration within the reservoir ended prior to the application of the nitrogen gas. The diffuser was connected to a liquid nitrogen dewar by vinyl tubing, control valve and regulator. A moderate flow of nitrogen created a gentle water circulation within the tank.

Phosphorus in the system occurred with addition of 42.585 mls of a 1000ppb phosphorus stock solution from the GBWP experiment for a target reservoir concentration of 75ppb phosphorus. In order to establish an environment for anammox to develop, ammonia and nitrite needed to be present. Ammonium chloride (117.902 g, purity 96.5%)) and sodium nitrite (59.629 g, purity 97.0%)) were added to achieve a concentration of 70 mg/l for both ions. Over the course of the experiment, additional ammonium chloride and sodium nitrite additions occurred based on water quality results.

Upon completion of the experiment, sampling of the sand in the fluidized sand filter occurred for bacterial analysis. Samples were collected using sterilized pipets and sample bags (Nasco Whirl-Pak Bags, Ethylene oxide sterilized, Mfg. # 99100013.00). Removal of the oolitic aragonite sand from the filter occurred for drying. Spreading

of the sand on trays occurred to dry it with laminar airflow from a fan. Mixing of the sand occurred often during the drying period to allow complete evaporation of the water. Once dry, weighing of the sand occurred, with the subtraction of the weight of the gravel and tray. The calculated percent loss of sand used this formula: Percent Sand Loss = ((Original sand weight - used sand weight)/ Original sand weight) x 100.

4.3.2 Water Quality Testing

The sampling port directly above the circulation pump outlet was the collection point for water samples. Sampling consisted of purging approximately 2 liters of water from the sample port, one liter at a time. Return of this water to the reservoir occurred by pouring it into the side trap on the reservoir. Collected water samples occurred from the second liter remove. Testing of the samples occurred weekly for pH, NH_4+, NO_2-, PO_4- and alkalinity by the Water Quality Laboratory of the National Aquarium Baltimore (www.aqua.org). The laboratory referenced the following methods from the manual *Standard Methods for the Examination of Water and Wastewater, 21st Edition* to analyze the parameters (2510, 4500- H+, 4500- NH_3, 4500- NO_3, 2320 and 4500- P, respectively). Samples for nitrate were taken at the sample time and analyzed by the Molecular Characterization and Analysis Complex laboratory at the University of Maryland Baltimore County using method 4500- NH_3/ 4110 from the manual *Standard Methods for the Examination of Water and Wastewater*.

Over the course of the experiment, additional ammonium chloride (Sigma-Aldrich, ACS reagent, 96.5%) and sodium nitrite additions (Sigma-Aldrich, #237213-500G, ACS reagent, ≥ 97.0%) occurred based on water quality results. These additions occurred by way of the fill port on the side of the reservoir tank.

4.3.3 Statistical Analysis

Calculated averages include ± standard deviation where appropriate. The calculation of the standard deviations occurred by using the STDEV.S formula in EXCEL.

4.4 Results

4.4.1 Water Quality

System preparation occurred from September 4, 2018 through October 2, 2018. Addition of Crystal Sea® Marinemix occurred on four separate days with additions of 19.77 kg, 0.542 kg, 0.542 kg and 0.600 kg. The experiment setup, water quality monitor, computer, FSF and saltwater mix addition was started circulating on September 4, 2018 for a period of 48 days to test operation of components and establish the salinity. The saltwater anammox experiment started on October 19, 2018 with the addition of phosphorus stock solution, and the ammonium and nitrite compounds. The aeration ended, the seeded media added, and nitrogen gas purging occurred at this time. The experiment ended on July 15, 2018, 270 days duration. The YSI 6-Series multi-probe 6600 V2-4 Sonde collected data from the starting date until June 20, 2018, 25 days before the end of the experiment. The data collection

ending due to a full memory capacity of the monitor, unbeknown to the researcher. Two additional dissolved oxygen readings occurred on June 27, 2018 and June 28, 2018, as the unit was still measuring conditions, days 252 and 253 respectively.

Four 250 l nitrogen dewars were used over the course of the experiment to maintain dissolved oxygen levels below 0.5 mg/l (0.03 mM). The first nitrogen dewar ran from the first day, (October 18, 2019) until it emptied on day 6. The second nitrogen dewar was added on day 12 continued until day 54, when it was found empty. A third nitrogen dewar was added on day 63 until day 216 and used only when dissolved oxygen reading showed an increase. A fourth nitrogen dewar added on day 236 until day 244.

On the last day of the experiment, the measurement of the water flowing through the

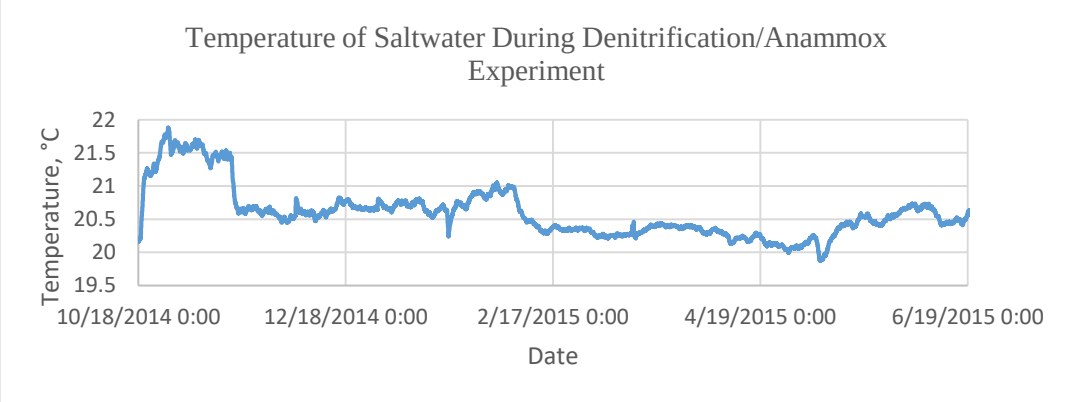


Figure 4.3 Temperature during saltwater denitrification/anammox experiment, °C.

fluidized sand filter occurred. The flow averaged 4.65 ± 0.05 lpm.

The temperature during the experiment remained very stable with a range from 19.87°C to 21.88°C. The average temperature was 20.59 ± 0.38°C, with a median value of 20.54°C (Figure 4.3).

The dissolved oxygen measured in the reservoir range from 0.01 to 0.44 mM during the project with an average of 0.03 ± 0.06 mM (Figure 4.4).

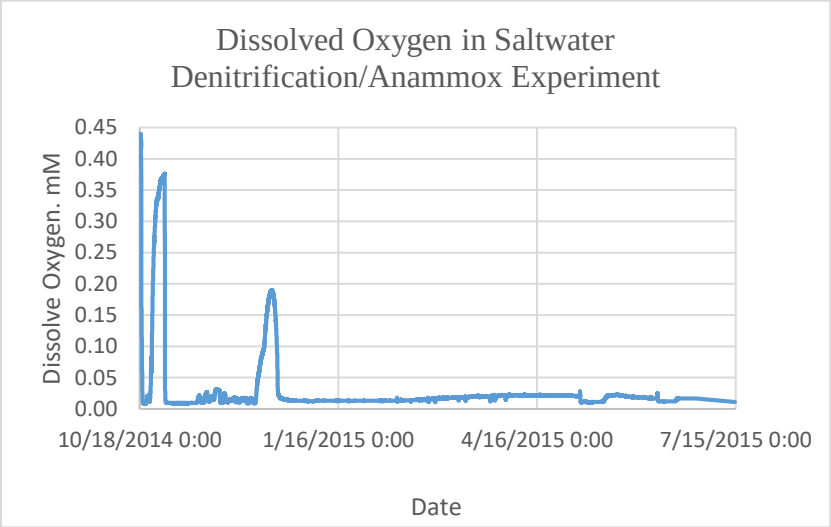


Figure 4.4 Dissolved oxygen concentration within reservoir of the saltwater denitrification/anammox for the 271-day duration of the experiment.

The standard deviation indicates strong variation among the data. Three high peaks that occurred during the experiment are the main driver for the variation. The first peak was the starting oxygen concentration in the system, which decreased below 0.06 mM by day 2. The second peak happen between days 6 and 12 with a high reading of 0.38 mM. The third peak occurred between days 55 and 63, with a high point of 0.19 mM. Both the second and third peaks happened directly after the nitrogen dewar ran out of gas. Once a full nitrogen dewar was reinstalled on the system, the dissolve oxygen level quickly receded. Review of Figure 4.4, if one does not consider the three dissolve oxygen peaks, the majority of the time the oxygen levels were very low and stable. The median value of 0.02 mM gives an indication of this stability, in spite of a higher average with a deviation almost twice its value.

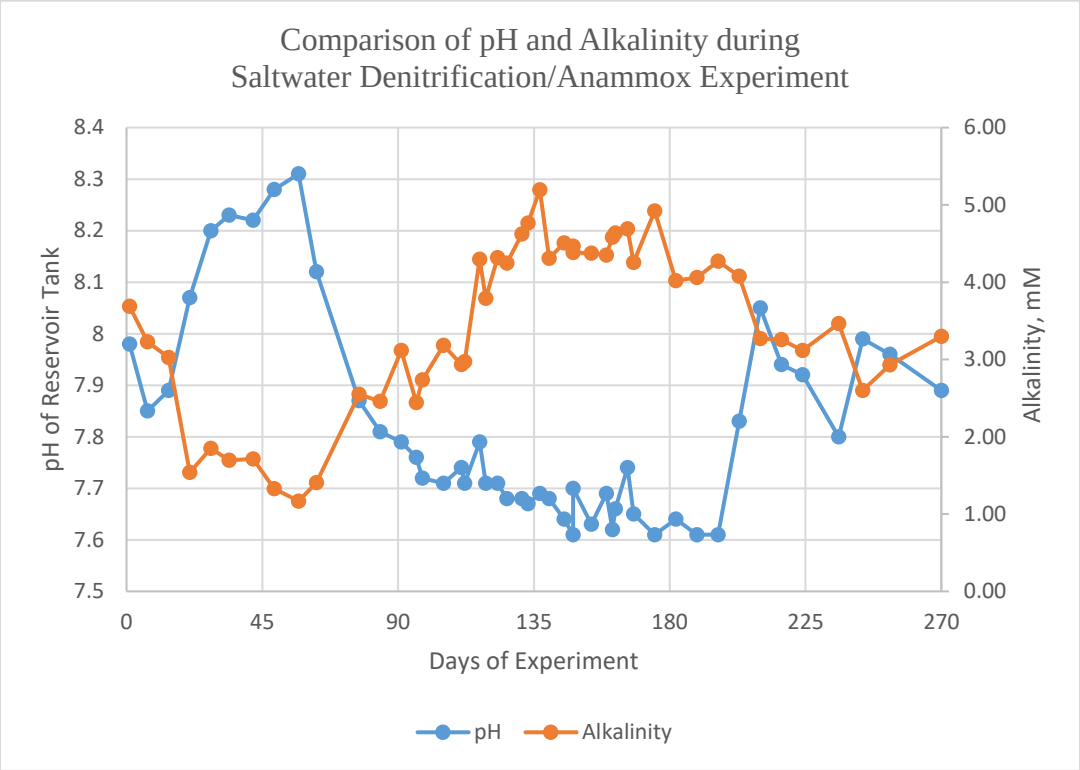


Figure 4.5 Comparison of the pH and alkalinity readings during the saltwater denitrification/anammox experiment.

The salinity ranged from 30 to 31.8 ppt. with an average of 31.3 ± 0.3 ppt. The median value is 31.3 ppt. shows a reasonably stable salinity. The first 28 days show the greatest changes with values ranging from 30.0 to 31.1 ppt.

The pH and alkalinity changes during the first 203 days show strongly opposite trends which reverse after day 77 (Figure 4.5). In the first 21 days, both pH and alkalinity show a declining trend. After which, the pH rose from a low of 7.85 at that point to a high of 8.31 on day 57. The alkalinity declined to its lowest point (1.17 mM) on day 57, the same day that the pH peaked. Day 57 is notable in that the pH never reached this high for the remainder of the experiment and the alkalinity never reach as low a point either. After day 57, the two parameters switched in their trends. The pH

started to drop and continued to remain low until day 196. The alkalinity began to rise after day 57 to a high of 5.20 mM on day 137, at which point it remained relatively stable. On day 186, the alkalinity started decreasing. From day 210 to the end of the experiment on day 270, both parameters remained stable.

Overall, the pH ranged from 7.61 to 8.31 with an average of 7.8 ± 0.20. The median value is 7.74. The alkalinity showed a range from 1.17 to 5.20 mM, with an average of 3.45 ± 1.09 mM. The median value, 3.46 mM, very similar with the average, in spite of the large variation in range.

The phosphorus ranged from a low of 0.096 to 0.889 µM with an average of 0.288 ± 0.154 µM with a median value of 0.254 µM. At the beginning of the experiment, the addition of phosphorus from a stock solution occurred with a concentration of 2.42 µM. The first sampling showed a phosphorus concentration of 0.889 µM, 63% below the addition rate. After the first measurement, the phosphorus concentration never rose above 0.519 µM (Figure 4.6). The phosphorus concentration remained below this threshold with minimal variation, with the exception of the period between days 112 to 196. During this period, the readings show fluctuations between measurements (Table 4-1). The changes in the P concentrations before and after this period are a magnitude different with respect to averages.

The phosphate readings reflect similar changes as the phosphorus readings (Figure 4.6). The phosphate measurements ranged from 0.295 to 2.727 µM with an average of 0.883 ± 0.472 µM. The median value was 0.779 µM. The initial phosphate of 2.727 µM dropped rapidly by day 14. As with the phosphorus readings, the

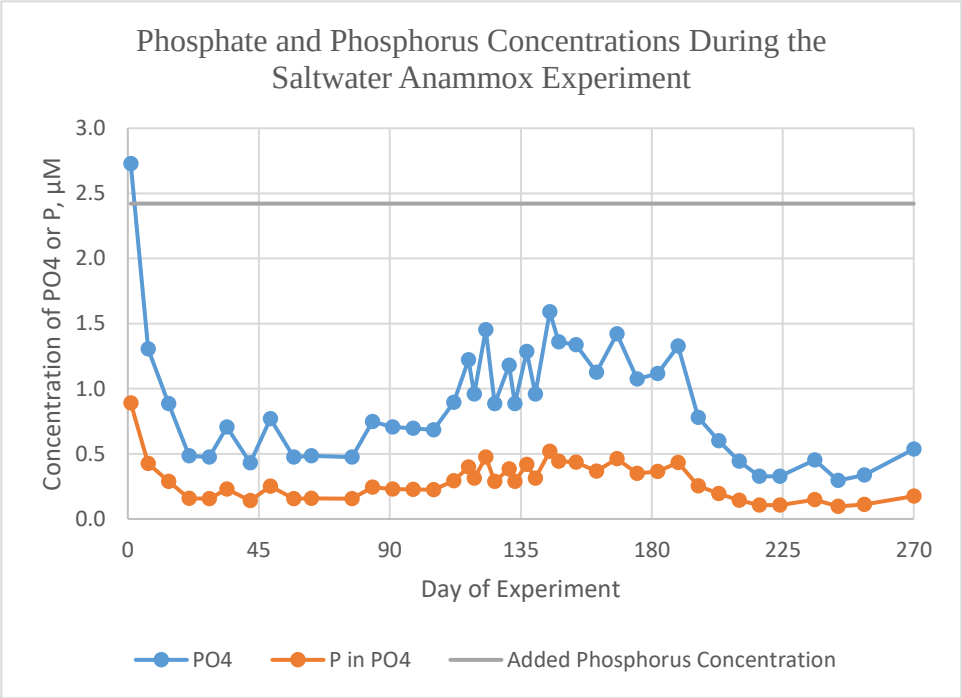


Figure 4.6 Phosphate and phosphorus concentrations during the saltwater anammox experiment. The added phosphorus concentration line is the amount of phosphorus added at the start, from a stock solution.

phosphate showed strong fluctuations between days 112 and 196 (Figure 4.6). The phosphate concentrations between these days are at or above the 0.779 µM level. At all other times the phosphate remained below this level with the exception of the first 14 days of the experiment, which shows a strong declining phosphate trend. The phosphate differences for days 0 to 111 show relatively similar standard deviation as with days 112 to 196 (Table 4-1). The first three measurements of phosphate and phosphorus up to day 21 are indicative of removal by the OAS sand. Removal of the first three phosphate readings from the calculation due to the large drop in the first 14 days, the standard deviations without the first three readings shows less variation than days 112 to 196. The changes in both the phosphorus and the phosphate between days 112 and 196 occurred during a period in which there was noteworthy changes

with the pH and alkalinity values (Figure 4.5). In a similar period, the alkalinity began increasing above the average of 3.45 ±1.09 mM on day 112 and remained above the average until day 203. The pH on day 91 dropped below the average of 7.8 ± 0.20 and remained there

Table 4-1 Average differences between consecutive phosphate and phosphorus readings during saltwater denitrification/anammox experiment ±standard deviations.

Days	P Difference ±std. dev., µM	PO_4 Difference ±std. dev., µM	PO_4 Difference ±std dev., µM (without first 3 readings)
0 to 111	0.048 ±0.142	0.146 ±0.436	-0.025 ±0.224
112 to 196	-0.0017 ±0.119	-0.005 ±0.366	
197 to 270	0.010 ±0.066	0.030 ±0.204	

until day 203 with a pH of 7.83. One can infer that the changes in pH and alkalinity between days 112 to 196 resulted in the fluctuations in phosphate and phosphorus during this same time. Initially, the declines in the first three weeks are by the OAS. Following this the changes are likely pH mediated, effecting the binding of the phosphorus to the OAS.

The laboratory analyzed water samples for calcium on days 140 and 270 with values for 6.68 and 8.74 mM, respectively. The pH for these two days were 7.68 and 7.89. Additions of NH_4Cl and $NaNO_2$ occurred to add nitrogen to the system, where it resided as total ammonium nitrogen (TAN), nitrite and nitrate. The TAN

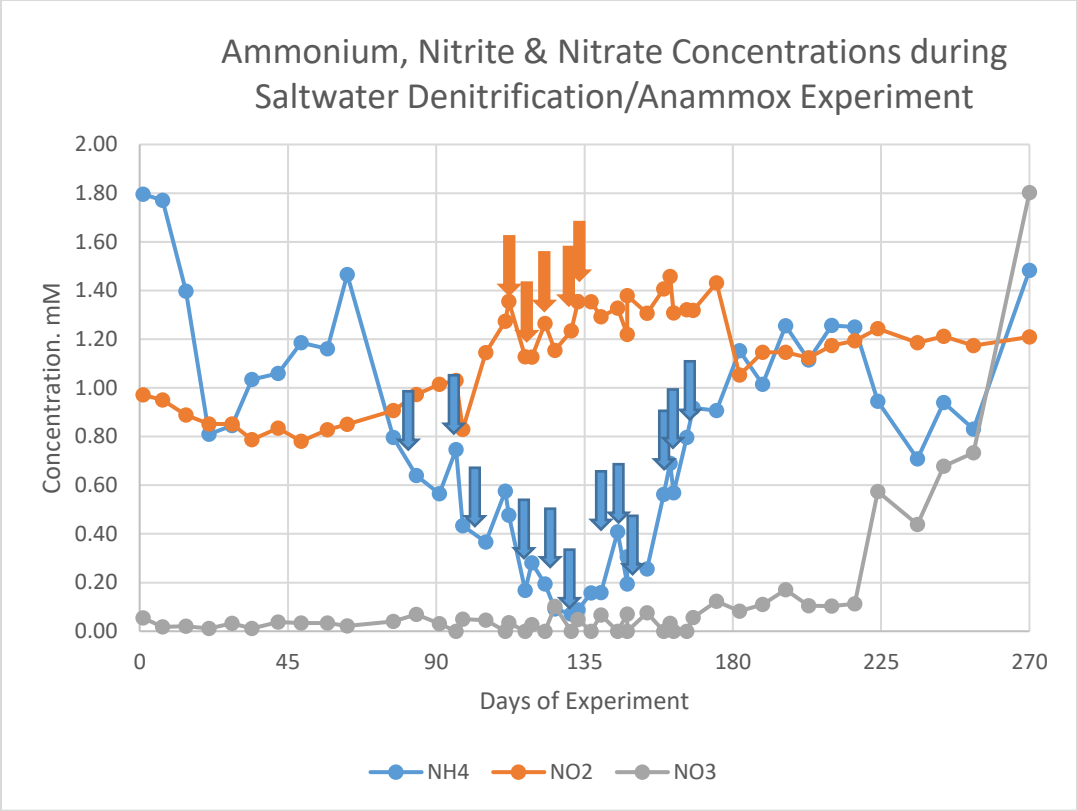


Figure 4.7 The TAN, nitrite and nitrate concentrations during the saltwater denitrification/anammox experiment with beginning additions of 117.9 g NH_4Cl and 59.6 g $NaNO_2$ on day one. Blue arrows mark the addition of 59.9 g of NH_4Cl on days 81, 95, 102, 117, 123, 131, 145, 148, 159, 161 and 166. The one blue arrow on day 134 represents the addition of 39.4 g of NH_4Cl. The five orange arrows mark additions of 77.2 g $NaNO_2$ on days 111, 117, 123 and 131. On day 134, an orange arrow marks the addition of 50.8 g $NaNO_2$.

concentrations ranged between 0.07 to 1.80 mM, with an average of 0.76 ± 0.46 mM and a median value of 0.80 mM. The TAN graph in Figure 4.7 displays the changing nature of the compound during the experiment. Twelve additions of NH_4Cl occurred between days 81 and 166 (Figure 4.7). On days 81, 95, 102, 117, 123, 131, 145, 148, 159, 161 and 166 the addition was 59.9 g of NH_4Cl. On day 134, the addition was 39.4 g of NH_4Cl.

The nitrite ranged from 0.78 to 1.46 mM, with an average of 1.14 ± 0.19 mM and a median value of 1.17 mM. The nitrite measurements varied but showed an increased

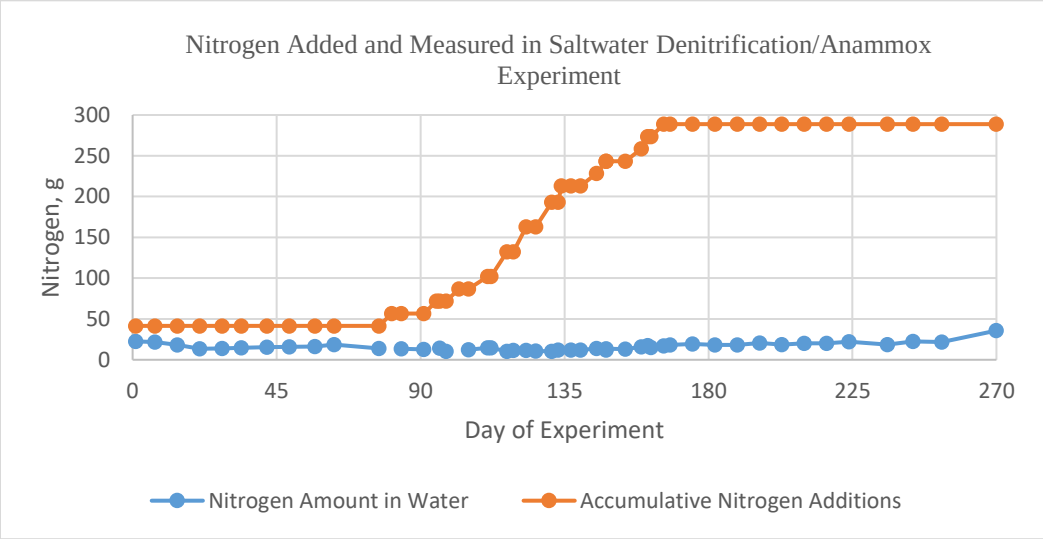


Figure 4.8 The daily measured nitrogen in the saltwater denitrification/anammox experiment, resulting from TAN, nitrite and nitrate. Daily accumulative nitrogen from NH_4Cl and $NaNO_2$ additions.

concentration by day 270. Five additions of $NaNO_2$ occurred during experiment. Four additions were 77.2 g $NaNO_2$ on days 111, 117, 123 and 131. On day 134, there was an addition of 50.8 g $NaNO_2$.

