Microbial Resource Management in Indoor Recirculating Shrimp Aquaculture Systems

by

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Abstract

Indoor recirculating aquaculture systems (RAS) for the production of shrimp are a potentially sustainable alternative to traditional pond culture systems in terms of water conservation and reduced impact on receiving water quality. RAS systems consist of a shrimp production tank and one or more biofilters for water treatment. Microorganisms in the biofilters have a critical role in maintaining water quality in the production tank. Therefore, a better understanding of microbially mediated nitrogen transformation processes in indoor RAS can help improve performance through appropriate operational modifications. Furthermore, a reduction in commercial shrimp feed may be possible through the use of biofilter biofilm as a supplemental feed source for shrimp.

The microbial community was characterized in the trickling filter of a local (Okemos, MI) indoor, zero-discharge RAS used in the production of Pacific white shrimp, *Litopanaeus vannamei*. Ammonium oxidizing archaea and nitrite-oxidizing nitrospiras were the dominant nitrifying microbes in this system. Clone libraries and quantitative polymerase chain reaction were used to identify and quantify the ammonium-oxidizers and nitrite-oxidizers in the system.

A laboratory-scale indoor, zero-discharge RAS was designed and operated under simulated intensive growth conditions, i.e., at least 100 shrimp per m^2 of tank area. The ammonium load to the system was increased to simulate shrimp growth and additional waste production over time to investigate the effect of the ammonium concentration on the population abundance of ammonia-oxidizers and nitrite-oxidizers in the biological aerated filters of this system. A correlation between ammonium concentration and niche differentiation of ammonium oxidizers was not observed but the abundance of ammonia oxidizing bacteria increased, with increasing ammonium concentration. Also *Nitrospira* nitrite oxidizing bacteria (NOB) were more abundant than *Nitrobacter* NOB.

The laboratory-scale indoor, zero-discharge RAS was operated to examine the impact of biofilter biofilm as a supplemental feed source on shrimp growth and survival. Supplementing commercial shrimp feed with RAS biofilter biofilm is a viable way to reduce feed costs and improve the sustainability of RAS. Additional research is needed to optimize the level of biofilm supplmentation for increased shrimp growth.

Chapter 1: Introduction

1.1 Background

Aquaculture, which is the cultivation or farming of aquatic animals and plants in fresh, brackish, and marine environments (Pillay & Kutty, 2005), has been the most rapidly growing animal food producing sector, with an annual increase of at least 8% since 1985 (Food and Agriculture Organization, 2012). The demand for farmed seafood has been increasing, since production from wild fish harvest is relatively stable or declining, the global population is increasing, and the per-capita seafood consumption has been predicted to increase from to about 1.5 kg per person per year by 2025 (Food and Agriculture Organization, 2008). As with all food production systems, there are drawbacks to aquaculture. The major downside is that aquaculture as a global food production system has a negative impact on the environment (Goldburg & Triplett, 1997, Boyd & Clay, 1998, Naylor *et al.*, 2000). Negative impacts include destruction of coastal mangrove forests or wetlands, use of fish meal or fish oil from wild-caught fish for feed, accidental release of non-native species, and discharge of excess nutrients causing eutrophication in receiving water bodies. If the aforementioned environmental impacts of aquaculture are reduced or eliminated, aquaculture can have a net positive impact on the environment.

Americans are eating more seafood. Since 1980, fish consumption has increased more than 50% (Blisard *et al.*, 2002). The U.S. Food and Drug Administration (U.S. FDA) reported that imports account for more than 75 % of total U.S. fish consumption (Allshouse *et al.*, 2004), and the U.S. imports billions of dollars more in edible seafood than it exports. According to the Food and Agriculture Organization of the United Nations (Food and Agriculture Organization, 2005), edible U.S. seafood production is dominated by oysters and clams (shellfish) and channel catfish (fish). By contrast, shrimp are the largest import item. Most of this shrimp is from Asia as the Asia-Pacific region produces 88% of all farmed species of shrimp and prawns (Food and Agriculture Organization, 2008). In 2001, for example, imported shrimp was estimated at 883 million pounds and was approximately worth $3.6 billion dollars (Allshouse *et al.*, 2004).