The nitrate, though not added, ranged from 0.01 to 1.80 mM, with an average of 0.17 ± 0.33 mM and a median value of 0.06 mM. With the value of the median lower than the average (64.7% lower), indicates that the high average is driven by the elevated nitrate readings in the last 46 days of the experiment and the majority of readings were low. Over the first 217 days, the nitrate concentration stayed no higher than 0.17 mM. After day 217, the nitrate values increased steadily until the end of the experiment.

In order to assess the possible presence of anammox occurrence in the system, the comparison of nitrogen in the system from TAN, nitrite and nitrate against the additions of nitrogen from NH_4Cl and $NaNO_2$ occurred (Figure 4.8). The total

amount of accumulative nitrogen added to the system from additions totals 288.79 g. The daily total measured nitrogen in the system ranged from 10.31 to 35.75 g, with an average of 16.13 ± 4.65 g and a median value of 14.93 g. The maximum measured nitrogen value occurred on the final day of the experiment. The average daily total nitrogen in the water between days 81 and 166 was 13.04 ± 2.00g, showing little variation, even though during this time there were thirteen additions of nitrogen from NH_4Cl and $NaNO_2$. The nitrogen additions therefore were rapidly assimilated or otherwise deposited in a nitrogen sink.

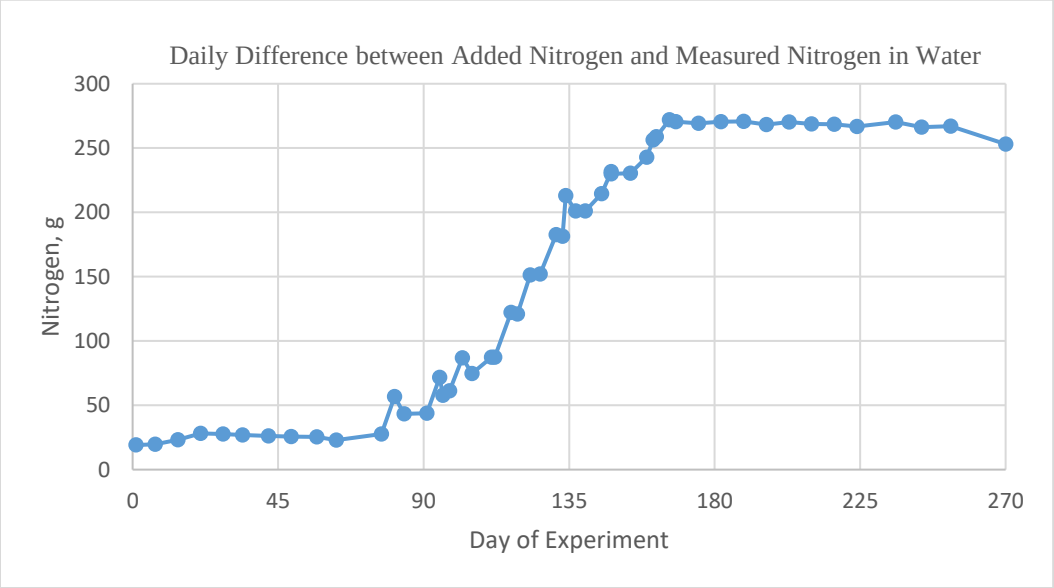


Figure 4.9 The daily difference between the additions of nitrogen in the form on NH_4Cl and $NaNO_2$ and the amount measured in the saltwater as NH_4, NO_2 and NO_3 for the saltwater denitrification/anammox experiment.

The difference between the measured nitrogen in the water, and that accumulated on day 270 shows a loss of 253.04 g N, presenting an 87.62% loss of N (Figure 4.9). Over the first 77 days, the initial nitrogen additions remained higher than the measured nitrogen in the water and stable with an average of 25 ± 3.2 g N. The

subsequent nitrogen additions show an increasing difference with an average value of 153 ± 77.0 g N between days 81 and 166. The nitrogen increased from 56.66 g to 271.95 g during this period. From day 168 to the end of the experiment, the nitrogen difference averaged 268 ± 4.7 g N, showing little variation.

The comparison of ratio of NH_4/NO_2 to the total nitrates measured in the system occurred in Figure 4.10. The ratio remained above the optimal ratio of one for anammox (Fux, 2003, Zhang et al., 2008) during the beginning and end of the experiment. The ratio ranged from 0.06 to 1.86, averaging 0.73 ±0.50 and a median value of 0.66. As seen in Figure 4.10 and noted by the relatively large average standard deviation, the ratio varied considerably throughout the experiment. The changing NH_4/NO_2 ratios did not appear to influence the rate of nitrate production over the first 217 days. Separation of the NH_4/NO_2 ratio and NO_3 amounts into discrete day ranges enable a better understand of the relationships (Table 4-2). The average nitrate amount shows a slow, but steady increase from the beginning to the end of the experiment as seen in Figure 4.10 and

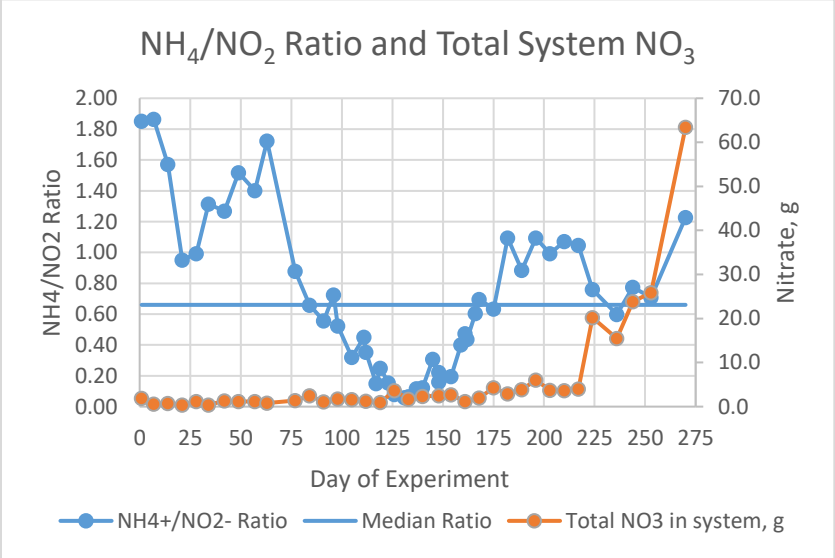


Figure 4.10 The daily ratio of NH_4 to NO_2 present in the marine denitrification/anammox trial. The median line of the NH4/NO2 ratio (0.66). The total system daily nitrate concentrations.

Table 4-2. At the same time, the NH_4/NO_2 ratios oscillated above and below the median ratio value and the target ratio of one for anammox. The period between days 85 to 168 corresponds to the same interval that numerous nitrogen additions transpired. The NH_4/NO_2 ratio reached its lower individual point (ratio = 0.06, days 131 and 133) during this time, as well as the lowest averaged value for the selected ranges (Table 4-2, 85 to 168 days). Even with these conditions, the nitrate value changed very little compared with the period day 1 to day 84. Comparison of that information with days 225 to 270, show a different reaction. The NH_4/NO_2 ratio averaged 0.83 ±0.28 with an increasing average nitrate amount of 32.1 ±21.3 g. With over a doubling of the NH_4/NO_2 ratio (0.32 to 0.83) from days 85-168 to 225-270, the nitrate amount increased by 16 times (1.9 to 32.1). The average NH_4/NO_2 ratio from days 85-168 to 169-224 increased by approximately 3 times (0.32 to 0.95) and resulted in an increase in the average nitrate of 3.2 times (1.9 to 6.1). Changes in the NH_4/NO_2 ratio do not directly affect the production of nitrate within the marine denitrification/anammox system.

Table 4-2 Average NH_4/NO_2 ratio and NO_3 with standard deviation over select ranges of day for the marine denitrification/anammox experiment

Days	1 to 84	85 to 168	169 to 224	225 to 270
Average NH_4/NO_2 ratio	1.33 ±0.40	0.32 ±0.21	0.95 ±0.17	0.83 ±0.28
Average NO_3	1.1 ±0.6	1.9 ±0.8	6.1 ±5.8	32.1 ±21.3

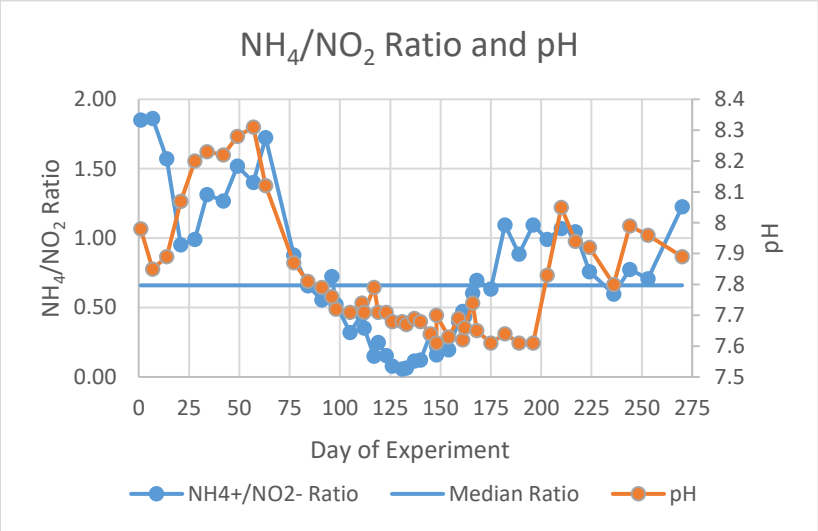


Figure 4.11 Comparison of the similarity of the NH_4/NO_2 ratio with the changing pH reading during the marine denitrification/anammox experiment

The similarity of the NH_4/NO_2 ratio to the changing pH readings shows a strong correlation indicating a probable cause and effect relationship (Figure 4.11).

4.4.2 Microbiome Analysis

The listing of phylum, classes, orders and families detected on the aragonite sand occurs on Table 4-3. The largest grouping (46.7%) occurred for the Unassigned; other category, followed by *Actinobacteria*, *Proteobacteria* and *Bacteroidetes*, with percentages of 28.0%, 22.0% and 3.3%, respectively. The analysis located two families within the *Actinobacteria*: *Microtrichaceae* and *Mycobacteriacease* representing 2.7% and 3.3% of the total identified groups. No family determinations occurred in the *Bacteroidetes* and *Proteobacteria* phylum.

Table 4-3 Results of the microbiome analysis on the marine aragonite sand in the denitrification/anammox experiment. Detected bacterial groupings according to phylum, class, order and family, along with percentages per phylum.

Percent Phylum	Phylum	Class	Order	Family
28.0%	Actinobacteria	Acidimicrobiia	Microtrichales	Microtrichaceae
		Actinobacteria	Corynebacteriales	Mycobacteriacease
3.3%	Bacteroidetes	Bacteroidia		
22.0%	Proteobacteria	Alphaproteobacteria	Rhizobiales	
		Gammaproteobacteria		
46.7%	Unassigned; Other			

4.5 Discussion

Establishment of conditions beneficial to denitrification/anammox bacteria is vital for the removal of nitrogen by denitrification or anammox reactions to take place. The study met the conditions of temperature, oxygen, pH, nitrogen compounds and bacteria for this current work. Temperatures for denitrification can occur over a broad range depending on the denitrifiers established in the filter (Timmons & Ebeling, 2010). The denitrification temperatures reportedly range from 14.6°C to 27.2°C (Lee et al., 2000, Singer et al., 2008, Tsukuda et al., 2015, Zhou et al., 2019). Published works have reported that temperatures in this range are acceptable for anammox to take place (Cema et al., 2011, Jin et al., 2012). The observed temperatures were

within limits recommended for denitrification and anammox (Timmons & Ebeling, 2010, Cema et al., 2011, Jin et al., 2012).

The dissolved oxygen concentrations ranged further than expected from 0.01 to 0.44 mM due to unanticipated loss of nitrogen purging. The high oxygen readings occurred at the initial startup and two times the nitrogen supplies were exhausted. Once nitrogen purging resumed, the oxygen levels quickly reduced. The median oxygen concentration (0.02 mM) value indicated that a low concentration remained conducive for anaerobic conditions to exist the majority of the time. Referring to Figure 4.10, the plot of the total nitrate present in the system shows no change during oxygen increases supporting that little to no long-term impact affected the residing anaerobic bacteria present. The total nitrate amount continued to be stable from day 60 until day 217. Tsukuda et al. (2015) showed denitrification can occur when the reactor dissolved oxygen concentration is <0.37 mg/l (<23 µM). Aerobic denitrification is reported at dissolved oxygen levels of 5.0 – 6.0 mg/l (0.31 – 0.38 mM), though denitrification is normally considered to occur in anoxic conditions with oxygen levels <0.2 mg/l (<12.5 µM) (Hong et al., 2019). Working with biodegradable plastic as an carbon source for denitrification, Zhang & Zhang (2018) observed nitrate removal rates of 30.6 g NO_3-N m^{-3} d^{-1} and 30.8 g NO3-N m^{-3} d^{-1} at starting oxygen concentrations of <1.0 mg/l and 4.8 mg/l. The oxygen in the low oxygen treatment decreased to <1.0 mg/l during the trial, attributed to bacterial respiration. The median dissolved oxygen concentration is consistent with those used in other anammox laboratory and sewage treatment work (Shivaraman &

Shivaraman, 2003, Kimura et al., 2011, Morales Aqualia et al., 2014, Yin et al., 2016).

The pH changed throughout the project but remained in the acceptable range for anammox activity of 6.5 to 8.5 (Yin et al., 2016). The ideal pH for denitrification occurs between 7 and 8, with possible diminished activity outside this range (Timmons & Ebeling, 2010). Zhang & Zhang (2018) working with biodegradable plastics as a carbon source observed denitrification proceeding at pH values of 6.5 to 7.8. Xu et al. (2018) showed low pH valves (5.68) decreased nitrate removal by denitrification by 34% in work using PHBV/PLA polymer blends for denitrification. At high nitrate concentrations (2700 mg/l), impeded denitrification occurs at pH values of 6.5 and 7.0 (Glass & Silverstein, 1998). The authors found nitrite concentrations increasing with rising pH values of 7.5, 8.5 and 9.0 (Glass & Silverstein, 1998). Qian et al. (2019) working with synthetic wastewater at pH's of 5, 7 and 9 caused denitrification to produce more nitrite with increasing pH values. By control of denitrification by higher pH and a low C/N ratio, partial-denitrification could be used to maintain a stable level of nitrite for anammox reactions (Qian et al., 2019). Published reports show the effective range for pH in anammox is 6.5-8.5 from work conducted on a sequence batch reactor (Yin et al., 2016). The observed pH in the present work is within the reported effective range for pH.

Nitrogen existed in the marine denitrification/anammox setup as TAN, nitrite as well as nitrate in the water column as evidenced by water samplings. The introduced nitrogen occurred with the initial NH_4Cl and $NaNO_2$ additions at the beginning of the

project. Numerous additional inputs of NH_4Cl and $NaNO_2$ happened during the middle portion of the experiment. The concentrations of the TAN and nitrite varied during the project (Figure 4.7). Observation of the period between days 90 and 180 in Figure 4.7 shows surprising similarities to the changes with the pH, alkalinity, phosphate and phosphorus graphs (Figure 4.5, Figure 4.6). The TAN decreased in concentration during this period. The nitrite values are elevated and show stronger changes between measurements than before or after this period. The nitrates shows some variation during this period, but not at the same scale as the nitrite. The occurrence of these changes during a similar period likely are the effects of the changing pH and alkalinity values.

Furthermore, the additions of NH_4Cl and $NaNO_2$ occurred between days 81 to 166. The period of additions overlaps with the changing pH and alkalinity periods (days 77-210) and the phosphate changes (days 112-196). The variability in the nitrogen complexes during the period of nitrogen compound additions is likely the result of the nitrogen additions. The variability in the pH and alkalinity measurement would seem unlikely effected by these additions. One could further surmise the phosphate variability is a probable effect of pH and alkalinity rather than the nitrogen additions. An important factor in anammox reactions is a consistent ratio between NH_4 and NO_2 with a value close to one. The anammox reactions utilize these ions in a one to one ratio, so maintenance of the NH_4/NO_2 ratio improves the effectiveness of the nitrogen removal. The NH_4/NO_2 ratio reached a high of 1.86 (Figure 4.10). The expectation would be that a low NH_4/NO_2 ratio (<1) would negatively affect nitrogen removal,

but the amount of nitrogen measured in the water, the difference between nitrogen added and measured and total nitrate amount does not show an impact to the nitrogen removal from the system (Figure 4.8, Figure 4.9, Figure 4.10).

After the initial dosing of NH_4Cl and $NaNO_2$ into the system at the start of the experiment, twelve NH_4Cl additions (days 81-166) and six $NaNO_2$ additions (days 111-134) occurred (Figure 4.7). The NH_4Cl additions showed no increases in the measured TAN level. The NH_4Cl additions appears to drive the TAN levels lower, when comparing TAN concentrations before and after the additions. In contrast, the NO_2 concentrations rose during the same period. The additions of $NaNO_2$ occurred during the higher NO_2 measurements. They did not appear to affect the NO_2 concentration appreciably; in viewing Figure 4.7 no strong response occurs. A rising of NO_2 levels concurrently with NH_4Cl additions presumes that nitrification is occurring converting the NH_4 into NO_2. The oxygen concentrations during this period are well within conditions acceptable to anaerobic bacteria (<0.03 mM). The seeded media added to the system likely contained active nitrifying bacteria, as originally occurring in an aerated biofilter. The seeding media, positioned just below the surface of the water, may have had sufficient oxygen in this water layer and in close proximity to the headspace at the water surface to support nitrification. The continuous monitoring of oxygen occurred with the probe position in the lower section of the water column. Measurement of oxygen levels close to the water surface did not occur.

The NH_4Cl additions resulted in a significantly lowering the NH_4/NO_2 ratio. On day 77 the NH_4/NO_2 ratio was 0.88, then decreased to 0.66 on day 166. During this time, the smallest recorded ratio values occurred with the lowest being 0.06 on days 131 and 133. When the additions ceased, the ratio started rising and passed the median ratio value (0.66) on day 168 and rose above the ratio of one on day 182.

Changes in pH, alkalinity, phosphate and phosphorus occurred at the same time as nitrogen additions occurred. Although pH started decreasing and alkalinity rising before the additions started, the lowest pH and the highest alkalinities values happened during this time. The two parameters also show stronger daily changes in their measurements than before or after this period (Figure 4.5). The phosphate concentrations and to a lesser degree the phosphorus concentrations showed variation thru this time as well (Figure 4.6). One would likely conclude that the occurrence of these parameter changes and variations are due to the nitrogen additions or because of them.

The NH_4/NO_2 ratio remained lower that ideal ratio of one for approximately 47% (127 days) of the study. The additions of NH_4Cl acted to lower the ratio, though a portion of the additions appears to form into NO_2 by nitrification. The result of an increase in NO_2 concentrations is the lowering of the NH_4/NO_2 ratio. Improved surveillance of the ratio during the experiment would have led to an opportunity to counter the imbalance in ions. The NH_4^+ ions overall averaged 0.76 ±0.46 mM and the NO_2^- ion averaged 1.14 ±0.19 mM. An increase in NH_4 additions after day 77 when the ratio was first lower than one, may have been sufficient to reach to a ratio

closer to one. Increased review of the NH_4/NO_2 ratio and proper additions would have raised the ratio, but the nitrate concentration over the first 217 days of the experiment does not show any response to a changing NH_4/NO_2 ratio.

The increase in the nitrate measurements that started after day 217 and continuing to the end of the experiment on day 270 show no clear reason for its increase. The pH, alkalinities, TAN, NO_2, dissolved oxygen, temperature were all relatively stable at that time. The NH_4/NO_2 ratio was between the median ratio value and one during this time. The causative condition making nitrate generation favorable is not forthcoming.

The understanding of the changing concentrations of phosphate and phosphorus in the system potentially could be a result of the existence of varying oxygen concentration zones within the experimental setup. In cyclic exposure to aerobic and anaerobic conditions, wastewater from domestic sewage can free phosphorus from bacteria, followed by bacterial up taken up in aerobic conditions as polyphosphate (Timmons & Ebeling, 2010). Timmons & Ebeling (2010) describe phosphorus removal by heterotrophic denitrification bacterial under aerobic or anoxic conditions without the need of alternating between oxygen levels. This phosphorus removal pathway occurs in both freshwater and saltwater RAS (Timmons & Ebeling, 2010).

Zeng et al. (2003) using a simultaneous nitrification, denitrification and phosphorous removal (SNDPR) in a sequencing batch reactor under aerobic and anaerobic conditions demonstrated phosphorus release in anaerobic conditions followed by uptake of phosphorus through the aerobic phase. The variability of the phosphorous

and phosphate concentrations during the experiment could be the result of this occurring, assuming that aerobic and anaerobic areas existed spatially in the experimental setup. The loss of nitrogen during SNDPR occurred not by the formation of N_2 gas, but by N_2O (Zeng et al., 2003). The occurrence of SNDPR in the current work may explain the variability of the phosphorus and phosphate concentrations and reveal another avenue for nitrogen loss from the system. Recently, Wang & He (2020) isolated a *Thauers* sp. strain SND5 from domestic wastewater showing it is capable of simultaneous nitrification, denitrification and phosphate removal using an internal carbon source of polyhydroxybutyrate (PHB), thus reducing the need for carbon in the denitrification process. Bacterial pathways exist for sequestering phosphate in anaerobic conditions suggesting a possible explanation for changing phosphate concentrations.

Throughout the denitrification/anammox project, there was 288.79 grams of nitrogen added to the system from the initial and ongoing additions of NH_4Cl and $NaNO_2$. At the end of the experiment, the marine water contained 35.75 gram of nitrogen in the form of TAN, NO_2 and NO_3. The difference of these two values reveals 253.04 grams of nitrogen unaccounted. These numbers show an 87.62% loss of nitrogen from the water.

One can consider two possible explanations for the missing nitrogen: assimilation by bacteria and formation of N_2. During the experiment, notice of bacterial films forming on the interior surfaces of sections of clear tubing used in a portion of the circulation plumbing with the FSF occurred. One could assume formation of

bacterial films on all surfaces exposure to the systems water (such as the tank surface, piping and filter) occurs. Bacteria growth requires a carbon source. Supplemental of carbon to the system did not occur, though it was likely present in the biofilms of the seeded media and the $CaCO_3$ in the OAS. Part of the decrease in sand weight may be the loss of carbon to denitrification activities. With these assumptions and estimations of film thickness and percent nitrogen makeup, an estimation of the amount of nitrogen sequestered in biofilms can occur.

Another explanation for a nitrogen sink would be the air space above the water surface. If denitrification or anammox activity were occurring within the system, one would expect a loss of nitrogen from the aqueous solution. Experience with denitrification systems indicates the possible observation of gas bubbles on the internal surfaces of the setup are composed of nitrogen gas. No witnessing of this phenomenon occurred in this application, though missing of the evidence may have occurred if not in sufficient quantities. In addition, no measurements of the gas profile in the headspace above the water occurred to ascertain any change in nitrogen composition. If partial denitrification occurred as in the work by Zeng et al. (2003), the nitrogen may not have been released at N2, but as N_2O.

The reason for the introduction of mature, seeded Kaldnes™ K1 filter media from a marine system operating at stable conditions for many years was to provide the denitrification/anammox system with media assumed to contain anaerobic bacteria. Other investigations within the facility, have confirmed the existence of denitrification bacteria on the Kaldnes™ K1 biofilter media (Zohar et al., 2005). The

same work identified the presence of the known anammox bacteria *Planctomycetes* species from biofilter media (Fux, 2003, Zohar et al., 2005, Shivaraman & Shivaraman, 2003, Oshiki et al., 2016). Tal et al. (2004) by use of 16S-rRNA gene sequences detected the presence of anammox activity within moving mixed bed biofilters, with some being within the IMET-ARC facility. The presence of the anammox bacteria in RAS bacterial consortia provides strong support of these same bacteria being present in the seeding media (Tal et al., 2004). The used media visually contained dark biofilms within the structure of the media particle. Biofilter media within an aerobic nitrification unit develop films with anaerobic bacteria residing at the deepest layers within the biofilm (Cema et al., 2011). The nitrifying bacteria growing on the surface of the biofilms compete for oxygen resulting in little being able to penetrate to the innermost layer of the biofilm (Cema et al., 2011). With this micro-ecology occurring on a biofilm coated aerobic media, explains how anaerobic bacteria can exist in an active biofilter, as well as, be seeding material for an anaerobic setup (Zohar et al., 2005, Cema et al., 2011).

Previous work with the OAS in CF and FSF showed the presence of the phylum *Planctomycetes* occurred on the OAS used in the freshwater, brackish and saltwater application, as well as, the unused naïve sand (Figure 2.21B). Presence of this anammox containing phyla in all four of these sand samples, supports the possibility that the phyla would be present in the current work, though confirmation of the anammox genera would need to confirmation. The total lack of the phyla from the anaerobic FSF is contrary to the expectations.

Between these two inoculation sources, the assumption is denitrification and/or anammox bacteria were present in the experimental system. Confirmation of the presence of anammox and denitrification bacteria in the freshwater denitrification experiment can be confirm by bacterial analysis of the sand remaining from the experiment as well as determining their presence in the biofilter of the source system. Categorizing of nearly half of the detected phylum occurred as unassigned or other in the microbiome analysis. Lacking the functionality of these bacterial groups, one could speculate these groupings conceivably contain bacteria with the ability to conduct denitrification or anammox activities. The phyla Proteobacteria and Bacteroidetes do contain denitrification bacteria genera though known genera were not revealed (Deng et al., 2020). Two families, *Microtrichaceae* and *Mycobacteriacease*, occurred in the *Actinobacteria* phylum. The *Microtrichaceae* family contains filamentous species commonly isolated from wastewater treatment plants (Levantesi et al., 2006). *Mycobacteriacease* is predominately disease agents in animals and humans (Johansen et al., 2020). The genus Mycobacterium are well known as being responsible for tuberculous, but they also are separated into non-tuberculous mycobacteria (NTM) types (Johansen et al., 2020). The NTM types include true pathogens, opportunistic pathogens and saprophytes (Johansen et al., 2020). In light of the current study, Hedgecock et al. (1962) confirm nitrite and nitrate utilization by *Mycobacteria* with the reduction of nitrate to nitrite in many cases. Though the *Mycobacteria* studied by Hedgecock et al. (1962) were not confirmed present in the current study, the possibility of *Mycobacteria* reduction of

nitrate to nitrite could explain the portion of the nitrite concentration occurring in the system.

The genera *Brocadia*, *Kuenenia*, *Anammoxoglobus*, *Jettenia* and *Scalindua* of the phylum *Planctomycetes* contain bacteria known to mediate anammox activity (Jetten et al., 2009). The microbiome analysis lacked any findings of these genera or the phylum associated with anammox activity. The existence of these genera may have occurred at levels not detectable by the analysis. If so, their ability to perform anammox activity would likely be minimal and ineffective in nitrogen removal. Denitrification occurs by a broad range of gram-positive and gram-negative bacteria as well as *Archaea* (Zumft, 1997). The *Proteobacteria* phylum contain aerobic heterotrophic bacteria known to be responsible for denitrification activities. The microbiome analysis detected two genera in *Proteobacteria*, *Gammaproteobacteria* and *Alphaproteobacteria*. The *Gammaproteobacteria* do include bacteria associated with denitrification as evidenced by sediment cores exhumed from mangroves in Brazil (Luvizotto et al., 2019). Known denitrification activities do occur with the genera *Alphaproteobacteria* (Cua & Stein, 2014). Cua & Stein (2014) working with *Sphingomonas wittichii* RW1, an *Alphaproteobacterium*, show its ability in certain conditions to process NO_2^- to N_2O. Incomplete denitrification to N_2O instead of N_2 is a plausible consequence in the currently study. The *Rhizobiales* of the *Alphaproteobacteria* are usually associated with terrestrial nitrogen-fixing bacteria in close relationships with plants (Knelman et al., 2018). However, a limited number of genera in the order *Rhizobiales* exist in marine environments (Yosef et al., 2008). In

light of the absence of known taxonomic groupings, including anammox bacteria in the OAS, the loss of nitrogen from the system most likely did not occur by anammox bacteria, but proceeded through denitrification or partial denitrification by classes within the *Proteobacteria* phylum.

In conclusion, conditions existing in the test system were sufficient for denitrification and anammox bacteria to live and develop. The absence of detected anammox associated bacteria on the oolitic aragonite sand point to other pathways for the nitrogen removal. Denitrification is the likely pathway via *Alphaproteobacteria* and *Gammaproteobacteria* bacteria. The introduction of nitrogen inputs to the denitrification/anammox system resulted in the decrease of the NH_4/NO_2 ratios. The nitrogen inputs led to nitrite increases, a simultaneous decrease in pH and rise in alkalinity and increase of phosphate in the water column. The inputs did not appear to effect the nitrate concentrations directly. The denitrification/anammox test setup shown the capability of removing 87.62% of the nitrogen added to the system indicating a functional arrangement for nitrogen removal.

Chapter 5: **Growth of the Pacific Oyster (*Crassostrea gigas*) and the Eastern Oyster (*Crassostrea virginica*) in Recirculating systems containing Oolitic Aragonite Sand**

5.1 Abstract

Oysters play an important ecological part of the estuarine and coastal environments, while also showing increases in bivalve aquaculture along the eastern United States and elsewhere. The impact of oyster diseases such as Demo, MSX, OsHV-1 and *Vibrio sp.*, concerns all life stages: larvae, juveniles and adults. Using oysters in land-based RAS as opposed to the historical culturing practices show promise in the industry. This study determined effects of aragonite sand on the survivability and growth of Eastern and Pacific oysters in RAS. Culture of both species of oysters occurred in simple RAS using either aragonite or silica sand in a fluidized sand filter, in order to determine response on shell and tissue growth. The water quality occurred within ranges being acceptable for oysters for alkalinity, calcium, TAN, nitrite, pH and salinity. Fluorometer readings showed active feeding occurred up to 120 minutes. The Eastern and Pacific oysters did respond dissimilarly to the conditions of the RAS arrangements. The survival in Eastern oysters was as high as ≥80%, in contrast, the Pacific oysters had survival rates of ≤56 percent. Eastern oysters had an average survival 64% higher than that of the Pacific oysters. Significant whole weight increases occurred with the Eastern oyster, but not the Pacific oysters. Both species showed significant increases in shell length, width and height within the control and treatment tanks. Only the Pacific oysters showed a significant increase in

the shell height of the control group as compared to the treatment group. No significant differences exist between the control and the treatment groups with length and width measurements. With each species, the shuck oyster meat (either wet or dry), decreased in weight or remained unchanged when compared to the initial subsample group. Both species showed shell increases, while at the same time lost tissue weight, perhaps due to temperature and water quality stress resulting in reduced somatic growth and limit shell development. The results of this work do not demonstration any differences between the control tanks with silica sand or the treatment tanks using oolitic aragonite sand as substrates in a fluidized sand filter in comparisons for survival, whole weight, shell dimensions and shucked meat weights.

5.2 Introduction

Oysters play an important ecological part of the estuarine and coastal environments. Recent work reveals that how Pacific oysters can benefit eelgrass (*Zostera marina*) beds from the eelgrass wasting disease caused by the pathogen *Labyrinthula zosterae* by filtering it out of the water (Groner et al., 2018). Eastern Oysters are capable of removing 700 – 5,500 pounds of nitrogen per year per 1 million harvestable-sized oysters relating the tremendous impact they have on coastal environments (US EPA, n.d., *Chesapeake Bay Total Maximum Daily Load (TMDL) | US EPA*, n.d.). Lai et al. (2020) using a modeling method which included denitrification, sediment burial and oyster harvest to determine annual nitrogen removal from the Mobile Bay, Alabama estimated the Eastern oyster reefs removed 34,911 ±5,032 kg N per year.

National Marine Fisheries Service Office of Science and Technology Fisheries of the United States 2018 Current Fishery Statistics No. 2018 report the value of U.S. commercial landings in 2018 are at $5.6 billion (National Marine Fisheries Service, 2020). In 2017, the report notes the US aquaculture production was valued at $1.47 billion with oysters representing 36.5 million pounds of the estimated 625.7 million pounds of total aquaculture production, valued at $186 million.

Although oyster and other bivalve aquaculture show a strong ability to remove nutrient from coastal waters that can lead to eutrophic conditions, local residents and businesses often are against such operations within sight of their locations (Rice, 2001). In spite of this resistance, oyster aquaculture is on the increase. In the Chesapeake Bay, the establishment of oyster hatcheries support oyster aquaculture growth (T. R. Williamson, 2014, T. R. Williamson et al., 2015). Hatcheries such as the Aquaculture and Restoration Ecology Laboratory at the Horn Point facility of the University of Maryland's Center of Environmental Science (2003), as well as the Aquaculture Genetics and Breeding Technology Center of the Virginia Institute of Maine Science (1998), are instrumental in seed production and strain development for increased oyster aquaculture in the region (T. R. Williamson et al., 2015). Long standing aquaculture regulations in Maryland restricted the development of oyster aquaculture in the Chesapeake Bay, (Parker et al., 2020). The revision of the states oyster's regulations occurred in 2009 to encourage oyster aquaculture along with new financing options for oyster aquaculture development through the Maryland Agriculture and Resource Based Industry Development Corporation (Parker et al.,

2020). Because of these two initiatives, the Maryland harvest of oyster increased from 3,340 bushels in 2012 to in excess of 74,000 bushels in 2018 (Parker et al., 2020).

Additional drawbacks exist to the current productions methods use for oysters. Exposure to diseases and parasites is a major concern. The impact of oyster diseases concerns all life stages, larvae, juveniles and adults (Barbosa Solomieu et al., 2015). The oyster disease Demo (*Perkinsus marinus*) results in significant decreases in growth rate of infected Eastern oysters and produce higher mortality in the second year of growth, than the first (Paynter & Burreson, 1991). In addition, oysters show greater growth in higher salinity, though the prevalence of *P. marinus* infected oyster is greater as well posing a problem in site selection, better growth vs less *P. marinus* infection (Paynter & Burreson, 1991). An introduction of parasites, bacterial and viral infections to larval and juvenile oyster populations in hatcheries can cause significant losses and once establish in a facility require persistent effort to remove or control (Elston et al., 2008). Barbosa Solomieu et al. (2015) report of Pacific oyster (*Crassostrea gigas*) mortalities occurring worldwide between 2008 to 2012 from numerous pathogen with the predominance of ostreid herpesvirus (OsHV-1) and *Vibrio sp*. implicating environmental stressors as a attributing factor on the oyster and pathogens. An issue that commercial oyster farmers, as well as other stakeholders, are concerned with is the illegal harvest of oysters (Bashore et al., 2012). In a review of citations issued between 1959 to 2010, shows that 73.9% of the citations were for surpassing catch limits, undersized or unculled oysters (Bashore et

al., 2012). Holding oysters in secured recirculation systems, as opposed to bottom culture in leased beds, would likely lessen the risk of theft. Land based location of shellfish operations may be a more acceptable solution.

Hurricane Katrina in 2005, the Deep Water Horizon oil spill in 2010 and freshwater influx from the Bonnet Carre Spillway in 2011 have decimated the Mississippi oyster industry leading the Mississippi Governors Oyster Council to choose an inland location to rebuild their hatchery facility, (Walker, 2017). Using a RAS system with artificial saltwater, the facility proved effective in producing 500 million eyed-larvae for cage culture operations on the coast (Walker, 2017).

Using oysters in land based or novel ways beyond the historical culturing practices is gaining acceptance in the industry. As an example, in a traditional Hawaiian Moli'I fishpond on Kualoa Farm in Oahu, growing triploid Pacific Oysters to manage algae growth as well as to produce a marketable product occurs (De Guzman, 2020). However, not a RAS, the pond is an isolated enclosure and the farm does use depuration tanks with artificial saltwater to clean oysters for 48 hours before being sold (De Guzman, 2020). Gortan (2021) reports of an Australian oyster company, SmartOyster, which holds live oysters in chilled and filtered tanks after harvesting from cages in the field (Gortan, 2021). The advantage is the oysters retain their good quality, they can harvest from the field when conditions are good, the company has stock ready for sales, reduces revenue losses and reduces the workload (Gortan, 2021). The use of live oyster holding systems, referred to as wet storage, is a known method to maintain harvested stock and prevent contamination due to harmful algae

blooms and harvest closures, though currently with limited use in commercial operations (Buzin et al., 2015). Development of permitting procedures, methodologies and implementation plans have occurred by the Washington State Department of Health for Onshore Wet Storage of Oysters (www.doh.wa.gov/CommunityandEnvironment/Shellfish/CommercialShellfish) and the New South Wales Food Authority in Australia (Food Safety Program for wet storage of shellfish, foodauthority.nsw.gov.au 2020). In France, Buzin et al. (2015), working on an oyster-wet storage RAS showed that *Crassostrea gigas* in a wet storage system provided sufficient nitrifying capacity on their shells to counter excretions of total ammonia nitrogen production. Use of the wet storage method is a way to limit environmental stressors on oyster, keeping them in good condition for sell up to 5 weeks periods (Buzin et al., 2015).

As an example of a flow-through integrated treatment of effluent, a finfish operation (Southern Flounder *Paralichthys lethostigma* at University North Carolina Wrightsville Aquaculture facility Aquaculture Facility in Wrightsville Beach) directed wastewaters to an upwelling culture system holding Eastern Oyster (*Crassostrea virginia*) for a 4 week period in an effort to decrease nutrient releases (Myers, 2006). Though the idea is promising, this work showed no reductions in effluent nutrients, nor growth in the oysters (Myers, 2006).

Wang (2003) from the University of Hawaii developed a design for a shrimp/algae/oyster recirculating integrated system, which relies on the effluent waste of the shrimp production component to generate algae that in turn feeds the oysters or

another algae consuming animal such as bivalve seed or artemia. The design suggests moving away from bacterial based treatment components as in traditional RAS systems to algae based design, which use system nutrients as a resource for additional production (J. K. Wang, 2003).

Qiu et al. (2017) constructed and demonstrated a successful oyster larval (*Crassostrea angulata*) culture system taking into account oyster swimming rates, maintenance, suitable algae species for culture and water quality. Confined culture of oyster larval certainly requires significant labor and oversight of the setup, but does provide greater control and stability over the culture conditions leading to improved larval production.

Fish Information & Services Worldnews reported in 2016 of the startup of the first completely land-based oyster farm in the Netherlands. The reason for developing the new enterprise according to the owners is to control all aspects of the oyster culture process, as well as ensure protection from the Ostreid herpesvirus-1, which is a serious disease effecting spat and juvenile oysters throughout Europe and elsewhere (Fuhrmann et al., 2016).

The consideration of land-based marine aquaculture systems incorporating more than one species for efficient operation and environmental concerns is occurring (Bunting & Shpigel, 2009). Looking at this integrational aquaculture approach from an economic perspective Bunting & Shpigel (2009) showed a worthwhile internal rate of return over ten years when growing a premium product and certain capital and labor considerations are met for an system including seabass (*Dicentrarchus labrax*),

microalgae, *C. gigas* and clams (*Ruditapes philippinarum*). Bunting & Shpigel (2009) pose the idea of adding oyster and clam components to existing fish operations may a useful approach in embracing integration of aquaculture systems. Another potential consideration of land-based oyster use is that of reduction of total suspended solids (TSS) in the effluent of marine RAS operations (Y. Zhou et al., 2014). Zhou et al. (2014) exploited the filter feeding behavior of the Pacific oyster (*Crassostrea gigas*) and the Blue mussel (*Mytilus galloprovincialis*) to reduce the TSS in effluent flows from a marine RAS system. The results show TSS reductions of 21.3 ±3.7% and 14.1 ±2.6%, for the oysters and mussels respectively (Y. Zhou et al., 2014). The inclusion of filter feeding organisms in treating of the marine aquaculture effluent have a positive effect in reducing organic matter, nitrogen and phosphorus nutrients (Y. Zhou et al., 2014). The use of oysters as a component in integrated multi-trophic aquaculture systems (IMTA) have occurred with limited success (Li et al., 2019). Use of small and large juvenile Pacific oysters (*Crassostrea gigas*) in a IMTA system showed survival, consumption of algae, but lacked growth resulting from supposed stress and/or lack of sufficient nutrients (Li et al., 2019). Culture and use of oysters in RAS and IMTA appear possible, though additional work on the needs of the inhabitants and balance of nutrients throughout the systems needs attention (Li et al., 2019)(Yeo et al., 2004).

As the impetus to study the effect of oolitic aragonite sand on oyster growth rates started with an unexpected, anecdotal observation in an oyster holding system. At the Institute of Marine and Environmental Technology's Aquaculture Research Center,

Eastern Oysters housed in a small recirculation system for a principal investigator at the Institute. In an effort to maintain a stable pH and alkalinity levels within the system, the installation of a fluidized sand filter holding oolitic aragonite sand occurred. The filter did maintain the water quality parameters as intended; however, the husbandry staff noticed shell growth on the edges of most of oysters in the system. The investigator and the owner of a local commercial oyster farm noted the growth was similar to that seen during the summer time on the farm. With the use of the oolitic aragonite sand in the oyster culture tanks, the expectation would be increased growth as opposed to tanks without a calcium carbonate sand. This observation triggered the current work in this paper.

With the understanding of the importance of oyster culture, seeing the efforts being applied to RAS systems and considering our experiences in oyster husbandry, the use of oolitic aragonite sand to stabilize water quality and possibly improve growth performance in Pacific and Eastern oysters housed in RAS systems is an intriguing idea. The experimental objectives of this work is to 1) determine if oysters are able to survive and show growth in a simple RAS, 2) determine if culture with oolitic aragonite sand has a positive effect on growth and 3) determine if these potential improvements occur with both the Eastern and Pacific oysters.

5.3 Methods

5.3.1 Oyster Sources

Hatchery-produced diploid juvenile Eastern oysters (*Crassostrea virginica*) (average length ± standard deviation, 36.6 ±5.2mm) were provided by True Chesapeake Oyster Company from a nursery at St. Mary's Creek, MD (38º07'07"N, 76º20'47"W). Similarly hatchery-produced diploid juvenile Pacific oysters (*Crassostrea gigas*) (27.6 ±5.9mm) were provided by the Hog Island Oyster Company from a nursery at Eureka, California (40º47'38"N, 124º11'33"W). Initially, stocking of oysters in two separate holding tanks occurred, according to species, upon arrival for acclimation to facility conditions. The acclimation systems maintained salinity at 31.9 ±1.9ppt. The receiving temperature for the oyster was 11°C, which gradual increased to 20°C in 14 days. Placement of oysters on a mesh platform kept them off the bottom.

Oysters of both species were randomly sorted to control and treatment tanks. Each tank held 50 Eastern Oysters and 50 Pacific Oysters. The tanks used an egg crate platform positioned approximately 6 cm below the 50% volume level height with a raised divider separating both species.

The experiment ran from January 9, 2019 through July 9, 2019 for 182 days. Mortalities were oysters with open shells. Determination of survival in each tank at the conclusion of the study occurred with the following calculation:

> Percentage survival = ((Starting oyster count - ending oyster count)/Starting oyster count) x 100.

At the end of the study, all oysters were bagged per control or treatment tank and refrigerated for dry weight analysis of shucked meats

5.3.2 System

A group of six cone-bottomed, cylindrical fiberglass tanks (Gemini Fiberglass Inc. model # CBT-50, 24" diameter x 30" height with 1-1/2" bottom drain and 30° cone angle) each filled with 160 liters of 30 ppt. artificial saltwater produced according to in-house specifications (Patent WO 02/074076) was used for the experiment (Figure 5.1). The arrangement used triplicate tanks for the controls and the experimental tanks (Figure 5.2).

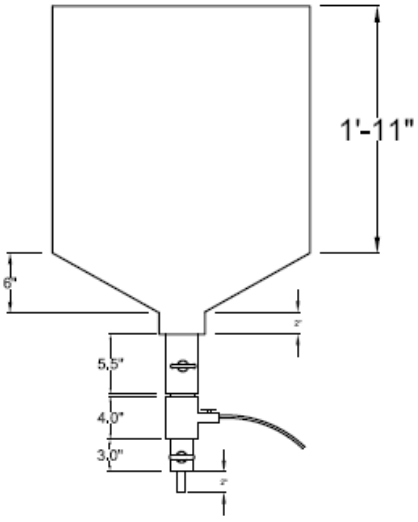


Figure 5.1 A single fiberglass tank with bottom drain valve, connection to pump and main shutoff.

Placement of the solid standpipe in the bottom tank drain aided in control of partial water changes. Weekly water exchanges occurred by opening drain valve, draining 50% of the water due to the height of the standpipe, then refilling from a reservoir tank filled with the 30 ppt. water at ambient temperature. Checking for mortalities occurred daily at feedings and weekly during the water exchange. An open shell, and lack of closure once touched indicated morbid animals. Positioning of an aeration diffuser at the center bottom of each tank created a moderate flow within the tanks.