Consequently, most of the safety violations for seafood imports are related to shrimp and prawns (Allshouse *et al.*, 2004). For example, in 2001 U.S. FDA detention data for seafood products imported from 130 countries listed 6,405 violations. The vast majority of these (83.6%) were for adulteration (which deals with safety, packaging integrity, or sanitation problems), and *Salmonella* accounted for 34 percent of all adulteration violations (Allshouse *et al.*, 2004). Approximately 25% of all U.S. FDA seafood import detentions in 2001, and more than half of the violations for *Salmonella* were for shrimp and prawns (farm raised and wild caught) (Allshouse *et al.*, 2004).

Pacific white, or whiteleg, shrimp (*Litopeneaus vannamei*) is the most popular shrimp species cultured. Pacific white shrimp culture production in the U.S. averaged approximately 2,800 metric tons per year from 1990-2010 (Figure 1.1), which is less than one percent of the global average yearly production (Food and Agriculture Organization, 2013). These data suggest there is great potential for the U.S. to increase domestic shrimp production, which would have a positive impact on food safety and trade.

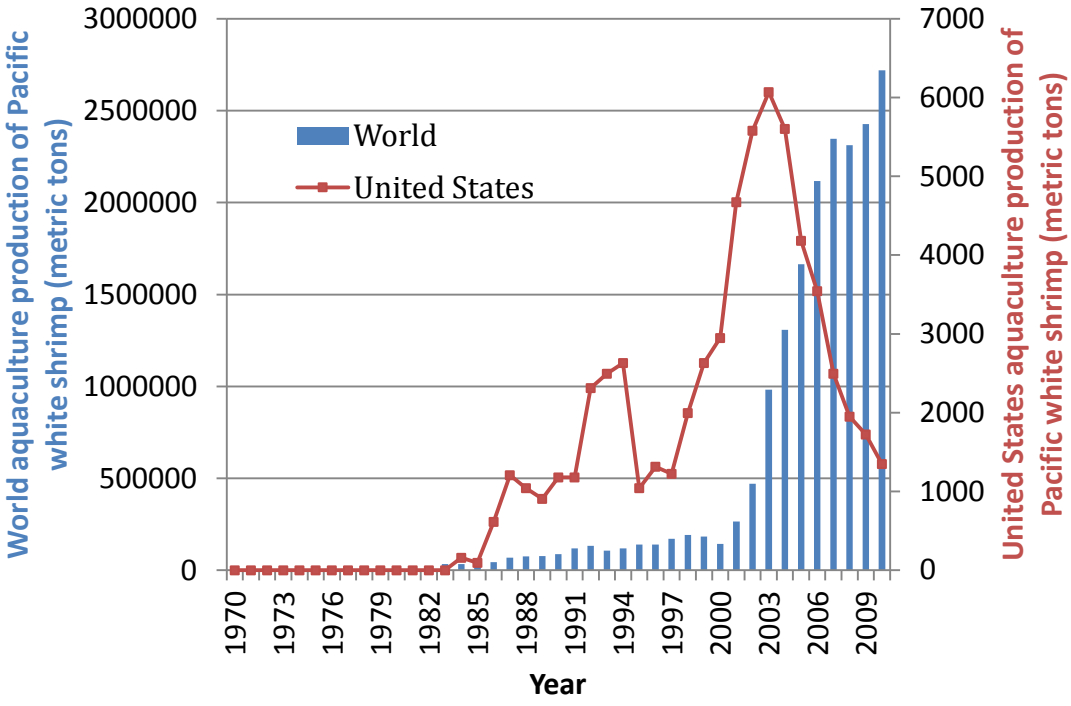

Figure 1.1: World and United States aquaculture production of Pacific whiteleg shrimp from 1970-2010. Data source, Food and Agriculture Organization, 2013.

Pacific white shrimp is the species of choice for commercial shrimp farming in the U.S. From 1950-1972, shrimp farming research and development in the U.S. were aimed at native *Penaeus* (or *Litopenaeus*) species found in the coastal waters of South Carolina and Texas (Hopkins *et al.*, 1995). In 1974, a study was published on the very successful intensive culture of Pacific white shrimp and since that time research and development of intensive shrimp production in the U.S. has focused on Pacific white shrimp (Hopkins *et al.*, 1995). Currently, Pacific white shrimp are farmed at production levels seven times higher than other shrimp species (Food and Agriculture Organization, 2013).