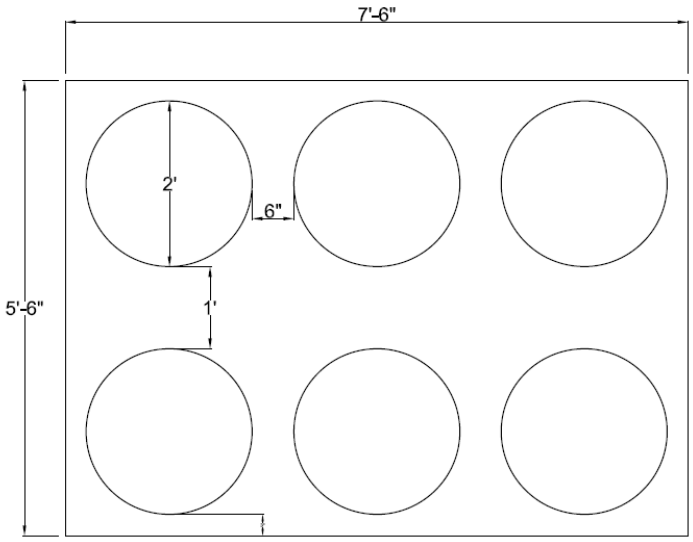


Figure 5.2 Layout of three control tanks using a fluidized sand filter with silica sand and three-treatment tank using oolitic aragonite sand.

Fabrication of fluidized sand filters (FSF) occurred from clear schedule 40 PVC pipe (73 mm in diameter x 610 mm long, inside diameter of 62.1 mm) for each tank (Figure 5.3). Placement of a coupling on the lower end of the filter, fitted with a solid PVC circular piece inserted into one end made it watertight. The upper end of the filter used a female adapter and plug. Installation of a ½" barbed fitting on the side of the filter just above the PVC solid section was the water inlet. Placement of a ½" barbed fitting on the upper end of the filter functioned as the outlet of the filter. A disc cut from blue (high density) Matala® filter media pads (Pentair, item # FM98) of

approximately 38.1 mm thick was placed above the location of the bottom ½" barbed fitting where it entered the filter, as a false bottom. On top of the filter media disc, 100 ml of inert aquarium gravel that passed through a #6 sieve (3360 µm open space) and was retained on a #8 sieve (2380 µm open space) was placed. The gravel had a density of 1.61 kg/l. Use of the gravel allowed distribution of the inlet water evenly across the cross-sectional area of the filter and prevented the sand from blocking the inlet line. On top of the gravel, the addition of 1000 grams of either unused oolitic aragonite sand (Sandy Cay Development Company Ltd, Nassau Bahamas), density 1.66 kg/l for the treatment tanks or Sakrete® Natural Play sand 1.48 kg/l density (Model # 40100301) for the control tanks occurred.

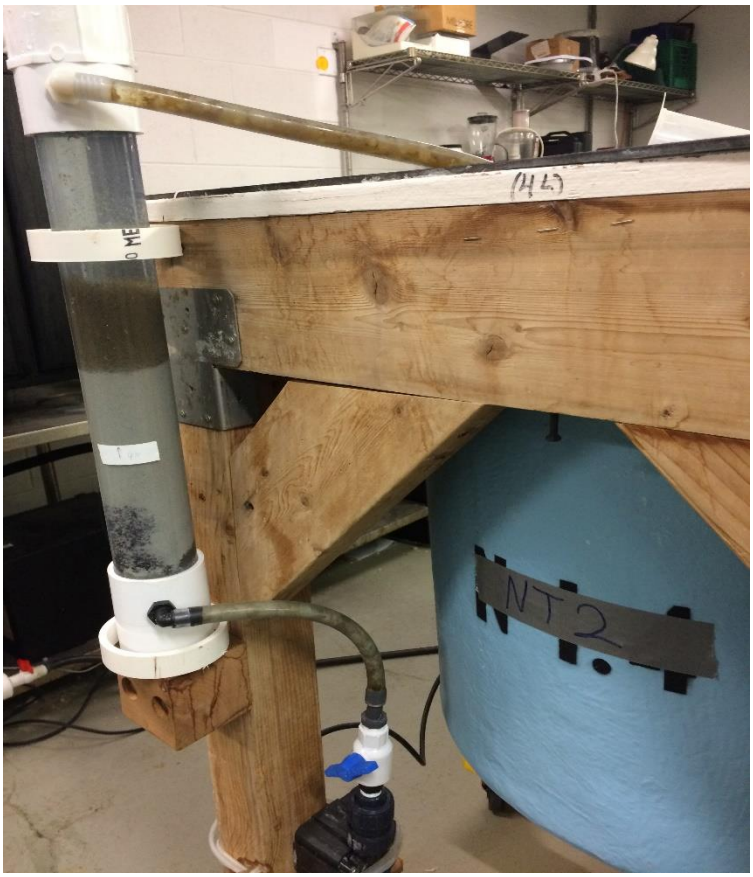


Figure 5.3 Image of standard layout for control and experimental tanks for oyster culture. Note fluidized filter filled with oolitic aragonite sand, pump intake at filter bottom and filter outlet at top

Use of an external pump (Supreme Aqua-Mag Magnetic Drive Pumps by Danner Manufacturing, Inc. models 2, 3 and 5) occurred to circulate tank water from the bottom center drain of the tank through the FSF. A control and check valves on the outlet of the pump allowed for flows adjustments. Recirculation flow rates were

determined by timing the length of time it took fill a 2-liter beaker. Flows were calculated according to the formula, Flow Rate, lpm = (volume in liters collected in beaker/ time in seconds) x 60. Periodic check and adjustment of flows to confirm equal rates occurred. Initially, the flow rate through the fluidized sand filter was set at 4.0 lpm resulting in a cross-sectional velocity in the fluidized sand filter of 2.2 cm/s.

During the experiments, monitoring of the sand level in the FSF occurred. The starting height of the sand in each filter was marked after the sand was original added. On day 182, recheck of sand heights occurred.

No active temperature control occurred with the tanks, allowing them to reach ambient temperature due to the room temperature. Temperatures were recorded daily using and an alcohol thermometer from day 1 until day 49. After day 49, the use of a Helect Infrared Thermometer (-50°C - 550°C range, Model Number H1020) occurred. Siphoning of solids from the tank bottom occurred during weekly water change.

5.3.3 Oyster Husbandry and Water Quality

The feeding recommendations by Reed Mariculture, Inc. in the document *Using Shellfish Diet® and other Instant Algae® 1800 Feeds*, suggests a rate of 0.03 – 0.06 ml Shellfish Diet per gram of wet meat weight per day for conditioning of brood stock. Using the manufactures rate, measured wet meat weights of sampled oysters and the count with each tank, the rate of feed should be 1.2 to 2.4 mls per tank per

day. The set daily feeding rate of six mls per control and treatment tanks were 5 to 2.5 times beyond that recommended rates for brood stock, ensuring sufficient algae to maintain biological growth. During the acclimation period, oysters were fed with the Reed Mariculture® Shellfish Diet 1800®, 20 mls per daily feeding. With all feedings, powering off the circulation pumps for a minimum of 1 hour occurred to allow the oysters to feed ad libitum, after that time circulation resumed.

Weekly water samples from each treatment and control tank occurred for testing of salinity, pH, total ammonia nitrogen, nitrite and alkalinity by the ZooQuatic Laboratory LLC. The laboratory referenced the following methods from the manual *Standard Methods for the Examination of Water and Wastewater, 21st Edition* to analyze the parameters (2510, 4500- H+, 4500- NH_3, 4500- NO_3, and 2320, respectively). The control and treatment tanks were sampled for ion concentrations, only once on day 7 of the experiment for: Li+, Na+, MG++, K+, Ca++, Sr++, F-, Cl-, Br- and SO_4-. Ion samples were analyzed by the Molecular Characterization and Analysis Complex laboratory at the University of Maryland Baltimore County using method 4110 B from the manual *Standard Methods for the Examination of Water and Wastewater*. The University facility analyzed ion concentrations by suppression conductivity on a Dionex Ion chromatography unit.

In order to verify the oysters were consuming the algae, confirmation occurred by fluorometer measurements. A fluorometer measures chlorophyll levels in samples, which is an indirect measurement of the presence of algae. By measuring chlorophyll (algae) from the point of algae addition to the tanks at feeding, the decrease in

measurements indicates the loss of algae from the water column and therefore a decrease in algae. Even though the circulation pumps in the tanks were stopped during feeding, moderate aeration remained, providing mixing and suspension of the algae partials.

Fluorometer readings were taken five times using Turner Designs AquaFluor® Handheld Fluorometer and Turbidimeter (P/N: 998-0851 Revision 2.0) to determine algal suspension and oyster uptake. Before the addition of feed, fluorometer readings occurred in each tank. Zeroing of the meter occurred by subtraction of this initial value from those taken. Readings started with the addition of the algae feed with readings occurring at 10-minute intervals. Subtraction of the zero reading from the measured reading occurred to determine algal suspension level in tank.

5.3.4 Oyster Measurements

At tank stocking, the weighing of blotted dried oysters occurred to the nearest 0.001 g. Measurements of the height, width and length occurred by Vernier calipers to the nearest 0.1 mm. At the conclusion of the study, weight and shell measurements with all live oysters occurred as at the beginning, as well as wet and dry shucked meats. Dry weight analysis of shucked oysters meats occurred by blotting dry on a paper towel, weighing to the nearest 0.0001 grams and drying in a 105°C drying oven for a minimum of 24 hours. Placement of the dried shucked meats in a desiccator occurred after drying for cooling to ambient temperature. Triplicate weightings of the samples occurred on a Mettler AM100 scale. The samples were returned to the desiccator

between weighing's. Averages of the three weighs determined the averaged dry weight of the sample.

5.3.5 Statistical Analysis

Use of the statistical program R-Studio (version 3.6.2) occurred to compare means between replicates and treatments in all three studies using one-way ANOVA. Recording of the level of significance occurred for each comparison. Where ANOVA differences existed, application of a Tukey, post-hoc test, occurred to elucidate were the differences occurred. Calculated averages include ± standard deviation where appropriate. Calculating standard deviations used the STDEV.S formula in EXCEL.

5.4 Results

The initial flow rate and sand expansion proved to be too strong and resulted in gradual removal of sand from the filters of all six tanks. On day 24, notice of sand accumulating on all the tank bottoms occurred during siphoning of the solids. Adjustment of flow rates to a rate of 3.5 lpm (velocity of 1.9 cm/s) occurred to curtail sand losses. Additional sand was added to each of the tanks 111.7g, 63.8g, 114.2 g and 98.6g, 114.3g, 155.2g to the control tanks and treatment tanks number's 1,2 and 3, respectively. From this point forward, flow rates remained at 3.5 lpm. No significant differences occurred between flow rates, ANOVA [$F(5,222) = 0.1396$, $p = 0.98$].

The loss of sand in the tank 1 on day 170 lead to the replacement of the Matala® filter material due to clogging. Return of the siphoned and rinsed loss sand to the FSF occurred.

At the completion of study, recording of sand levels occurred (Table 5-1). Control tank 1 loss 10.7% of its sand with no losses in control tanks 2 and 3. As noted, control tank 1 experienced sand loss twelve days previously. All treatments tanks showed sand losses, 1.6%, 4.8% and 20.0% for tanks 1, 2 and 3, respectively. The loss of oolitic aragonite sand may be due to channeling like control tank 1 experienced or a loss of the finer grained partials of the sand mix. The sand loss appears not to be due to sand densities, as the OAS density is greater than the silica play sand.

Table 5-1 Measured and calculate loss of sand from fluidized sand filters for control tanks (C) and treatment tanks (T) at completion of Eastern and Pacific Oyster Growth Experiment

Tanks =	C1	C2	C3	T1	T2	T3
Decrease in Sand Height, mm	23.8	0	0	3.2	9.5	39.7
Volume of sand loss, milliliters	72.1	0.0	0.0	9.6	28.8	120.2

During the study, feeding occurred on 161 days out of the 182-day duration of the experiment.

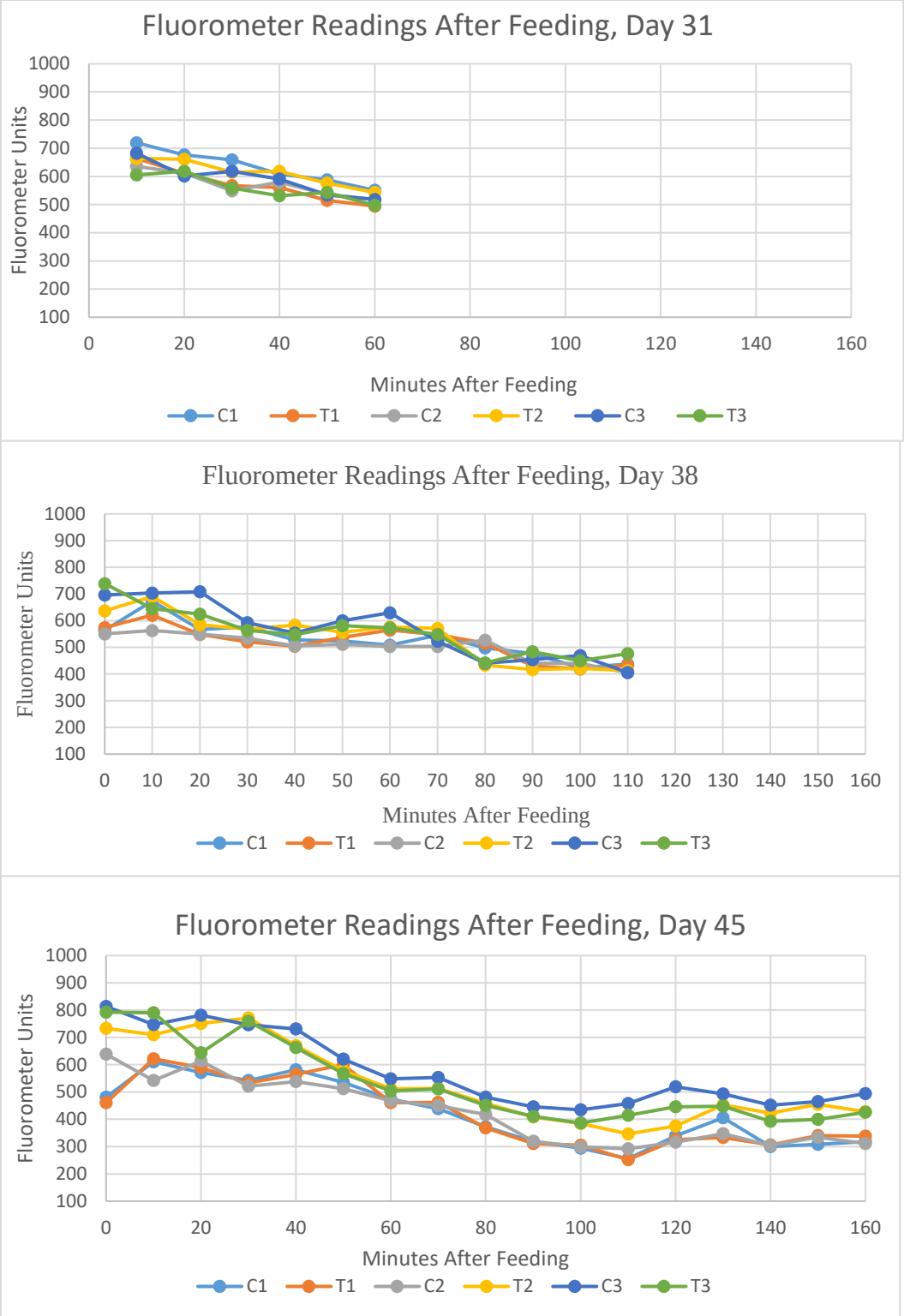


Figure 5.4 Fluorometer measurement taken after feeding for days 31, 38, and 45

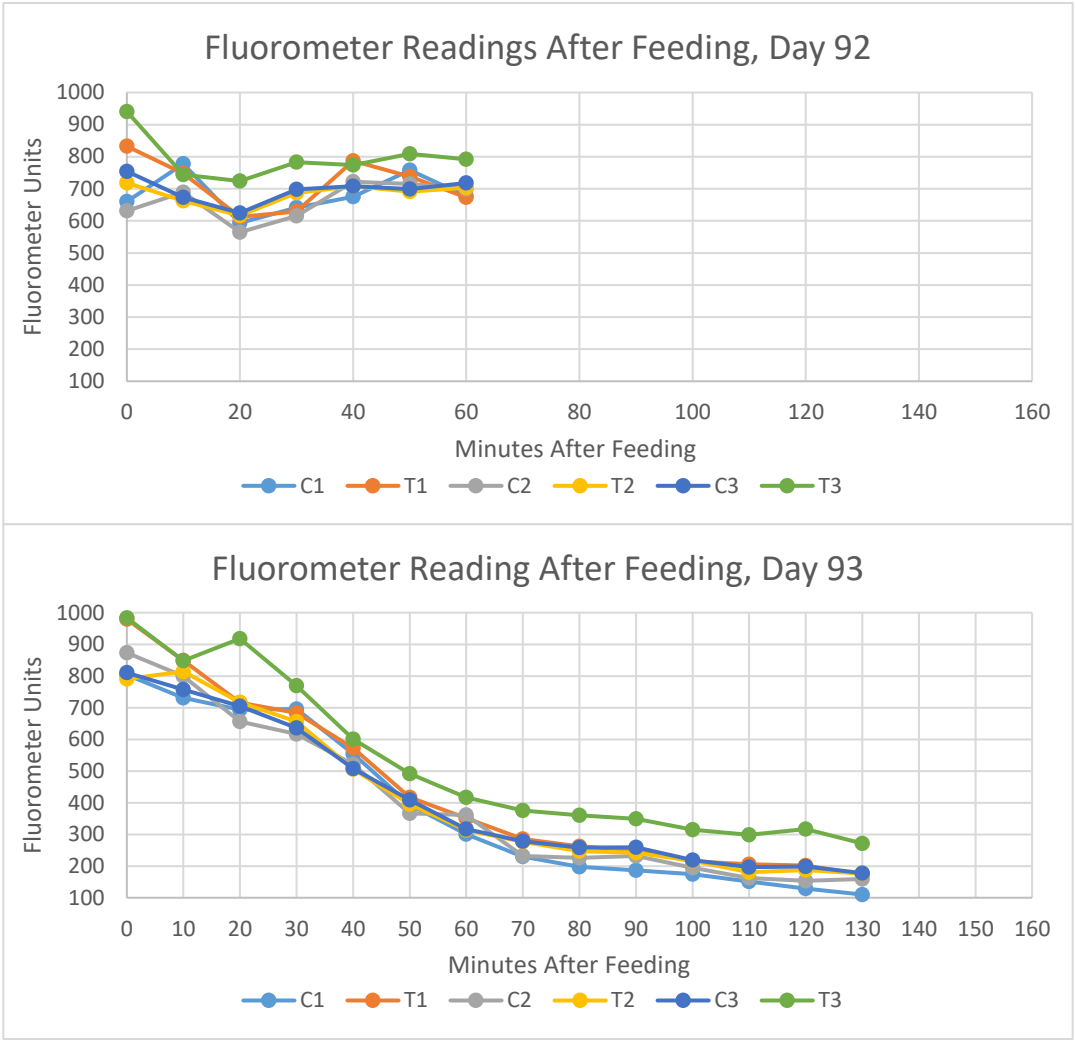


Figure 5.5 Fluorometer measurement taken after feeding for days 92 and 93

Timed fluorometer readings occurred on days 31, 38, 45, 92 and 93 (Figure 5.4, Figure 5.5). The timed periods ranged from 60 to 160 minutes. All fluorometer graphs reveal declining values from the starting measurements (Figure 5.4, Figure 5.5). Measurements on day 92 only showed a decline for the first 20 minutes followed by an initial rise in readings with the last 20 minutes reasonably stable. The average last readings of day 92 was 710.4 fluorometer units, which was the highest

ending readings of all five testing days. The graph profile from day 92 is different from the other four test days. There is no outstanding reason for this variation, though the difference occurred within all six tanks. The other testing days showed the last average fluorometer readings of 520.3, 428.5, 385.8 and 178.1, for days 31, 38, 45 and 93, respectively. The three long timed periods (day 38- 110 minutes, day 45 – 160 minutes, day 93 – 130 minutes) showed declining values ending between 100 - 120 minutes. After this period, the readings remained stable. This is a possible indication the rate of feeding decreased or stopped after about 2 hours. Significant differences exist between the five sets of fluorometer readings according to an ANOVA [$F(5,330) = 2.32$, $p < 0.05$]. However, a Tukey test of the data show all p-values of all pair-wise comparisons to be above >0.05. The steady decline in fluorometer readings indicates active oyster filtering of the water column.

5.4.1 Comparison of water quality parameters between tanks and treatments

The salinity values in all six experimental tanks shows a slight variation over the first 30 days followed by decreased variability after this point. The tanks received a weekly 50% water change using replacement water from a common reservoir. Any variation in the tank salinities is likely due to variations in the replacement water reservoir. Salinities averaged 31.3 ± 1.8 ppt (Table 5-2). No significant salinity differences exist between the three control tanks and the three treatment tanks ANOVA [$F(5,153) = 0.737$, $p = 0.60$].

Table 5-2 Overall average ±standard deviation, minimum value and maximum values for salinity, pH, TAN, NO2, alkalinity and calcium measurements for three control tanks and three treatment tanks combined for Eastern and Pacific oyster study on effect of aragonite sand.

	Salinity, ppt	pH	TAN, µM	NO2, µM	Alkalinity, mM	Calcium, mM
Average =	31.3 ±1.8	8.22 ±0.21	14.02 ±27.22	13.85 ±29.83	3.77 ±0.52	7.35 ±0.76
Min. Values=	26.1	7.61	1.66	0.65	1.94	6.17
Max. Values=	34.7	8.43	179.62	259.10	5.22	8.74

No significant differences occur between pH values in all six experimental tanks ANOVA [F (5,153) =0.2589, p=0.93]. The pH values averaged 8.22 ± 0.21 for all tanks, (Table 5-2). All tanks showed a strong decline in pH over the first 15 days. The pH values over the first three weeks, as well as the whole time period show similar values in all tanks.

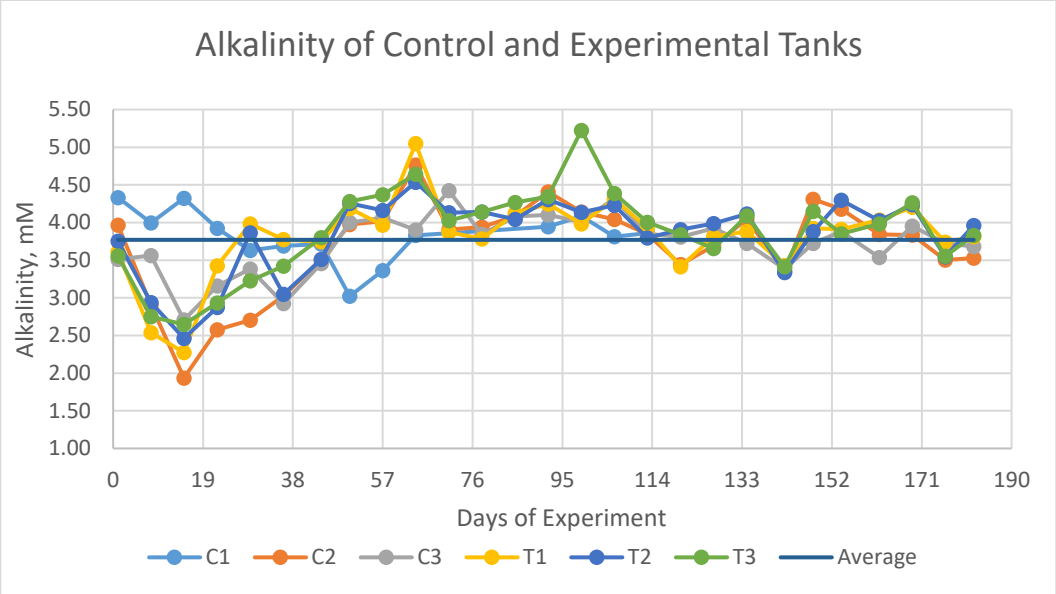


Figure 5.6 The alkalinity values from the control and treatment tanks during the culture of Eastern and Pacific oysters with and without oolitic aragonite sand.

The alkalinity values average 3.77 ±0.52 mM for all tanks over the course of the experiment (Table 5-2). Over the first 15 days, the alkalinities decreased in value

similar to the pH decline (Figure 5.6). This alkalinity drop affected all tanks strongly with the exception of control tank 1. The alkalinity in this control tank showed a gradual decline over a period of 50 days. The alkalinity in control tank 1 rose above 3.3 mM on day 57. All other tanks reached this point by day 44.

The similar timing and strength of the decreases in both pH and alkalinities graphs indicate a connection between the two events. There are two points, which the alkalinity levels seemed to spike for some tanks, day 64 and 99. On day 64, all tanks increased above 4.58 mM with the exception of control tanks 1 and 3, which remained near the overall average alkalinity. Then, on day 99 the alkalinity in treatment tank 3 showed a sharp increase to 5.22 mM, the highest reading measured. Even with the variation detected, no significant differences exist between the set of alkalinity values in all six experimental tanks ANOVA [$F(5,153) = 0.661$, $p = 0.65$]. The TAN concentrations averaged 14.02 ±27.22 µM (Table 5-2, Figure 5.7). Table 5-3 shows the day, tank and value of the TAN spikes. These spikes are 2.72 to 12.96 times the average of all the tanks. The total ammonium nitrogen concentrations during the experiments did not vary significantly between the control and treatment tanks ANOVA [$F(5,153) = 0.800$, $p = 0.55$].

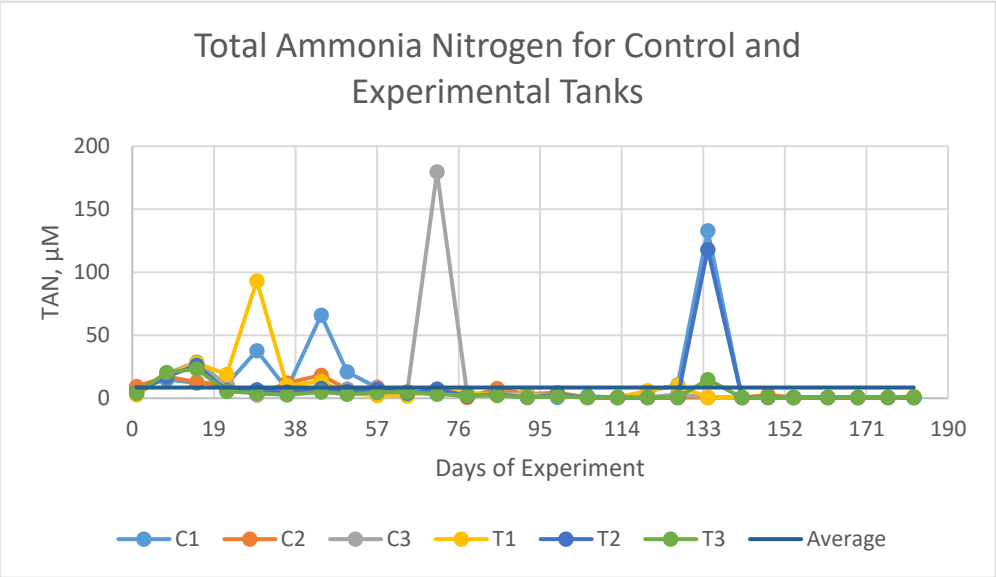


Figure 5.7 The total ammonia nitrogen (TAN) values from the control and treatment tanks during the culture of Eastern and Pacific oysters with and without oolitic aragonite sand.

Table 5-3 The day and concentration of increased TAN levels for the control and treatment tanks during trials with Eastern and Pacific oysters with and without oolitic aragonite sand.

Day	C1	C3	T1	T2
29	0.68	-	1.68	-
44	1.19	-	-	-
71	-	3.24	-	-
134	2.4	-	-	2.13

The nitrite values show substantial variation throughout the experiment with an overall average of 13.85 ±29.83 µM (Table 5-2). All tanks experienced one or more nitrite spikes during the experiment with a number of spikes matching tanks and days that TAN spikes occurred (Figure 5.8, Table 5-4). No significant differences exist between control and treatment tank for nitrite levels ANOVA [F (5,153) =0.474, p=0.80]. The observed nitrite and TAN spikes before and on day 71 are likely due to

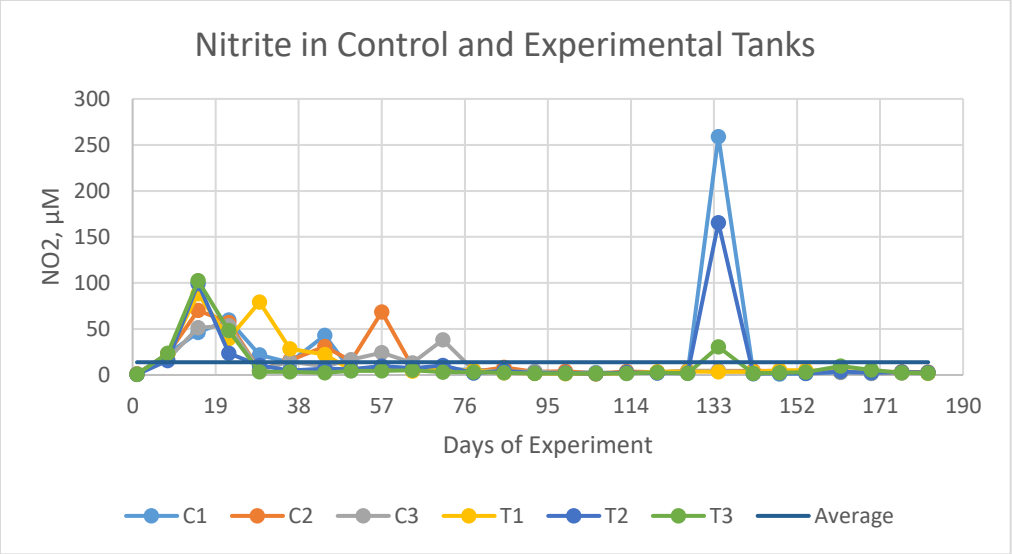


Figure 5.8 The nitrite values from the control and treatment tanks during the culture of Eastern and Pacific oysters with and without oolitic aragonite sand.

Table 5-4 The day and concentration of increased nitrite levels for the control and treatment tanks during trials with Eastern and Pacific oysters with and without oolitic aragonite sand. Data surrounded by a bold border indicate tanks and days of a corresponding TAN spike occurring.

Day	C1	C2	C3	T1	T2	T3
15	2.13	3.23	-	4.06	4.57	4.71
22	2.75	-	-	-	-	-
29	-	-	-	3.64	-	-
44	1.98	1.42	-	-	-	-
57	-	3.15	1.11	-	-	-
71	-	-	1.75	-	-	-
134	11.92	-	-	-	7.61	1.4

nitrogen cycling as mortalities remained low ranging from 0-7 dead per tank (≤7%).

The elevated nitrite and TAN levels on day 134 likely stem from oyster mortalities.

The mortalities in control tank 1, treatment tank 2 and 3 were 33%, 3% and 16%, respectively.

Both the TAN and nitrite concentrations varied widely during the first 50 days of the experiment. During this same period, the pH and alkalinity (Figure 5.6) show unexpected declines and recoveries. The speculation is the development of the biological filtration capacity in the tanks consumed alkalinity over the first four weeks resulting in a pH decline.

Testing of calcium levels occurred eight times during the experiment. Significant calcium level differences occur between control and treatment tanks ANOVA [$F(5, 42) = 5.552$, $p<0.05$]. A Tukey test shows control tank one is significantly different from treatment tanks one, two and three, $p<0.05$. Control tank one values trend above

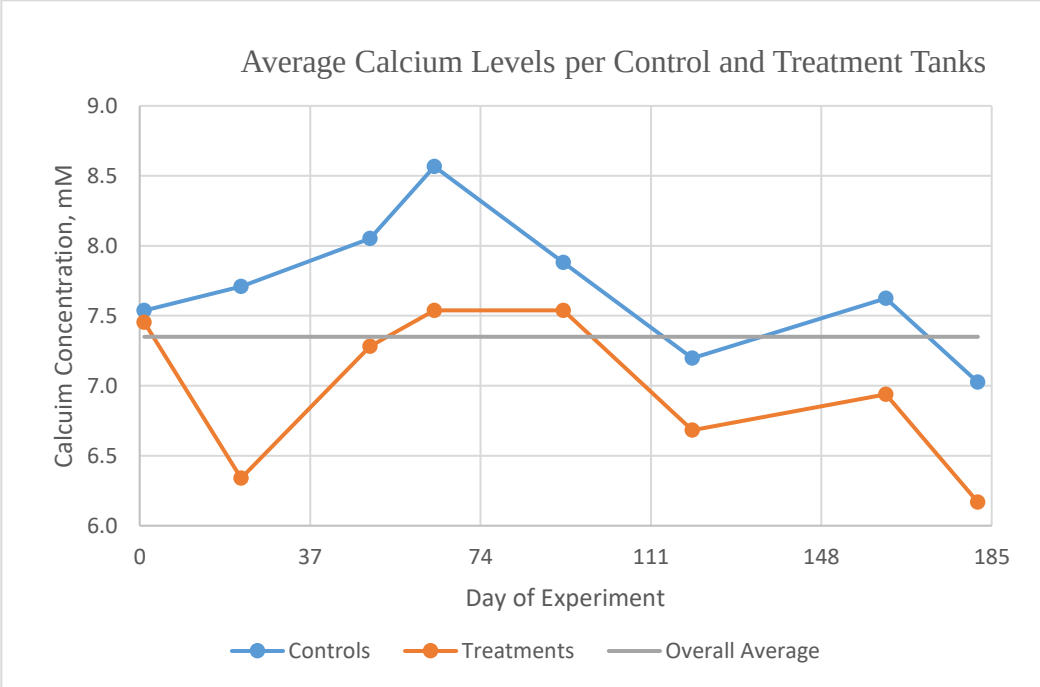


Figure 5.9 The average calcium concentration in the control and treatment tanks during the culture of Eastern and Pacific oysters with and without oolitic aragonite sand.

the overall average readings during the complete experiment. All other control to treatment tank comparisons show no significant differences, $p>0.19$. Even though no significant differences exist between the control groups and the treatment group, there does appear to be a trend with the data. In order to smooth out some on the variability in the calcium data, the three control tanks and the three treatment tanks were averaged (Figure 5.9). Using the overall average calcium value, 62.5% of the control tank readings occur above the average (Figure 5.9). Of the treatment tanks, 70.8% of their readings are below the average (Figure 5.9). No significant differences exist between the control tanks, $p>0.08$. The treatment tanks calcium levels have no significant differences, $p>0.50$. The control tank averages show an increase over the first 64 days, followed by a steady decline. The treatment tank averages have an initial drop in calcium with an increase to above average for days 64 and 92. After day 92, the average calcium levels decreased in treatment tanks. From day 64, both groupings started showing declines in the calcium levels.

Sampling for ion analysis occurred only on day 7 of the study (Table 5-5). Ions were similar between the control and treatments with the exception of strontium and bromine. Both of these ions were lower in the treatment tanks than the control tanks. The OAS in the treatment FSF possibly adsorbed the strontium and bromine. The ion measurements from the control and treatment tanks were slightly lower than is typically found in the saltwater supply for the facility. The slight decrease of values is likely due to salinity. The salinity in the control and treatment tanks averaged 31.3 ±1.8 ppt., whereas the salinity used in the facility ranges from 35 to 37 ppt.

Table 5-5 Ion analysis of control and treatment tanks on day 7 of Eastern and Pacific oyster aragonite study. Average of the three tanks in the control and treatment groups with standard deviation occurred to these values. The ions with a (*) beside them indicate a lack of overlap with value ±standard deviations between the control and treatment groups.

Tanks	Li^{+}	Na^{+}	Mg^{++}	K^{+}	Ca^{++}
Control	0.3 ±0.1	9218 ±343	991 ±64	305 ±25	323 ±19
Treatment	0.3 ±0	8596 ±685	908 ±64	294 ±29	292 ±17

	Sr^{++}*	F^{-}	Cl^{-}	Br^{-}*	$SO4^{-}$
Control	8.1 ±0.4	0.7 ±0.1	15650 ±543	58.1 ±2.3	2166 ±146
Treatment	7.2 ±0.4	0.5 ±0.1	15158 ±1284	52.3 ±1.3	2015 ±147

5.4.2 Oyster Survival

The Eastern oysters performed better in survival than the Pacific oysters. The survival rate of the Eastern oysters at the conclusion of the study ranges from 40% to 90% across both control and treatment tanks, with an overall average of 77%. The survival of the Pacific oysters ranged from 2% to 56%, with an average of 35% (Table 5-6).

Table 5-6 Percent survival in control and treatment tanks holding Eastern and Pacific Oysters.

	Control Tanks			Treatment Tanks			Species Average
Oysters	1	2	3	1	2	3	
Eastern	40%	88%	84%	90%	80%	80%	77%
Pacific	2%	56%	40%	42%	40%	32%	35%

Table 5-6 shows five Eastern oyster groups with survival of ≥ 80%. One tank of Pacific oysters had a survival of 56%. The remaining six tanks had survivals of less

than 50% (five Pacific oyster tanks and one Eastern Oysters). The survival between control tanks and treatment tanks per species shows no significant differences in either species ANOVA [$F(1, 4) = 0.648$, $p = 0.46$] and ANOVA [$F(1, 4) = 0.107$, $p = 0.76$] for Eastern and Pacific oysters respectively. The survival between the two species (grouping control and treatment tanks per species) is significantly different in the survival between the Eastern and Pacific oysters, ANOVA [$F(1, 10) = 15.486$, $p < 0.05$]. These findings overall reveal that the conditions of the experiments were more favorable to the Eastern Oysters, than the Pacific Oysters. However, the acclimation period and transportation stresses may play an important part on survivability.

5.4.3 Whole Wet Weights

The Eastern oysters starting wet weights show no significant differences with the initial subsample, the control and treatment tanks, ANOVA [$F(2, 315) = 0.749$, $p = 0.47$]. The wet weights of the Eastern oysters did increase between the initial subsample starting weights and the ending weights in both the control and treatment tanks. Eastern oysters increased in weight significantly by the end of the study for both the control and treatment tanks, ANOVA [$F(2, 546) = 17.343$, $p < 0.05$]. Pair-wise comparison in a Tukey test reveals no significant differences between the Eastern Oyster control and treatment tanks ending whole weights ($p = 0.85$). This finding would denote the OAS provided no measureable differences, than the silica sand with respect to weight increases. A significant difference does occur between

the starting whole weights and the ending weights of the control and treatment tanks, ($p<0.05$, for both). The increase in weight with both groupings suggests that culture conditions and feed supply was acceptable for growth with the Eastern oyster.

The Pacific oyster starting wet weights show no differences between the initial subsample, the control and treatment tanks ANOVA [$F(2, 315) = 0.562$, $p=0.57$]. The Pacific oyster show little change between the initial starting weights and the ending weights in the control and treatment tanks. The starting and the ending averages actually show a slight decrease in the ending weights, though no significant differences occur between the Pacific oyster starting whole weights and the ending weights in the control and treatment tanks ANOVA [$F(2, 421) = 0.822$, $p=0.44$].

5.4.4 Measurements: Length, Width, Height

5.4.5 Pacific Oysters – Starting Measurement Comparisons between Tanks

The Pacific oysters showed significant differences in the length between tanks from the start of the experiment, ANOVA [$F(5, 294) = 3.28$, $p<0.05$]. A Tukey test shows length comparisons between all Pacific Oyster tanks reveals only one significant difference, Control tank 1 and Treatment tank 2, $p<0.05$ (Table 5-7). All other Tukey

Table 5-7 The average ±standard deviation starting and ending shell length, width and height measurements (mm) for the Pacific oysters in control tanks (C) and treatment tanks (T).

	Length		Width		Height	
Tanks	Starting	Ending	Starting	Ending	Starting	Ending
C1	34.8 ±4.8	38.2 ±4.8	20.0 ±2.5	23.0 ±2.4	8.8 ±2.0	8.6 ±1.4
C2	36.9 ±4.9	39.3 ±5.0	21.9 ±2.3	24.0 ±2.6	9.3 ±1.5	10.7 ±1.9
C3	36.2 ±5.6	40.0 ±4.5	20.5 ±2.7	22.7 ±3.9	8.4 ±1.1	8.8 ±1.1
T1	36.5 ±4.8	39.1 ±4.3	21.9 ±2.1	24.0 ±1.9	8.7 ±1.3	8.8 ±1.2
T2	38.9 ±4.9	40.5 ±4.8	21.5 ±2.3	22.9 ±4.0	8.8 ±1.3	8.8 ±1.4
T3	36.5 ±5.6	39.0 ±4.7	21.2 ±2.5	23.3 ±2.4	8.3 ±1.4	8.6 ±1.4
Overall averages	36.6 ±5.1	39.3 ±4.7	21.2 ±2.4	23.3 ±2.9	8.7 ±1.5	9.1 ±1.4

pair-wise comparisons show no significant differences, $p>0.11$. Significant differences exist in the width of Pacific oyster shells from the start ANOVA [$F(5, 294) = 5.192$, $p<0.05$]. A Tukey test shows significant Pacific oyster width differences existed between tanks: Control 1: Control 2, $p<0.05$; Control 1: Treatment 1, $p<0.05$; Control 2: Control 3, $p<0.05$ (Table 5-8). The Pacific oysters show a significant shell height difference from the start of the study ANOVA [$F(5, 294) = 2.402$, $p<0.05$]. A Tukey test of the Pacific oyster heights from the starting

measurements show a significant difference exists between only two tanks: control tank 2 and treatment 3 ($p<0.05$).

5.4.6 Pacific Oyster - Comparison of starting and ending shell measurements

All tanks showed an apparent increase in shell lengths (Table *5-7*). Significant length differences do occur between starting and ending measurement in the Pacific oyster control and treatment tanks ANOVA [$F(3, 540) = 16.015$, $p<0.05$] (Table 5-7, Table 5-8).

Table 5-8 Significant differences in Pacific oyster length, width and height dimensions between comparisons of starting and ending measurements for Pacific Oysters. Significance determined by one-way ANOVA followed by pair wise comparisons by Tukey post-hoc test, $p<0.05$.

Length		Width		Height	
Starting Control	Ending Control	Starting Control	Ending Control	Starting Control	Ending Control
Starting Treatment	Ending Treatment	Starting Treatment	Ending Treatment		
Starting Control	Ending Treatment	Starting Control	Ending Treatment	Ending Control	Ending Treatment
Starting Treatment	Ending Control	Starting Treatment	Ending Control	Starting Treatment	Ending Control

The width of the Pacific oyster shells show increases from the starting measurements (Table 5-7). Significant differences occur between Pacific oyster shell widths between starting measurements and ending measurements ANOVA [$F(3, 540) = 30.936$, $p<0.05$]. Table 5-7 and Table 5-8 shows significant increases in shell length and width of Pacific oysters as determined by Tukey test on pair wise comparisons. The differences are the same for both length and widths (Table 5-8). Starting

measurements in length and width increased for the control and treatment tanks during the study.

Less change occur with the shell height between starting and ending measurements. Significant differences exist between Pacific oyster shell heights in the control and treatment tanks at the start and end of the experiment ANOVA [$F(3, 540) = 8.867$, $p<0.05$]. In Table 5-8, a Tukey test shows increases in the shell heights between starting control and final control tanks, but not with the treatment starting and ending measurements. The significant differences in shell height in the control tanks appears driven by control tank 2, which showed greater changes than the other two tanks.

5.4.7 Eastern Oysters– Starting Measurement Comparisons between Tanks

The randomly selected Eastern oysters showed starting lengths and widths variation from the beginning (Table 5-9). Significant differences existed in the length and width measurements of the Eastern oysters from the start of the experiment ANOVA

Table 5-9 The average ±standard deviation starting and ending shell length, width and height measurements (mm) for the Eastern oysters in control tanks (C) and treatment tanks (T).

	Length		Width		Height	
Tanks	Starting	Ending	Starting	Ending	Starting	Ending
C1	28.1 ±4.3	31.9 ±3.8	22.3 ±1.8	24.7 ±1.6	8.4 ±1.7	9.7 ±1.6
C2	28.7 ±5.2	32.8 ±5.7	21.9 ±2.2	27.1 ±3.5	9.2 ±1.9	11.9 ±2.0
C3	25.3 ±4.6	30.8 ±4.2	20.4 ±2.4	25.4 ±3.1	8.9 ±1.8	9.4 ±1.7
T1	30.0 ±6.9	33.4 ±7.3	22.3 ±2.8	25.6 ±3.0	9.1 ±1.6	9.9 ±1.4
T2	28.4 ±6.4	32.3 ±6.9	22.0 ±2.8	26.7 ±3.9	9.3 ±2.2	9.8 ±1.9
T3	25.0 ±5.9	32.0 ±7.2	19.3 ±2.6	24.7 ±2.3	8.9 ±1.7	9.5 ±1.7
Overall averages	27.6 ±5.6	32.2 ±5.8	21.4 ±2.4	25.7 ±2.9	9.0 ±1.8	10.0 ±1.7

[F (5, 301) =6.458, p<0.05], ANOVA [F (5, 301) =13.135, p<0.05], respectively. Using a Tukey test, the significant differences between control and treatment tanks with respect to length and width are displayed (Table 5-10). Treatment tanks 1 and 2 show longer lengths than Control tank 3 or Treatment tank 3. With the shell widths, Treatment tank 1 and 2 are longer than Control tanks 1 and 3. Treatment tank 3 shows smaller widths than Control tanks 1 and 2, as well as, Treatment tanks 1 and 2. No significant differences in the height of the Eastern oyster shells exists at the beginning of the experiment ANOVA [F (5, 294) =1.481, p=0.20] (Table 5-9).