Farmers can replicate the complete life cycle of Pacific white shrimp (Figure 1.2). The shrimp hatchery cycle begins with broodstock, or adult shrimp that can reproduce. Spawning occurs and the shrimp are reared through the larval stages. Nurseries are used to increase shrimp size for stocking growout systems and/or acclimate post larval (PL) shrimp to farm conditions. Growout systems are where PL shrimp are grown to a marketable size. The supply of large quantities of PL shrimp from hatcheries has allowed the expansion of shrimp aquaculture because it reduced the reliance on capture fisheries for PL shrimp delivery. A specific-pathogen-free (SPF) shrimp population was established in Hawaii, and it has been a source of SPF stocks to hatcheries in the U.S. (Hopkins *et al.*, 1995). Commercial shrimp farms in the U.S. are located in several states, including Texas, South Carolina, Florida, and Arizona (U.S. Marine Shrimp Farm Program, 2010).

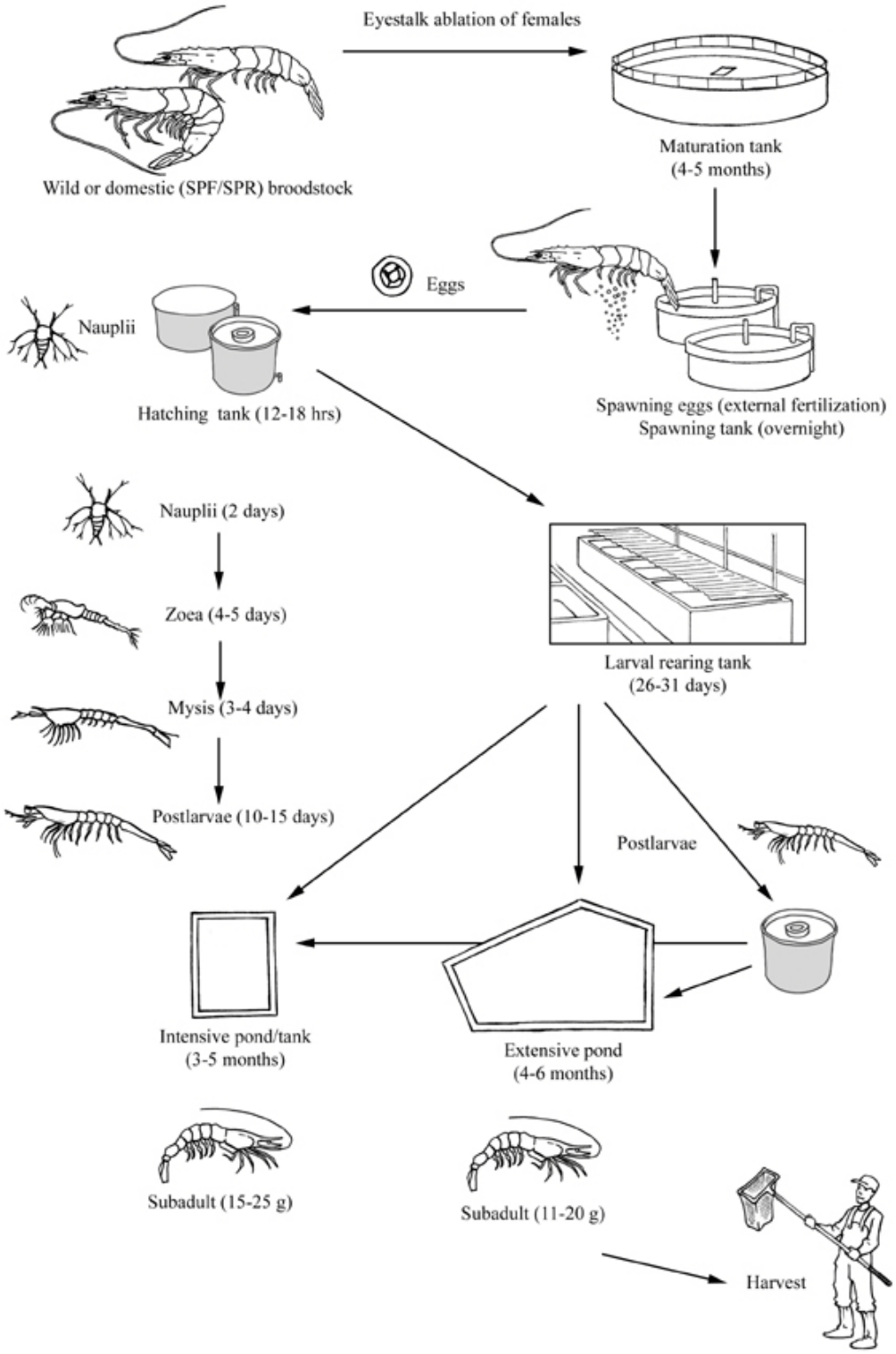

Figure 1.2: Pacific white shrimp or Pacific whiteleg shrimp (*Litopenaeus vannamei*) production cycle. (Source: FAO Cultured Aquatic Species Information Programme [online]. Last updated August 17, 2006. Accessed March 24, 2010. http://www.fao.org/fishery/culturedspecies/Litopenaeus_vannamei/en)

Shrimp aquaculture in the U.S. must comply with environmental regulations and take into account the economics of shrimp markets. Accordingly, the expansion of shrimp aquaculture in the U.S. must proceed in a sustainable and environmentally sensitive manner (Clay, 1997). Factors to consider in the sustainability of shrimp aquaculture are (Clay, 1997): site selection, eliminate dependency on the capture of wild PL shrimp, efficient use of feed, reduction of carbon and nitrogen loads to receiving waters, and reduced water usage. Systems proposed for sustainable shrimp farming include recirculating aquaculture systems (RAS) and the biofloc system (BFS). At present, nearly all worldwide shrimp aquaculture production, ranging from extensive to intensive, occurs in outdoor, flow-through ponds (FAO, 2007). In the U.S., shrimp culture occurs in ponds because RAS operations, either indoors or outdoors, typically are too expensive (Whetstone *et al.*, 2002) to compete with imported shrimp. To be economically competitive with shrimp imports, U.S. shrimp farmers must culture shrimp at high stocking densities and/or have high-value markets for their product. Indoor RAS allow shrimp farms to be located inland, away from valuable coastal property and close to specialty high-value niche markets, such as gourmet food stores or restaurants that want a supply of fresh fish/shellfish (Losordo *et al.*, 1998).

1.2 Overall goal of dissertation and description of content