Table 5-10 Significant differences in Eastern oyster length and width measurements between tanks at the start of the study. No significant differences occurred with the heights. Significance determined by one-way ANOVA followed by pair wise comparisons by Tukey post-hoc test, p<0.05.

Length		Width	
Treatment Tk 1	Control Tk 3	Treatment Tk 1	Control Tk 3
Treatment Tk 1	Treatment Tk 3	Treatment Tk 2	Control Tk 3
Treatment Tk 2	Treatment Tk 3	Treatment Tk 3	Control Tk 1
Control Tk 2	Control Tk 3	Treatment Tk 3	Control Tk 2
		Treatment Tk 3	Treatment Tk 1
		Treatment Tk 3	Treatment Tk 2

5.4.8 Eastern Oysters- Comparison of starting and ending shell measurements

The ending length, width and heights of Eastern oysters increased in all tanks from starting values (Table 5-9). The Eastern oysters showed significant shell length increases between the starting and ending measurements ANOVA [F (3,574) =29.633, p<0.05]. A Tukey test shows significant shell length differences in the Eastern Oysters (Table 5-11). The starting lengths for both the control and the treatment tanks increased by the end of the study. The width differences for the Eastern oysters increased between starting and ending measurements ANOVA [F

(3,574) =109.43, $p<0.05$]. A Tukey test showed no significant differences in shell width of the Eastern Oysters with the final measurement in the control tanks and treatment tanks ($p>0.72$). Significant differences do occur between starting and ending width measurement within both the control tanks and treatment tanks (Table 5-11).

Significant differences exist between starting and ending height measurements in both the control and treatment tanks ANOVA [F (3,567) =19.853, $p<0.05$]. A Tukey test shows significant differences exist in shell height between: starting and ending measurement for control tanks and treatment tanks for Eastern oysters (Table 5-11).

Table 5-11 Significant differences in Eastern oyster length, width and height measurements between comparisons of starting and ending measurements for Eastern Oysters. Significance determined by one-way ANOVA followed by pair wise comparisons by Tukey post-hoc test, $p<0.05$.

Length		Width		Height	
Starting Control	Ending Control	Starting Control	Ending Control	Starting Control	Ending Control
Starting Treatment	Ending Treatment	Starting Treatment	Ending Treatment	Starting Control	Ending Treatment
Starting Control	Ending Treatment	Starting Control	Ending Treatment	Ending Control	Ending Treatment
Starting Treatment	Ending Control	Starting Treatment	Ending Control	Starting Treatment	Ending Treatment

5.4.9 Shucked-Meat Wet Weight

Measurement of the starting wet and dry weights of the shucked meat occurred with an 18-oyster subsample of both species. The results indicated the average individual wet weights of the Eastern and Pacific Oyster were 0.2929 and 0.5079 grams, respectively (Table 5-12).

Table 5-12 Wet weight characteristics of shucked oyster meat from all the surviving individuals in control tanks (C, play sand in fluidized filter) and Treatment tanks (T, oolitic aragonite sand). The Initial column is initial wet weight of the shucked meats.

	Eastern Oysters						
	Initial	C1	C2	C3	T1	T2	T3
Average =	0.2929	0.2990	0.3171	0.2427	0.3304	0.3615	0.2762
Min.=	0.1346	0.1358	0.0678	0.0891	0.0900	0.1350	0.1138
Max.=	0.4710	0.5362	0.9193	0.9889	1.3201	2.0710	1.0893
n =	18	20	44	42	45	40	40

	Pacific Oysters						
	Initial	C1	C2	C3	T1	T2	T3
Average =	0.5079	0.0552	0.2005	0.2146	0.1699	0.1608	0.1893
Min.=	0.2206	0.0552	0.0448	0.0686	0.0644	0.0566	0.0798
Max.=	0.8704	0.0552	1.0498	0.5270	0.4912	0.2969	0.3158
n =	18	1	28	20	21	20	16

In Table 5-12, the listing of the values for the average, minimum and maximum shucked-meat weights occur for the surviving oysters at the completion of the experiment for both species. Statistical analysis results indicate no significant differences between the final wet weights of the shucked meat in all six Eastern oyster tanks, controls and treatment tanks, ANOVA [$F(5,225) = 0.795$, $p = 0.55$]. In addition, there is no significant differences between the initial wet weight of the shucked Eastern oyster meat with those of the controls and treatments groups [$F(2,246) = 1.414$, $p = 0.24$]. Averaging the shucked wet weights of the control tanks and the treatment tanks and comparing those values with the initial shucked wet weight values shows control tanks decreased by 2.3% and the treatment tanks increased by 21.9% (Table 5-13). The count of the initial subsample of oysters (n=18) may of reduced the strength of the comparison with the ending shucked wet weights. A

larger number in the initial subsample could have improved the statistical power of comparisons. The higher averaged shucked wet weights for the Eastern Oyster treatments is driven by outliers that are 4.0, 5.7 and 11.4 times the average wet weights for treatment tanks 1, 2 and 3 respectively (Table 5-12). The Eastern Oyster shuck wet weights show no differences between initial, control and treatment groupings.

The shucked wet weights of Pacific oysters show no significant difference between all tanks of the controls and treatment tanks, ANOVA [$F(5,100) = 0.565$, $p = 0.73$] (Table 5-12). Significant differences exists between the initial, control and treatment groupings of the Pacific oyster shucked meat wet weights ANOVA [$F(2,121) = 36.326$, $p < 0.05$]. A Tukey test of the shucked meat wet weights show no significant differences between the control and treatment groups of the Pacific oysters, ($p = 0.53$). The test does show significant differences between the initial wet weights with those of the treatment and the control group, $p < 0.05$ for both comparisons. The Pacific Oysters experienced strong percentage decreases in the average shucked wet weights of 69.1% and 65.9% for control and treatment tanks, respectively. This in a strong indicator that the culture conditions in the experimental tanks were not favorable for Pacific Oysters survival and growth.

Table 5-13 Average and percentage change from initial values for wet weight of shucked oyster meat from all the surviving Eastern and Pacific oyster individuals in control tanks and Treatment tanks. The Initial column is initial wet weight of the shucked meats. A negative sign indicates an increase.

	Initial	Control Tanks	Percent Decrease	Treatment Tanks	Percent Decrease
Eastern Oyster Average =	0.2929	0.2863	2.3%	0.3570	-21.9%
Pacific Oyster Average =	0.5079	0.156766	69.1%	0.1733	65.9%

5.4.10 Shucked Meat Dry Weights

Using the shucked dry weight data for the initial Eastern Oyster subsample (n=18) and the surviving oysters in the control and treatment tanks, a significant difference exist between weights, ANOVA [$F(2,246) = 72.883$, $p<0.05$]. A follow up Tukey test shows no differences between the control and treatment groups, but a significant difference does occur between the initial subsample and control tanks, as well as, the initial and treatment tanks, $p<0.05$for both comparisons (Table 5-14). The average dry weight values of the control and the treatment tanks are 65.9% and 64.7%, respectively, lower than the initial dry weights of the subsample, likely due to limited number of subsample. The average dry shucked weight values are much lower than the shucked wet weight values. The stronger decline in the dry weight percentages signals that the culture conditions of the Eastern oysters may not have been sufficient to enlist visceral growth, as suggested by whole wet weights.

Table 5-14 Dry weight characteristics of shucked oyster meat from all the surviving individuals in control tanks (C, play sand in fluidized filter) and Treatment tanks (T, oolitic aragonite sand). The Initial column is initial wet weight of the shucked meats. Note that one individual represents the information in the C1 Pacific Oysters tank.

Eastern Oysters

	Initial	C1	C2	C3	T1	T2	T3
Average =	0.0959	0.0327	0.0359	0.0294	0.0347	0.0339	0.0331
±Std Dev.	0.0287	0.0105	0.0197	0.0156	0.0194	0.0294	0.0205
Min.=	0.0498	0.0134	0.0049	0.0070	0.0079	0.0118	0.0127
Max.=	0.1502	0.0524	0.1097	0.1000	0.0962	0.1796	0.1156

Pacific Oysters

	Initial	C1	C2	C3	T1	T2	T3
Average =	0.0777	0.0042	0.0153	0.0153	0.0134	0.0113	0.0183
±Std Dev.	0.04036	n/a	0.01577	0.00778	0.00932	0.00512	0.0058
Min.=	0.0088	0.0042	0.0044	0.0047	0.0048	0.0050	0.0044
Max.=	0.1723	0.0042	0.0710	0.0371	0.0440	0.0233	0.0280

Significant differences of the shucked meat dry weights of the Pacific Oyster exist between the initial, control and treatment tanks ANOVA [$F(2,121) = 95.744$, $p<0.05$]. Tukey test shows significant differences between the initial shucked dry weight values and the control and treatment tanks, $p<0.05$ for both comparisons (Table 5-14). The test shows no differences between the control and the treatment tanks, $p=0.95$. The averaged control and treatment tanks are 85.1% and 81.6%, respectively, lower than the initial values.

5.5 Discussion

The Eastern and Pacific oysters responded dissimilarly to the conditions of the simple RAS arrangements. Although variations did occur with the water parameters, no detected statistically significant differences occurred with flow, fluorometer readings,

temperature, salinity, pH, alkalinity, TAN and nitrite between the control and treatment tanks.

Even though lacking significance, an explanation for the initial rapid pH drop and subsequent recovery to day 36 is not clear. Whatever the cause for such pH changes, it can be said that it effected each tank equally. In the current work, the oolitic aragonite sand does not show any indication of modifying the pH any more than the control tanks with silica sand.

The reasoning for the lower average calcium levels in the treatment tanks than the control tanks is unexpected. The use of the oolitic aragonite sand, a calcium carbonate compound, in the treatment tanks one would presume the calcium concentrations would be elevated above tanks containing only crystalline silica sand. The present data does not show reveal a significant increase of calcium in tank waters exposed to oolitic aragonite sand, contrary to expectations.

Lowe et al. (2017) report Eastern Oysters sized >75 mm in length experiences growth between 10 -30°C. Beyond this range, the growth rates decrease significantly. The reported optimal temperature of the Pacific Oyster is 19°C to 20°C (Cassis et al., 2011, Bougrier ' et al., 1995, Gray, 2016). Wiltshire (2007) reported Pacific Oyster larval have a temperature range from 20 to 30°C, with adults able to handle a wider range for short periods of time. The temperatures experienced in this study were at and above 19°C. The oysters in this worked experienced temperatures within the acceptable range of both species.

The large mortalities seen in the oysters, especially the Pacific oysters, could be a result of the temperatures in the culture waters. Even though the experimental temperatures were within the published ranges of both species, perhaps the source temperatures were lower and the acclimation period not adequately long enough for the oyster to adjust to the higher temperatures, adding stress. Increasing the acclimation period with adjusted temperature may have been more successful. With this consideration, using culture temperatures in the 14°C to 18°C range may have placed reduced stress on the oysters, resulting in a higher survival percentage.

The salinities were in the range of the oyster species. The Eastern Oyster preferred salinity range is 14-28 ppt., though it can tolerate salinities beyond this range, 5-40 ppt. (Shumway, 1996). Casas et al. (2018) suggest the ideal salinity for the species is between 15-25 ppt. when considering oxygen consumption and clearance rates. Pacific oysters have preferred range of 25 to 30 ppt., though they can handle a broader range (Wiltshire, 2007). The calcium measurements showed no significant differences existing within the control or treatment groups and between the control tanks and the treatment tanks. Although not significant over the 182 days of the project, the averaged calcium values for the control tanks trended above the overall average calcium concentration as compared to the treatment tanks, which ranged more below the average calcium value. Although the observation was not significant by the end of the experiment, the possible lower calcium levels in the treatment tank may be a result of uptake by oysters for shell growth. The water quality results are within ranges report from a RAS operation holding Eastern Oysters for alkalinity,

calcium, TAN, nitrite, pH and salinity (Kuhn et al., 2013). The TAN and nitrite spikes in this current work exceeded the measured maximums report by Kuhn et al. (2013). As a whole the water quality parameter are within ranges consider acceptable to both species of oysters.

The leveling of the fluorometer readings between 110 to 120 minutes indicate a conclusion of a feeding period by the oysters. Fluorometer readings indicate a feeding duration of about two hours is occurring, though pump circulation remained off for a minimum of one hour. Even though tank circulation restarted after approximately one hour, the lack of a solids filter would allow algae to remain present for feeding. Prolonging the feeding period to at least two hours, may have provided additional needed nutrients to the oysters to help in survival and growth. Though the calculated amount of algae added to the system was more than required, assuming sufficient feed was present for the oysters needs. The continued decline in fluorometer readings for 120 minutes implies the oysters did require additional algae due to this behavior. Unfortunately, no visual confirmation of this feeding behavior occurred. However, the fluorometer readings between the control and the treatment tanks were similar, inclusion of a tank without oysters as a control could have conduced if settlement of the algae was a factor in the declining fluorometer readings or not. If algal settlement was a factor, the reduction of the quantity of suspended algae may have provided insufficient feed impacting growth. Use of additional daily feeding periods, longer period or continuous feeding, may have provided better opportunity for the oysters to feed, resulting in better tissue growth.

In previous work with scallops, the use of continuous feeding proven to be successful for that species. The technique involved the use of an elevated reservoir tank filled with system water and a daily amount of the Reed Mariculture Shellfish Diet®. The aerated reservoir tank delivered a suspension to the culture tanks over a 24-hour period, allowing the bivalves the opportunity to feed continuously. Attributing poor tissue growth to the fed diet seems a plausible explanation. The use of suitable live algal species, along with the Shellfish Diet®, likely would have provided a better quality diet than a commercial diet alone. In addition, direct and repetitive observations of feeding oysters would assure algal removal from the water column occurred by the oysters. Adoption of this feeding method with the Eastern and Pacific oysters in the current work might have provided sufficient nutrients for increase somatic growth and survival in both species.

The survival of the Eastern Oyster is glaringly better than that of the Pacific Oysters. Eastern oysters had an average survival rate 64% higher than that of the Pacific oysters. The survival of the Eastern Oysters in the current work is low when compared to that reported by Kuhn et al (2013) with a survival of 99% with zero-water exchange in a RAS, though the system included biofiltration, a protein skimmer and a UV unit is sharply different in complexity. The used fluidized sand filters in the current study provided ample surface area for nitrification bacterial colonization. However, the inclusion of the life support equipment (protein skimming and UV disinfection) of Kuhn et al. (2013) may of improved conditions leading to greater survival.

In contrast, the Pacific oysters had survival rates of ≤56 percent in all tanks. Pacific oyster mortality of <1% occurred during a 35 day study of TAN concentrations in RAS representing a better outcome than the current study (Buzin et al., 2015), which ran much longer (182 days). The noted water parameter anomalies (alkalinity, TAN, nitrite, calcium) that may have contributed to the low survival of both species in control tank one. The unusual changes in this tank indicates a problem was occurring that produced unfavorable conditions for both species. The possibility that undetected dead oysters occurred in the control tank 1, effecting water quality, is a conceivable explanation.

The improvement of the life-support system, water exchange rate and oyster sampling might have afforded conditions for enhanced survival and growth. Relying on settlement of solids and weekly removal by siphoning might have added to the nitrogen load on the tanks due to the long holding time. Use of more active, daily solids removal methods, like a fine mesh filter bag or pad, would have provided increased solids removal. The weekly water exchanges might have been insufficient with the limited filtration utilized. The simple set up could have been more effective with increased water exchanges. With improved life-support, a weekly water exchange could have been adequate. The delay in finding and removing dead oyster most likely added to poorer water quality than would otherwise occur. Close observance of open shells during feeding would have helped detect dead oysters sooner. Sampling of the oysters only occurred at the start and the end of the experiment. Conducting regular subsampling of oysters every 2 – 3 weeks for

weights and measurements could have alerted if mortalities were occurring and increasing in frequency. As with the survival of the oysters, the weights and growth of the oysters differed between species. A lack of a measureable change between the starting weights and the ending weight points to probable unfavorable conditions for the growth of Pacific oysters.

In spite of the random selection and stocking of oysters of both species in the control and treatment tanks, significant differences occurred with the oysters at the beginning of the study. The Eastern oysters showed differences with the starting length and width of the shell, but not with the shell heights. The Pacific oyster had differences between tanks with the starting length, width and height measurements.

Comparison of the starting and ending shell measurements show differences. The Eastern oysters showed growth between starting and ending measurements in shell length, width and heights. The second control tank showed strong growth in shell height, likely driving the increases over the other two control tanks. These increases all were significant for the controls and the treatment groups. However, there were no significant differences in final sizes of all measurements between the control and treatment groups.

The Pacific oysters, like the Eastern oysters, showed increases in the shell length, width and height between the starting and ending measurements. The differences in length, width and height were significantly different for both the control and treatment groups. No significant differences occurred with the ending length and width comparisons between the control and the treatment groupings. The Pacific

oysters did show significant differences in ending shell height between the control and treatment groups. The control group showed a higher shell height than the treatment group. The difference appears driven by control tank number 2, which showed a strong growth in shell height as compared to all other tanks.

In all of the shell dimensions of both species showed increases between the starting measurements and the ending ones, which were also significant. The only significant difference between the control group and the treatment group was that of shell height in the Pacific Oyster. This difference showed that the control group increase was greater than the treatment increases.

The results showing growth in the shell dimensions in all groups, contrasts with that of the oyster meats. Comparisons of the shuck meats from the oysters (wet and dry weights) occurred between the initial subsample group, the control and treatment groupings for both species. The Eastern Oyster showed no significant differences in the wet shucked meat weights between tanks, nor between the initial subsample, control and treatment groupings. The Pacific Oysters also showed no significant differences between the tanks of the control and treatment tanks. A significant difference did occur between the initial subsample and both the control and treatment groupings of the Pacific Oysters. These differences represented a large loss of wet weight as compared to the initial subsample.

The dry shucked meat comparisons show no significant differences existed between the control and treatment groupings for both species. However, significant differences do occur between the initial subsamples and the control and treatment groupings for

both species. The control and treatment group's dry shuck meat weights of both species were lower than the initial subsample group. The dry weight percentage declines are lower than the wet weight declines, similar to that seen in the Eastern Oysters. These results further convey that the Pacific Oysters were in conditions outside their range for optimal growth. With both the Eastern and Pacific oysters, the shuck oyster meat (either wet or dry), decreased in weight or remained unchanged when compared to the initial subsample group. The oysters of both species showed increases in the length, width and height of their shells, while at the same time lost tissue weight both on a wet and on dry weight bases. Work on juvenile Eastern Oysters (3-5 mm shell height) showed negative effects with repeated exposure to the stressors of low oxygen levels and elevated temperatures (Donelan et al., 2021). The size of the Eastern Oysters in this study were larger and lacked exposure to these same stressors, but they did experience TAN and nitrite spikes, considered a stressor on the oysters. With this stress, the nitrogen spikes that incurred may be sufficient in magnitude to result in reduced somatic growth and limit shell development.

The results of this work do not demonstration any differences between the control tanks with silica sand or the treatment tanks using oolitic aragonite sand as substrates in a fluidized sand filter in comparisons for survival, whole weight, shell dimensions and shucked meat weights. The difference of shell height in the Pacific Oyster was significantly different between the control and treatment tanks, but the difference was greater height in the control tanks.

In addition, no definitive water quality differences between control and treatment groups with respective to temperature, salinity, pH, alkalinity, TAN, nitrite and calcium occurred. The survival rate between groupings also did not show any significant differences.

The conditions of the experimental tanks supported conditions allowing growth of the shell dimension for both species and significant whole weight growth for the Eastern oysters. On closer examination of the wet oyster tissue, shows a reduction in the Pacific oyster and remaining the same in the Eastern oysters. The dry shucked oyster meats show declines in both species. Based on the percentage survival of both species, the Eastern oyster performed better than the Pacific oyster.

Bibliography

Ahmed, N., Thompson, S., & Glaser, M. (2019). Global Aquaculture Productivity, Environmental Sustainability, and Climate Change Adaptability. *Environmental Management*, *63*(2), 159–172. https://doi.org/10.1007/s00267-018-1117-3

Alou, M. T., Ndongo, S., Frégère, L., Labas, N., Andrieu, C., Richez, M., Couderc, C., Baudoin, J. P., Abrahão, J., Brah, S., Diallo, A., Sokhna, C., Cassir, N., La Scola, B., Cadoret, F., & Raoult, D. (2018). Taxonogenomic description of four new Clostridium species isolated from human gut: 'Clostridium amazonitimonense', 'Clostridium merdae', 'Clostridium massilidielmoense' and 'Clostridium nigeriense.' *New Microbes and New Infections*, *21*, 128–139. https://doi.org/10.1016/j.nmni.2017.11.003

Badiola, M., Mendiola, D., & Bostock, J. (2012). Recirculating Aquaculture Systems (RAS) analysis: Main issues on management and future challenges. *Aquacultural Engineering*, *51*, 26–35. https://doi.org/10.1016/j.aquaeng.2012.07.004

Barbosa Solomieu, V., Renault, T., & Travers, M. A. (2015). Mass mortality in bivalves and the intricate case of the Pacific oyster, Crassostrea gigas. *Journal of Invertebrate Pathology*, *131*, 2–10. https://doi.org/10.1016/j.jip.2015.07.011

Bartelme, R. P., McLellan, S. L., & Newton, R. J. (2017). Freshwater recirculating aquaculture system operations drive biofilter bacterial community shifts around a stable nitrifying consortium of ammonia-oxidizing archaea and comammox

Nitrospira. *Frontiers in Microbiology*, *8*(JAN). https://doi.org/10.3389/fmicb.2017.00101

Bashore, C. J., Lane, H. A., Paynter, K. T., Naylor, M., Harding, J. R., & Love, D. C. (2012). Analysis of marine police citations and judicial decisions for illegal harvesting of eastern oysters (Crassostrea Virginica, Gmelin 1791) in the maryland portion of the chesapeake bay, United States, from 1959 to 2010. *Journal of Shellfish Research*, *31*(3), 591–598. https://doi.org/10.2983/035.031.0301

Bougrier ', S., Geairon, P., Deslous-Paoli, J. M., Bather, C., & Jonquikres, G. (1995). Allometric relationships and effects of temperature on clearance and oxygen consumption rates of Crassostrea gigas (Thunberg). In *Aquaculture* (Vol. 134). ELSEVIER.

Boyd, C. E., D'Abramo, L. R., Glencross, B. D., Huyben, D. C., Juarez, L. M., Lockwood, G. S., McNevin, A. A., Tacon, A. G. J., Teletchea, F., Tomasso, J. R., Tucker, C. S., & Valenti, W. C. (2020). Achieving sustainable aquaculture: Historical and current perspectives and future needs and challenges. In *Journal of the World Aquaculture Society* (Vol. 51, Issue 3, pp. 578–633). Blackwell Publishing Inc. https://doi.org/10.1111/jwas.12714

Boyd, C. E., Tucker, C. S., & Somridhivej, B. (2016). Alkalinity and Hardness: Critical but Elusive Concepts in Aquaculture. *Journal of the World Aquaculture Society*, *47*(1), 6–41. https://doi.org/10.1111/jwas.12241

Buhmann, A., & Papenbrock, J. (2013). Biofiltering of aquaculture effluents by

halophytic plants: Basic principles, current uses and future perspectives. *Environmental and Experimental Botany*, *92*, 122–133. https://doi.org/10.1016/j.envexpbot.2012.07.005

Bunting, S. W., & Shpigel, M. (2009). Evaluating the economic potential of horizontally integrated land-based marine aquaculture. *Aquaculture*, *294*(1–2), 43–51. https://doi.org/10.1016/j.aquaculture.2009.04.017

Bustos-Serrano, H., Morse, J. W., & Millero, F. J. (2009). The formation of whitings on the Little Bahama Bank. *Marine Chemistry*, *113*(1–2), 1–8. https://doi.org/10.1016/j.marchem.2008.10.006

Buzin, F., Dupuy, B., Lefebvre, S., Barillé, L., & Haure, J. (2015). Storage of Pacific oysters Crassostrea gigas in recirculating tank: Ammonia excretion and potential nitrification rates. *Aquacultural Engineering*, *64*(January), 8–14. https://doi.org/10.1016/j.aquaeng.2014.11.007

Casas, S. M., Filgueira, R., Lavaud, R., Comeau, L. A., La Peyre, M. K., & La Peyre, J. F. (2018). Combined effects of temperature and salinity on the physiology of two geographically-distant eastern oyster populations. *Journal of Experimental Marine Biology and Ecology*, *506*(December 2017), 82–90. https://doi.org/10.1016/j.jembe.2018.06.001

Cassis, D., Pearce, C. M., & Maldonado, M. T. (2011). Effects of the environment and culture depth on growth and mortality in juvenile Pacific oysters in the Strait of Georgia, British Columbia. *Aquaculture Environment Interactions*, *1*(3), 259–274. https://doi.org/10.3354/aei00025

Cema, G., Płaza, E., Trela, J., & Surmacz-Górska, J. (2011). Dissolved oxygen as a factor influencing nitrogen removal rates in a one-stage system with partial nitritation and Anammox process. *Water Science and Technology*, *64*(5), 1009–1015. https://doi.org/10.2166/wst.2011.449

Chen, S., Summerfelt, S., Losordo, T., & Malone, R. (2002). *Recirculating Systems, Effluents, and Treatments: Vol. Chapter 6*.

Chen, Shulin, Coffin, D. E., & Malone, R. F. (1997). Sludge production and management for recirculating aquacultural systems. *Journal of the World Aquaculture Society*, *28*(4), 303–315. https://doi.org/10.1111/j.1749-7345.1997.tb00278.x

Cherkas, O., Beuvier, T., Zontone, F., Chushkin, Y., Demoulin, L., Rousseau, A., & Gibaud, A. (2018). On the kinetics of phase transformations of dried porous vaterite particles immersed in deionized and tap water. *Advanced Powder Technology*, *29*(11), 2872–2880. https://doi.org/10.1016/j.apt.2018.08.008

Chertkov, O., Brown, P. J., Kysela, D. T., de Pedro, M. A., Lucas, S., Copeland, A., Lapidus, A., del Rio, T. G., Tice, H., Bruce, D., Goodwin, L., Pitluck, S., Detter, J. C., Han, C., Larimer, F., Chang, Y. J., Jeffries, C. D., Land, M., Hauser, L., … Brun, Y. V. (2011). Complete genome sequence of Hirschia baltica type strain (IFAM 1418 T). *Standards in Genomic Sciences*, *5*(3), 287–297. https://doi.org/10.4056/sigs.2205004

Chesapeake Bay Total Maximum Daily Load (TMDL) | US EPA. (n.d.). Retrieved April 28, 2021, from https://www.epa.gov/chesapeake-bay-tmdl

Chin, K., Liesack, W., & Janssen, P. H. (2001). Opitutus terrae gen. nov., sp. nov., toaccommodate novel strains of the division ‘Verrucomicrobia’ isolated from rice paddy soil. *International Journal of Sysematic and Evolutionary Microbiology*, 1965–1968.

Cho, S., Kambey, C., & Nguyen, V. K. (2020). Performance of anammox processes for wastewater treatment: A critical review on effects of operational conditions and environmental stresses. In *Water (Switzerland)* (Vol. 12, Issue 1). MDPI AG. https://doi.org/10.3390/w12010020

Chopin, T. (2006). Integrated Multi-Trophic Aquaculture What it is, and why you should care... and don’t confuse it with polyculture. *Northen Aquaculture*, 4–4.

Chopin, T., Cooper, J. A., Reid, G., Cross, S., & Moore, C. (2012). Open-water integrated multi-trophic aquaculture: Environmental biomitigation and economic diversification of fed aquaculture by extractive aquaculture. *Reviews in Aquaculture*, *4*(4), 209–220. https://doi.org/10.1111/j.1753-5131.2012.01074.x

Christianson, L. E., Lepine, C., Sharrer, K. L., & Summerfelt, S. T. (2016). Denitrifying bioreactor clogging potential during wastewater treatment. *Water Research*, *105*, 147–156. https://doi.org/10.1016/j.watres.2016.08.067

Cripps, S. J., & Bergheim, A. (2000). Solids management and removal for intensive land-based aquaculture production systems. *Aquacultural Engineering*, *22*(1–2), 33–56. https://doi.org/10.1016/S0144-8609(00)00031-5

Cua, L. S., & Stein, L. Y. (2014). Characterization of denitrifying activity by the alphaproteobacterium, Sphingomonas wittichii RW1. *Frontiers in Microbiology*,

5(AUG), 1–7. https://doi.org/10.3389/fmicb.2014.00404

D'Haese, P. C., Douglas, G., Verhulst, A., Neven, E., Behets, G. J., Vervaet, B. A., Finsterle, K., Lürling, M., & Spears, B. (2019). Human health risk associated with the management of phosphorus in freshwaters using lanthanum and aluminium. In *Chemosphere* (Vol. 220, pp. 286–299). Elsevier Ltd. https://doi.org/10.1016/j.chemosphere.2018.12.093

Dahal, R. H., & Kim, J. (2018). Fluviicola kyonggii sp. nov., a bacterium isolated from forest soil and emended description of the genus Fluviicola. *International Journal of Systematic and Evolutionary Microbiology*, *68*(6), 1885–1889. https://doi.org/10.1099/ijsem.0.002759

Davidson, J., May, T., Good, C., Waldrop, T., Kenney, B., Terjesen, B. F., & Summerfelt, S. (2016). Production of market-size North American strain Atlantic salmon Salmo salar in a land-based recirculation aquaculture system using freshwater. *Aquacultural Engineering*, *74*, 1–16. https://doi.org/10.1016/j.aquaeng.2016.04.007

De Guzman, M.-L. (2020). *Oyster culture sustains ancient Hawaiian shpond Farm supplies Aloha State with delicious oysters initially brought in to address emerging threat*. https://www.aquaculturenorthamerica.com/oyster-culture-sustains-ancient-hawaiian-fishpond/

Deng, M., Dai, Z., Senbati, Y., Li, L., Song, K., & He, X. (2020). Aerobic Denitrification Microbial Community and Function in Zero-Discharge Recirculating Aquaculture System Using a Single Biofloc-Based Suspended

Growth Reactor: Influence of the Carbon-to-Nitrogen Ratio. *Frontiers in Microbiology*, *11*(August), 1–11. https://doi.org/10.3389/fmicb.2020.01760

Dierssen, H. M., Zimmerman, R. C., & Burdige, D. J. (2009). Optics and remote sensing of Bahamian carbonate sediment whitings and potential relationship to wind-driven Langmuir circulation. In *Biogeosciences* (Vol. 6). www.biogeosciences.net/6/487/2009/

Donelan, S. C., Breitburg, D., & Ogburn, M. B. (2021). Context-dependent carryover effects of hypoxia and warming in a coastal ecosystem engineer. *Ecological Applications*. https://doi.org/10.1002/eap.2315

Driscoll, C., Whitall, D., Aber, J., Boyer, E., Castro, M., Cronan, C., Goodale, C., Groffman, P., Hopkinson, C., Lambert, K., Lawrence, G., & Ollinger, S. (2003). Nitrogen pollution: Sources and consequences in the U.S. Northeast. *Environment*, *45*(7), 8–22. https://doi.org/10.1080/00139150309604553

Ebeling, J. M. (2000). Engineering aspects of recirculating aquaculture systems. *Marine Technology Society Journal*, *34*(1), 68–78.

Edgcomb, V. P., Bernhard, J. M., Beaudoin, D., Pruss, S., Welander, P. V., Schubotz, F., Mehay, S., Gillespie, A. L., & Summons, R. E. (2013). Molecular indicators of microbial diversity in oolitic sands of Highborne Cay, Bahamas. *Geobiology*, *11*(3), 234–251. https://doi.org/10.1111/gbi.12029

Elston, R. A., Hasegawa, H., Humphrey, K. L., Polyak, I. K., & Hase, C. C. (2008). Re-emergence of Vibrio tubiashii in bivalve shellfish aquaculture: Severity, environmental drivers, geographic extent and management. *Diseases of Aquatic*

Organisms, *82*(2), 119–134. https://doi.org/10.3354/dao01982

Friedman, G. M. (1993). *Biochemical and uitrastructural evidence for the origin of whitings: A biologically induced calcium carbonate precipitation mechanism: Comment and Reply COMMENT*. http://pubs.geoscienceworld.org/gsa/geology/article-pdf/21/3/287/3514768/i0091-7613-21-3-287.pdf

Fuhrmann, M., Petton, B., Quillien, V., Faury, N., Morga, B., & Pernet, F. (2016). Salinity influences disease-induced mortality of the oyster Crassostrea gigas and infectivity of the ostreid herpesvirus 1 (OsHV-1). *Aquaculture Environment Interactions*, *8*, 543–552. https://doi.org/10.3354/aei00197

Fux, C. (2003). The Anammox Process for Nitrogen Removal from Waste Water The Fruitful Collaboration Between Microbiologists and Process Engineers. *Swiss Federal Institute for Environmental Science and Technology (EAWAG)*, 20–21.

Gichana, Z. M., Liti, D., Waidbacher, H., Zollitsch, W., Drexler, S., & Waikibia, J. (2018). Waste management in recirculating aquaculture system through bacteria dissimilation and plant assimilation. In *Aquaculture International* (Vol. 26, Issue 6, pp. 1541–1572). Springer International Publishing. https://doi.org/10.1007/s10499-018-0303-x

Glass, C., & Silverstein, J. (1998). Denitrification kinetics of high nitrate concentration water: pH effect on inhibition and nitrite accumulation. *Water Research*, *32*(3), 831–839. https://doi.org/10.1016/S0043-1354(97)00260-1

Gortan, R. (2021). *Why live tanks are the future of oyster storage*. The Fish Site.

https://thefishsite.com/articles/why-live-tanks-are-the-future-of-oyster-storage

Gray, M. W. (2016). *Ecophysiology of marine bivalves: physiological rate processes in dynamic environments Identification of habitats and growth predictions for aquaculture in Maine through remote sensing and field studies View project Native Oyster Feeding Physiology View project*. https://doi.org/10.13140/RG.2.1.3084.5043

Groner, M. L., Burge, C. A., Cox, R., Rivlin, N. D., Turner, M. O., Van Alstyne, K. L., Wyllie-Echeverria, S., Bucci, J., Staudigel, P., & Friedman, C. S. (2018). *Oysters and eelgrass: potential partners in a high pCO 2 ocean*.

Guerdat, T. C. (2012). *A Comprehensive Analysis of Marine Recirculating Aquaculture Effluent Treatment*. North Carolina State University.

Hamlin, H. J., Michaels, J. T., Beaulaton, C. M., Graham, W. F., Dutt, W., Steinbach, P., Losordo, T. M., Schrader, K. K., & Main, K. L. (2008). Comparing denitrification rates and carbon sources in commercial scale upflow denitrification biological filters in aquaculture. *Aquacultural Engineering*, *38*(2), 79–92. https://doi.org/10.1016/j.aquaeng.2007.11.003

Hedgecock, L. W., Costello, R. L., Hospital, V. A., Barracks, J., & Louis, S. (1962). *Utilization of Nitrate by Pathogenic and Saprophytic Mycobacteria*. 195-undefined. http://jb.asm.org/

Helene, L. C. F., Dall'Agnol, R. F., Delamuta, J. R. M., & Hungria, M. (2019). Mesorhizobium atlanticum sp. Nov., a new nitrogen-fixing species from soils of the Brazilian atlantic forest biome. *International Journal of Systematic and*

Evolutionary Microbiology, *69*(6), 1800–1806. https://doi.org/10.1099/ijsem.0.003397

Hobbie, S. E., Finlay, J. C., Janke, B. D., Nidzgorski, D. A., Millet, D. B., & Baker, L. A. (2017). Contrasting nitrogen and phosphorus budgets in urban watersheds and implications for managing urban water pollution. *Proceedings of the National Academy of Sciences of the United States of America*, *114*(16), 4177–4182. https://doi.org/10.1073/pnas.1618536114

Hong, P., Wu, X., Shu, Y., Wang, C. B., Tian, C., Gong, S., Cai, P., Donde, O. O., & Xiao, B. (2019). Denitrification characterization of dissolved oxygen microprofiles in lake surface sediment through analyzing abundance, expression, community composition and enzymatic activities of denitrifier functional genes. *AMB Express*, *9*(1). https://doi.org/10.1186/s13568-019-0855-9

Howarth, R. W. (2008). Coastal nitrogen pollution: A reveiw of sources and trends globally and regionally. *Harmful Algae*, *8*, 14–20.

Huang, Z., Jiang, Y., Song, X., Hallerman, E., Peng, L., Dong, D., Ma, T., Zhai, J., & Li, W. (2018). Ammonia-oxidizing bacteria and archaea within biofilters of a commercial recirculating marine aquaculture system. *AMB Express*, *8*(1), 1–12. https://doi.org/10.1186/s13568-018-0551-1

Husemann, C., Rodić, V., & Husemann, J. (2018). Phosphorus as a Bottleneck for a Sustainable Food Suply. *Contemporary Agriculture*, *67*(2), 177–182. https://doi.org/10.2478/contagri-2018-0025

Jetten, M. S. M., Niftrik, L. Van, Strous, M., Kartal, B., Keltjens, J. T., & Op Den

Camp, H. J. M. (2009). Biochemistry and molecular biology of anammox bacteria biochemistry and molecular biology of anammox bacteria M.S.M. Jetten et al. *Critical Reviews in Biochemistry and Molecular Biology*, *44*(2–3), 65–84. https://doi.org/10.1080/10409230902722783

Jia, B., Tang, Y., Tian, L., Franz, L., Alewell, C., & Huang, J. H. (2015). Impact of fish farming on phosphorus in reservoir sediments. *Scientific Reports*, *5*. https://doi.org/10.1038/srep16617

Jiang, D., Amano, Y., & Machida, M. (2017). Removal and recovery of phosphate from water by calcium-silicate composites-novel adsorbents made from waste glass and shells. *Environmental Science and Pollution Research*, *24*(9), 8210–8218. https://doi.org/10.1007/s11356-017-8503-x

Jin, R. C., Yang, G. F., Yu, J. J., & Zheng, P. (2012). The inhibition of the Anammox process: A review. In *Chemical Engineering Journal* (Vol. 197, pp. 67–79). https://doi.org/10.1016/j.cej.2012.05.014

Johansen, M. D., Herrmann, J. L., & Kremer, L. (2020). Non-tuberculous mycobacteria and the rise of Mycobacterium abscessus. *Nature Reviews Microbiology*, *18*(7), 392–407. https://doi.org/10.1038/s41579-020-0331-1

Ju, J. H., Kim, J. S., Lee, D. H., Jeon, J. H., Heo, S. Y., Seo, J. W., Kim, C. H., Park, D. S., & Oh, B. R. (2019). Thermomonas aquatica sp. Nov., isolated from an industrial wastewater treatment plant. *International Journal of Systematic and Evolutionary Microbiology*, *69*(11), 3399–3404. https://doi.org/10.1099/ijsem.0.003630

Kamennaya, N. A., Ajo-Franklin, C. M., Northen, T., & Jansson, C. (2012). Cyanobacteria as biocatalysts for carbonate mineralization. In *Minerals* (Vol. 2, Issue 4, pp. 338–364). MDPI AG. https://doi.org/10.3390/min2040338

Khan, M. D., Shakya, S., Thi Vu, H. H., Habte, L., & Ahn, J. W. (2021). Low concentrated phosphorus sorption in aqueous medium on aragonite synthesized by carbonation of seashells: Optimization, kinetics, and mechanism study. *Journal of Environmental Management*, *280*. https://doi.org/10.1016/j.jenvman.2020.111652

Kimura, Y., Isaka, K., & Kazama, F. (2011). Tolerance Level of Dissolved Oxygen to Feed into Anaerobic Ammonium Oxidation (anammox) Reactor. In *Journal of Water and Environment Technology* (Vol. 9, Issue 2).

Kioussis, D. R., Wheaton, F. W., & Kofinas, P. (1999). Phosphate binding polymeric hydrogels for aquaculture wastewater remediation. In *Aquacultural Engineering* (Vol. 19).

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M., & Glöckner, F. O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research*, *41*(1), 1–11. https://doi.org/10.1093/nar/gks808

Knelman, J. E., Graham, E. B., Prevéy, J. S., Robeson, M. S., Kelly, P., Hood, E., & Schmidt, S. K. (2018). Interspecific plant interactions reflected in soil bacterial community structure and nitrogen cycling in primary succession. *Frontiers in Microbiology*, *9*(FEB). https://doi.org/10.3389/fmicb.2018.00128

Kuenen, J. G. (2008). Anammox Bacteria: from discover to application. *Nature*, *6*(April), 320–326.

Kuhn, D. D., Angier, M. W., Barbour, S. L., Smith, S. A., & Flick, G. J. (2013). Culture feasibility of eastern oysters (Crassostrea virginica) in zero-water exchange recirculating aquaculture systems using synthetically derived seawater and live feeds. *Aquacultural Engineering*, *54*, 45–48. https://doi.org/10.1016/j.aquaeng.2012.10.008

Kumararaja, P., Suvana, S., Saraswathy, R., Lalitha, N., & Muralidhar, M. (2019). Mitigation of eutrophication through phosphate removal by aluminium pillared bentonite from aquaculture discharge water. *Ocean and Coastal Management*, *182*.

Lahav, O., Massada, I. B., Yackoubov, D., Zelikson, R., Mozes, N., Tal, Y., & Tarre, S. (2009). Quantification of anammox activity in a denitrification reactor for a recirculating aquaculture system. *Aquaculture*, *288*(1–2), 76–82. https://doi.org/10.1016/j.aquaculture.2008.11.020

Lai, Q. T., Irwin, E. R., & Zhang, Y. (2020). Estimating nitrogen removal services of eastern oyster (Crassostrea virginica) in Mobile Bay, Alabama. *Ecological Indicators*, *117*. https://doi.org/10.1016/j.ecolind.2020.106541

Land, L. S. (2012). Chesapeake Bay nutrient pollution: Contribution from the land application of sewage sludge in Virginia. *Marine Pollution Bulletin*, *64*(11), 2305–2308. https://doi.org/10.1016/j.marpolbul.2012.07.003

Larson, E. B., & Mylroie, J. E. (2014). A review of whiting formation in the Bahamas

and new models. *Carbonates and Evaporites*, *29*(4), 337–347. https://doi.org/10.1007/s13146-014-0212-7

Lazzari, R., & Baldisserotto, B. (2008). *NITROGEN AND PHOSPHORUS WASTE IN FISH FARMING* (Vol. 34, Issue 4).

Lee, P. G., Lea, R. N., Dohmann, E., Prebilsky, W., Turk, P. E., Ying, H., & Whitson, J. L. (2000). Denitrification in aquaculture systems: an example of a fuzzy logic control problem. In *Aquacultural Engineering* (Vol. 23). www.elsevier.nl/locate/aqua-online

Levantesi, C., Rossetti, S., Thelen, K., Kragelund, C., Krooneman, J., Eikelboom, D., Nielsen, P. H., & Tandoi, V. (2006). Phylogeny, physiology and distribution of "Candidatus Microthrix calida", a new Microthrix species isolated from industrial activated sludge wastewater treatment plants. *Environmental Microbiology*, *8*(9), 1552–1563. https://doi.org/10.1111/j.1462-2920.2006.01046.x

Li, M., Callier, M. D., Blancheton, J. P., Galès, A., Nahon, S., Triplet, S., Geoffroy, T., Menniti, C., Fouilland, E., & Roque d'orbcastel, E. (2019). Bioremediation of fishpond effluent and production of microalgae for an oyster farm in an innovative recirculating integrated multi-trophic aquaculture system. *Aquaculture*, *504*(October 2018), 314–325. https://doi.org/10.1016/j.aquaculture.2019.02.013

Li, X., Zhou, X., Yang, B., & Wena, Z. (2021). Recovery phosphate and ammonium from aqueous solution by the process of electrochemically decomposing

dolomite. *Chemosphere*, *262*. https://doi.org/10.1016/j.chemosphere.2020.128357

Lowe, M. R., Sehlinger, T., Soniat, T. M., & Peyre, M. K. L. (2017). Interactive effects of water temperature and salinity on growth and mortality of eastern oysters, crassostrea virginica: A meta-analysis using 40 years of monitoring data. *Journal of Shellfish Research*, *36*(3), 683–697. https://doi.org/10.2983/035.036.0318

Lu, J., Zhang, Y., Wu, J., & Wang, J. (2020). Nitrogen removal in recirculating aquaculture water with high dissolved oxygen conditions using the simultaneous partial nitrification, anammox and denitrification system. *Bioresource Technology*, *305*. https://doi.org/10.1016/j.biortech.2020.123037

Luvizotto, D. M., Araujo, J. E., Silva, M. D. C. P., Dias, A. C. F., Kraft, B., Tegetmeye, H., Strous, M., & Andreote, F. E. R. N. A. N. D. O. D. (2019). The rates and players of denitrification, dissimilatory nitrate reduction to ammonia (Dnra) and anaerobic ammonia oxidation (anammox) in mangrove soils. *Anais Da Academia Brasileira de Ciencias*, *91*. https://doi.org/10.1590/0001-3765201820180373

Ma, B., Bao, P., Wei, Y., Zhu, G., Yuan, Z., & Peng, Y. (2015). Suppressing nitrite-oxidizing bacteria growth to achieve nitrogen removal from domestic wastewater via anammox using intermittent aeration with low dissolved oxygen. *Nature*, *5*, 1–9. https://doi.org/10.1038/srep13048

Manning, D. W. P., Rosemond, A. D., Benstead, J. P., Bumpers, P. M., & Kominoski,

J. S. (2020). Transport of N and P in U.S. streams and rivers differs with land use and between dissolved and particulate forms. *Ecological Applications*, *30*(6). https://doi.org/10.1002/eap.2130

Margolis, S., & Rex, R. W. (1971). *Endolithic Algae and Micrite Envelope Formation in Bahamian Oolites as Revealed by Scanning Electron Microscopy*. http://pubs.geoscienceworld.org/gsa/gsabulletin/article-pdf/82/4/843/3443311/i0016-7606-82-4-843.pdf

Millero, F., Huang, F., Zhu, X., Liu, X., & Zhang, J. Z. (2001). Adsorption and desorption of phosphate on calcite and aragonite in seawater. *Aquatic Geochemistry*, *7*(1), 33–56. https://doi.org/10.1023/A:1011344117092

Mirzoyan, N., Tal, Y., & Gross, A. (2010). Anaerobic digestion of sludge from intensive recirculating aquaculture systems: Review. In *Aquaculture* (Vol. 306, Issues 1–4, pp. 1–6). https://doi.org/10.1016/j.aquaculture.2010.05.028

Mitterer, R. M. (1972). Biogeochemistry of aragonite mud and oolites. *Geochimica et Cosmochimica Acta*, *36*, 1407–1422.