The long-term goal of the research presented in this dissertation is to improve the environmental sustainability of shrimp recirculating aquaculture systems (RAS) operated indoors. Microorganisms have a critical role in maintaining water quality and solid waste management in RAS. Therefore, a better understanding of the microbially-mediated nitrogen transformation processes in indoor RAS can help improve performance through appropriate operational modifications. Furthermore, a reduction in commercial shrimp feed may be possible through the use of microbial biomass from RAS biofilters as a supplemental feed source. The following is a description of the individual chapters in this dissertation:

Chapter 2 provides background information and a literature study on RAS and microbial biomass as a supplemental feed source.

In **Chapter 3,** the nitrifying microbial populations of a local indoor shrimp farm were analyzed. This farm utilized a zero-discharge RAS for culturing Pacific white shrimp. Ammonia oxidizing archaea and nitrite oxidizing *Nitrospira*-like bacteria were detected, as well as ammonia oxidizing bacteria. This study was published in the journal *FEMS Microbiology Ecology* (Brown *et al.*, 2013).

In **Chapter 4,** laboratory experiments were conducted with three RAS to evaluate the impact of increasing ammonia load on ammonia-oxidizing and nitrite-oxidizing microbes in biofilters.

In **Chapter 5,** microbial biomass from the biofilters of an indoor, zero-discharge RAS was evaluated as a supplemental feed source for Pacific white shrimp.

Chapter 6 provides conclusions for this study and recommendations for future research.

Appendix I is supplemental information for Chapter 3.

Appendix II is a summary of the residence time distribution analysis of the laboratory RAS. This analysis was done to describe the hydraulic characteristics of the experimental set-up.

Appendix III is supplemental information for Chapter 4.

Appendix IV is supplemental information for Chapter 5.

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Chapter 2: Literature Review

2.1 Introduction

As the human population increases, food production must also increase to meet demand. Aquaculture, or the farming of aquatic organisms, has been the most rapidly growing production sector, with an annual increase at least 8% since 1985 (Food and Agriculture Organization, 2012). The demand for farmed seafood has increased because production from wild fish harvesting is relatively stable or declining depending on fish type and because the global per-capita seafood consumption has increased (Food and Agriculture Organization, 2004, Food and Agriculture Organization, 2007, Food and Agriculture Organization, 2008).