Morales Aqualia, N., Ramon Vazquez-Padin, J., & Icaran, P. (2014). *Biofilm Reactor Technology and Design View project Reducing the pressure of fish canneries on the marine environment with novel effluent treatment and ecosystem monitoring (SEACAN) View project*. https://www.researchgate.net/publication/266262943

Morse, J. W., Gledhill, D. K., & Millero, F. J. (2003). CaCO3 precipitation kinetics in waters from the Great Bahama Bank: Implications for the relationship between Bank hydrochemistry and whitings. *Geochimica et Cosmochimica Acta*, *67*(15),

2819–2826. https://doi.org/10.1016/S0016-7037(03)00103-0

Mugg, J., Serrano, A., & Liberti, AngeloRice, M. A. (n.d.). *Aquaculture Effluents: A Guide for Water Quality Regulators and Aquaculturists*.

Myers, A. R. (2006). *RECIRCULATING AQUACULTURE SYSTEM INTEGRATION OF BIVALVE CULTURE FOR EFFLUENT NUTRIENT COMPOSITION REDUCTION*.

National Marine Fisheries Service. (2020). Fisheries of the United States 2018. *Current Fishery Statistics*, *2018*(2017), 1–167. https://www.fisheries.noaa.gov/national/commercial-fishing/fisheries-united-states-2018

Nemati, K. M. (2005). *Aggregates in Concrete*. https://doi.org/10.1201/9781482264647

Newell, N. D., Purdy, E. G., & Imbrie, J. (1960). Bahamian Oölitic Sand. In *Source: The Journal of Geology* (Vol. 68, Issue 5). https://www.jstor.org/stable/30059350?seq=1&cid=pdf-

Newton, R. J., & McLellan, S. L. (2015). A unique assemblage of cosmopolitan freshwater bacteria and higher community diversity differentiate an urbanized estuary from oligotrophic Lake Michigan. *Frontiers in Microbiology*, *6*(SEP). https://doi.org/10.3389/fmicb.2015.01028

Nitrogen and Water. (n.d.). Retrieved April 27, 2021, from https://www.usgs.gov/special-topic/water-science-school/science/nitrogen-and-water?qt-science_center_objects=0#qt-science_center_objects

O'Sullivan, L. A., Rinna, J., Humphreys, G., Weightman, A. J., & Fry, J. C. (2005). Fluviicola taffensis gen. nov., sp. nov., a novel freshwater bacterium of the family Cryomorphaceae in the phylum "Bacteroidetes." *International Journal of Systematic and Evolutionary Microbiology*, *55*(5), 2189–2194. https://doi.org/10.1099/ijs.0.63736-0

Oshiki, M., Satoh, H., & Okabe, S. (2016). Ecology and physiology of anaerobic ammonium oxidizing bacteria. In *Environmental Microbiology* (Vol. 18, Issue 9, pp. 2784–2796). Blackwell Publishing Ltd. https://doi.org/10.1111/1462-2920.13134

Park, S., & Yoon, J. H. (2013). Hirschia litorea sp. nov., isolated from seashore sediment, and emended description of the genus Hirschia. *International Journal of Systematic and Evolutionary Microbiology*, *63*(PART 5), 1684–1689. https://doi.org/10.1099/ijs.0.044297-0

Parker, M., Lipton, D., & Harrell, R. M. (2020). Impact financing and aquaculture: Maryland oyster aquaculture profitability. *Journal of the World Aquaculture Society*, *51*(4), 874–895. https://doi.org/10.1111/jwas.12702

Paynter, K. T., & Burreson, E. M. (1991). Effects of Perkinsus marinus Infection in the Eastern Oyster, Crassostrea virginica: I. Susceptibility of Native and MSX-Resistant Stocks. *Journal of Shellfish Research*, *10*(2), 417–423. http://scholar.google.com/scholar?hl=en&btnG=Search&q=intitle:Effects+of+Perkinsus+marinus+Infection+in+the+Eastern+Oyster,+Crassostrea+virginica:+I.+Susceptibility+of+Native+and+MSX-Resistant+Stocks#0

Purkis, S., Cavalcante, G., Rohtla, L., Oehlert, A. M., (Mitch) Harris, P., & Swart, P. K. (2017). Hydrodynamic control of whitings on Great Bahama Bank. *Geology*, *45*(10), 939–942. https://doi.org/10.1130/G39369.1

Qian, W., Ma, B., Li, X., Zhang, Q., & Peng, Y. (2019). Long-term effect of pH on denitrification: High pH benefits achieving partial-denitrification. *Bioresource Technology*, *278*(January), 444–449. https://doi.org/10.1016/j.biortech.2019.01.105

Qiu, T., Qi, J., Zheng, J., & Liu, Y. (2017). Design and performance of a recirculating aquaculture system for oyster larval culture. *Aquaculture Research*, *48*(12), 5699–5706. https://doi.org/10.1111/are.13392

Rice, M. A. (2001). *Environmental Impacts of Shellfish Aquaculture: Filter Feeding to Control Eutrophication*. Cape Cod Press.

Roalkvam, I., Drønen, K., Dahle, H., & Wergeland, H. I. (2021). A case study of biofilter activation and microbial nitrification in a marine recirculation aquaculture system for rearing Atlantic salmon (Salmo salar L.). *Aquaculture Research*, *52*(1), 94–104. https://doi.org/10.1111/are.14872

Robbins, L. L., Tao, Y., & Evans, C. A. (1997). Temporal and spatial distribution of whitings on Great Bahama Bank and a new lime mud budget. *Geology*, *25*(10), 947–950.

Sauthier, N., Grasmick, A., & Blancheton, J. P. (1998). BIOLOGICAL DENITRIFICATION APPLIED TO A MARINE CLOSED AQUACULTURE SYSTEM. *Wat. Res.*, *32*(6), 1932–1938.

Saviolo Osti, J. A., do Carmo, C. F., Silva Cerqueira, M. A., Duarte Giamas, M. T., Peixoto, A. C., Vaz-dos-Santos, A. M., & Mercante, C. T. J. (2020). Nitrogen and phosphorus removal from fish farming effluents using artificial floating islands colonized by Eichhornia crassipes. *Aquaculture Reports*, *17*. https://doi.org/10.1016/j.aqrep.2020.100324

Schleheck, D., Weiss, M., Pitluck, S., Bruce, D., Land, M. L., Han, S., Saunders, E., Tapia, R., Detter, C., Brettin, T., Han, J., Woyke, T., Goodwin, L., Pennacchio, L., Nolan, M., Cook, A. M., Kjelleberg, S., & Thomas, T. (2011). Complete genome sequence of Parvibaculum lavamentivorans type strain (DS-1 1). *Standards in Genomic Sciences*, *5*(3), 298–310. https://doi.org/10.4056/sigs.2215005

Schreier, H. J., Mirzoyan, N., & Saito, K. (2010). Microbial diversity of biological filters in recirculating aquaculture systems. In *Current Opinion in Biotechnology* (Vol. 21, Issue 3, pp. 318–325). https://doi.org/10.1016/j.copbio.2010.03.011

Sharrer, M. J., Tal, Y., Ferrier, D., Hankins, J. A., & Summerfelt, S. T. (2007). Membrane biological reactor treatment of a saline backwash flow from a recirculating aquaculture system. *Aquacultural Engineering*, *36*(2), 159–176. https://doi.org/10.1016/j.aquaeng.2006.10.003

Shearman, D. J., Twyman, J., & Zand Karimi, M. (1970). The genesis and diagenesis of oolites. *Proceedings of the Geologists' Association*, *81*(3), 561–564. https://doi.org/10.1016/S0016-7878(70)80015-3

Shinn, E. A., Steinen, R. P., Lidz, B. H., & Swart, P. K. (1989). *PERSPECTIVES:*

WHITINGS, A SEDIMENTOLOGIC DILEMMA l.

Shivaraman, N., & Shivaraman, G. (2003). Anammox-A novel microbial process for ammonium. *Current Science*, *84*(12), 1507–1508. https://about.jstor.org/terms

Shumway, S. E. (1996). Natural environmental factors. In *The Eastern Oyster: Crassostrea virginica* (Issue May, pp. 467–513).

Sibrell, P. L., & Kehler, T. (2016). Phosphorus removal from aquaculture effluents at the Northeast Fishery Center in Lamar, Pennsylvania using iron oxide sorption media. *Aquacultural Engineering*, *72–73*, 45–52. https://doi.org/10.1016/j.aquaeng.2016.04.003

Singer, A., Parnes, S., Gross, A., Sagi, A., & Brenner, A. (2008). A novel approach to denitrification processes in a zero-discharge recirculating system for small-scale urban aquaculture. *Aquacultural Engineering*, *39*(2–3), 72–77. https://doi.org/10.1016/j.aquaeng.2008.07.001

Skov, P. V. (2019). CO2 in aquaculture. In *Fish Physiology* (Vol. 37, pp. 287–321). Elsevier Inc. https://doi.org/10.1016/bs.fp.2019.07.004

Smil, V. (2000). PHOSPHORUS IN THE ENVIRONMENT: Natural Flows and Human Interferences. In *Annu. Rev. Energy Environ* (Vol. 25).

Staver, K. W., Brinsfield, & Russell B. (2001). *Agriculture and Water Quality on the Maryland Eastern Shore: Where Do We Go from Here?* https://doi.org/10.1641/0006

Suhr, K. I., Pedersen, P. B., & Arvin, E. (2013). End-of-pipe denitrification using RAS effluent waste streams: Effect of C/N-ratio and hydraulic retention time.

Aquacultural Engineering, *53*, 57–64. https://doi.org/10.1016/j.aquaeng.2012.11.005

Summerfelt, S. T. (2006). Design and management of conventional fluidized-sand biofilters. *Aquacultural Engineering*, *34*(3), 275–302. https://doi.org/10.1016/j.aquaeng.2005.08.010

Summerfelt, S. T., Zühlke, A., Kolarevic, J., Reiten, B. K. M., Selset, R., Gutierrez, X., & Terjesen, B. F. (2015). Effects of alkalinity on ammonia removal, carbon dioxide stripping, and system pH in semi-commercial scale water recirculating aquaculture systems operated with moving bed bioreactors. *Aquacultural Engineering*, *65*, 46–54. https://doi.org/10.1016/j.aquaeng.2014.11.002

Tal, Y, Yechezkel, E., van Rijn, J., & Schreier, H. J. (2004). Characterization and abundance of anaerobic ammonia oxidizing (anammox) bacteria in biofilters of recirculated aquaculture systems. *Proceedings of the 5th International Conference on Recirculating Aquaculture Cooperative Extension/Sea Grant*, *January*, 332–338.

Tal, Yossi, Schreier, H. J., Sowers, K. R., Stubblefield, J. D., Place, A. R., & Zohar, Y. (2009). Environmentally sustainable land-based marine aquaculture. *Aquaculture*, *286*(1–2), 28–35. https://doi.org/10.1016/j.aquaculture.2008.08.043

Tal, Yossi, Watts, J. E. M., & Schreier, H. J. (2006). Anaerobic ammonium-oxidizing (Anammox) bacteria and associated activity in fixed-film biofilters of a marine recirculating aquaculture system. *Applied and Environmental Microbiology*, *72*(4), 2896–2904. https://doi.org/10.1128/AEM.72.4.2896-2904.2006

The Issue | Nutrient Pollution | US EPA. (n.d.). Retrieved February 22, 2021, from https://www.epa.gov/nutrientpollution/issue

Timmons, M. B., & Ebeling, J. M. (2010). *Recirculating Aquaculture* (2nd ed.). Cayuga Aqua Ventures.

Troell, M., Joyce, A., Chopin, T., Neori, A., Buschmann, A. H., & Fang, J. G. (2009). Ecological engineering in aquaculture - Potential for integrated multi-trophic aquaculture (IMTA) in marine offshore systems. *Aquaculture*, *297*(1–4), 1–9. https://doi.org/10.1016/j.aquaculture.2009.09.010

True, B., Johnson, W., & Chen, S. (2004a). Reducing phosphorus discharge from flow-through aquaculture: III: Assessing high-rate filtration media for effluent solids and phosphorus removal. *Aquacultural Engineering*, *32*(1), 161–170. https://doi.org/10.1016/j.aquaeng.2004.08.004

True, B., Johnson, W., & Chen, S. (2004b). Reducing phosphorus discharge from flow-through aquaculture I: Facility and effluent characterization. *Aquacultural Engineering*, *32*(1), 129–144. https://doi.org/10.1016/j.aquaeng.2004.08.001

Tsukuda, S., Christianson, L., Kolb, A., Saito, K., & Summerfelt, S. (2015). Heterotrophic denitrification of aquaculture effluent using fluidized sand biofilters. *Aquacultural Engineering*, *64*, 49–59. https://doi.org/10.1016/j.aquaeng.2014.10.010

Turcios, A. E., & Papenbrock, J. (2014). Sustainable treatment of aquaculture effluents-What can we learn from the past for the future? In *Sustainability (Switzerland)* (Vol. 6, Issue 2, pp. 836–856). MDPI AG.

https://doi.org/10.3390/su6020836

US EPA, R. 03. (n.d.). *Chesapeake Bay Total Maximum Daily Load (TMDL)*. Retrieved April 28, 2021, from https://www.epa.gov/chesapeake-bay-tmdl

Van Kessel, M. A. H. J., Harhangi, H. R., Flik, G., Jetten, M. S. M., Klaren, P. H. M., & Op Den Camp, H. J. M. (2011). Anammox bacteria in different compartments of recirculating aquaculture systems. *Biochemical Society Transactions*, *39*(6), 1817–1821. https://doi.org/10.1042/BST20110743

Van Rijn, J. (2013). Waste treatment in recirculating aquaculture systems. *Aquacultural Engineering*, *53*, 49–56. https://doi.org/10.1016/j.aquaeng.2012.11.010

van Rijn, J., Tal, Y., & Schreier, H. J. (2006). Denitrification in recirculating systems: Theory and applications. *Aquacultural Engineering*, *34*(3), 364–376. https://doi.org/10.1016/j.aquaeng.2005.04.004

Vilella, S., Read, N., & Claericoates, J. (n.d.). *Manual on effluent treatment in aquaculture*. www.aquaetreat.org

Vincenzo, V., & Zonno, Z. (2006). Why treating aquaculture effluent water? Third Aquaetreat Training Course Third Aquaetreat Training Course Billund 28 Billund. In *Third Aquaetreat Training Course Third Aquaetreat Training Course Billund* (Vol. 28).

Virginia Administrative Code Title 4. Conservation And Natural Resources Agency 50. Virginia Soil And Water Conservation Board Chapter 85. Nutrient Management Training and Certification Regulations. (n.d.). Retrieved June 27,

2021, from https://law.lis.virginia.gov/admincode/title4/agency50/chapter85/section140/

Wahyuningsih, S., Effendi, H., & Wardiatno, Y. (2015). Nitrogen removal of aquaculture wastewater in aquaponic recirculation system The Declining of Rice Field Areas in Java Island amid continuing urban expansion and regional disparity View project Chironomidae View project. *AACL BioFlux*, *8*(4), 491–499. https://www.researchgate.net/publication/280632454

Walker, T. (2017). Inland RAS Helps To Restore Mississippi Oyster Industry. *Hatchery International*.

Wang, J. K. (2003). Conceptual design of a microalgae-based recirculating oyster and shrimp system. *Aquacultural Engineering*, *28*(1–2), 37–46. https://doi.org/10.1016/S0144-8609(03)00020-7

Wang, Q., & He, J. (2020). Complete nitrogen removal via simultaneous nitrification and denitrification by a novel phosphate accumulating Thauera sp. strain SND5. *Water Research*, *185*, 116300. https://doi.org/10.1016/j.watres.2020.116300

Wang, Y., Bott, C., & Nerenberg, R. (2016). Sulfur-based denitrification: Effect of biofilm development on denitrification fluxes. *Water Research*, *100*, 184–193. https://doi.org/10.1016/j.watres.2016.05.020

Ward, B. B. (2008). Nitrification in Marine Systems. In *Nitrogen in the Marine Environment* (pp. 199–261). Elsevier Inc. https://doi.org/10.1016/B978-0-12-372522-6.00005-0

Watten, B. J., Mudrak, V. A., Echevarria, C., Sibrell, P. L., Summerfelt, S. T., &

Boyd, C. E. (2017). Performance and application of a fluidized bed limestone reactor designed for control of alkalinity, hardness and pH at the Warm Springs Regional Fisheries Center. *Aquacultural Engineering*, *77*, 97–106. https://doi.org/10.1016/j.aquaeng.2017.03.003

Whitall, D., Castro, M., & Driscoll, C. (2004). Evaluation of management strategies for reducing nitrogen loadings to four US estuaries. *Science of the Total Environment*, *333*(1–3), 25–36. https://doi.org/10.1016/j.scitotenv.2004.05.006

Williamson, Timothy R., Tilley, D. R., & Campbell, E. (2015). Emergy analysis to evaluate the sustainability of two oyster aquaculture systems in the Chesapeake Bay. *Ecological Engineering*, *85*, 103–120. https://doi.org/10.1016/j.ecoleng.2015.09.052

Williamson, Timothy Robert. (2014). *DESIGN AND SUSTAINABILITY ANALYSIS OF OYSTER AQUACULTURE IN MARYLAND*. University of Maryland, College Park.

Wills, P. S., Pfeiffer, T., Baptiste, R., & Watten, B. (2016). Application of a fluidized bed reactor charged with aragonite for control of alkalinity, pH and carbon dioxide in marine recirculating aquaculture systems. *Aquacultural Engineering*, *70*, 81–85. https://doi.org/10.1016/j.aquaeng.2015.10.001

Wiltshire, K. (2007). *Ecophysiological tolerances of the Pacific oyster, Crassostrea gigas, with regard to the potential spread of populations in South Australian waters Prepared for PIRSA Marine Biosecurity*.

Windolf, J., Blicher-Mathiesen, G., Carstensen, J., & Kronvang, B. (2012). Changes

in nitrogen loads to estuaries following implementation of governmental action plans in Denmark: A paired catchment and estuary approach for analysing regional responses. *Environmental Science and Policy*, *24*, 24–33. https://doi.org/10.1016/j.envsci.2012.08.009

Xu, N., Wang, Y., Xu, X., Liu, C., Qian, J., & Feng, G. (2016). Mechanisms and applications of the synthesized fusiform aragonite for the removal of high concentration of phosphate. *Water, Air, and Soil Pollution*, *227*(2). https://doi.org/10.1007/s11270-016-2757-7

Xu, Z., Dai, X., & Chai, X. (2018). Effect of influent pH on biological denitrification using biodegradable PHBV/PLA blends as electron donors. *Biochemical Engineering Journal*, *131*, 24–30.

Yeo, S. E., Binkowski, F. P., & Morris, J. E. (2004). Aquaculture Effluents and Waste By-Products Characteristics, Potential Recovery, and Beneficial Reuse Recommended Citation. In *NCRAC Technical Bulletins North Central Regional Aquaculture Center* (Vol. 8). http://lib.dr.iastate.edu/ncrac_techbulletins

Yin, Z., Santos, C. E. D. dos, Vilaplana, J. G., Sobotka, D., Czerwionka, K., Damianovic, M. H. R. Z., Xie, L., Morales, F. J. F., & Makinia, J. (2016). Importance of the combined effects of dissolved oxygen and pH on optimization of nitrogen removal in anammox-enriched granular sludge. *Process Biochemistry*, *51*(9), 1274–1282. https://doi.org/10.1016/j.procbio.2016.05.025

Yosef, D. Z. Ben, Ben-Dov, E., & Kushmaro, A. (2008). Amorphus coralli gen. nov., sp. nov., a marine bacterium isolated from coral mucus, belonging to the order

Rhizobiales. *International Journal of Systematic and Evolutionary Microbiology*, *58*(12), 2704–2709. https://doi.org/10.1099/ijs.0.65462-0

Zeebe, R. E. (2012). History of seawater carbonate chemistry, atmospheric CO 2, and ocean acidification. In *Annual Review of Earth and Planetary Sciences* (Vol. 40, pp. 141–165). https://doi.org/10.1146/annurev-earth-042711-105521

Zeng, R. J., Lemaire, R., Yuan, Z., & Keller, J. (2003). Simultaneous Nitrification, Denitrification, and Phosphorus Removal in a Lab-Scale Sequencing Batch Reactor le. *Biotechnology and Bioengineering*, *84*(2), 170–178.

Zhang, L., Zheng, P., Tang, C. jian, & Jin, R. cun. (2008). Anaerobic ammonium oxidation for treatment of ammonium-rich wastewaters. In *Journal of Zhejiang University. Science. B* (Vol. 9, Issue 5, pp. 416–426). https://doi.org/10.1631/jzus.B0710590

Zhang, X., & Zhang, J. (2018). Effect of dissolved oxygen on biological denitrification using biodegradable plastic as the carbon source. *IOP Conference Series: Earth and Environmental Science*, *121*(3). https://doi.org/10.1088/1755-1315/121/3/032015

Zhou, B., Duan, J., Xue, L., Zhang, J., & Yang, L. (2019). Effect of plant-based carbon source supplements on denitrification of synthetic wastewater: focus on the microbiology. *Environmental Science and Pollution Research*, *26*(24), 24683–24694. https://doi.org/10.1007/s11356-019-05454-x

Zhou, Y., Zhang, S., Liu, Y., & Yang, H. (2014). Biologically induced deposition of fine suspended particles by filter-feeding bivalves in land-based industrial

marine aquaculture wastewater. *PLoS ONE*, *9*(9). https://doi.org/10.1371/journal.pone.0107798

Zohar, Y., Tal, Y., Schreier, H. J., Steven, C. R., Stubblefield, J., & Place, A. R. (2005). Commercially feasible urban recirculating Aquaculture: Addressing the marine sector. In *Urban Aquaculture* (pp. 150–171). CABI Publishing. https://doi.org/10.1079/9780851998299.0159

Zou, G., Papirio, S., Lakaniemi, A. M., Ahoranta, S. H., & Puhakka, J. A. (2016). High rate autotrophic denitrification in fluidized-bed biofilm reactors. *Chemical Engineering Journal*, *284*, 1287–1294. https://doi.org/10.1016/j.cej.2015.09.074

Zumft, W. G. (1997). Cell Biology and Molecular Basis of Denitrification †. *MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS*, *61*(4), 533–616. http://mmbr.asm.org/