Americans are shifting their dietary patterns to include consumption of more seafood. The U.S. demand for shrimp has continually increased since 1989 and is supplied primarily through imported shrimp. U.S. shrimp imports increased by more than 200% from 1989 to 2011 (Economic Research Service, 2013). During this time, the U.S. population increased from 248 to 308 million people with an associated per capita consumption of 15 to 16 pounds (6.8 to 7.3 kilograms) (Figure 2.1, National Marine Fisheries Service, 2012). The U.S. Food and Drug Administration (U.S. FDA) reported that seafood imports account for more than 75% of total U.S. fish consumption (Allshouse *et al.*, 2004). Shrimp are the largest seafood import item in the U.S. and come primarily from the Asia-Pacific region, which produces 88% of all farmed shrimp and prawns by mass (Food and Agriculture Organization, 2008). The majority of the shrimp imports to the U.S. come from Thailand, Ecuador, Vietnam, Indonesia, and China (Economic Research Service, 2013) and these countries do not have the same seafood safety standards as the U.S., which has led to concerns about seafood safety. For example in 2001, U.S. FDA detention data for seafood products imported from 130 countries listed over 6,000 violations; *Salmonella* contamination accounted for 34% of all adulteration, packing integrity or sanitation, violations (Allshouse *et al.*, 2004). Approximately 25% of all U.S. FDA seafood import detentions in 2001,

and more than half of the violations for *Salmonella*, were for shrimp and prawns (farm raised and wild caught) (Allshouse *et al.*, 2004). Therefore, increasing domestic shrimp production would have a positive impact on food safety and trade.

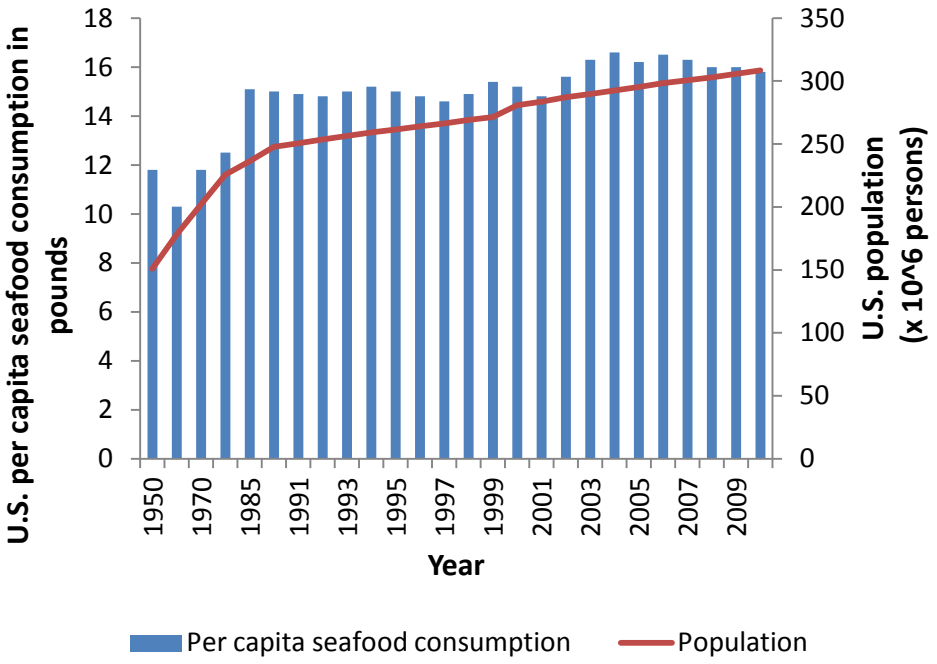

Figure 2.1: Per capita seafood consumption (left axis) and population (right axis) in the U.S. (Adapted from National Marine Fisheries Service, 2012)

The Pacific white shrimp (*Litopeneaus vannamei*), also called Pacific whiteleg shrimp, is the most popular cultured shrimp species in the world. It is native to the Pacific coast of Central and South America and has a complex life cycle that includes three larval stages. Pacific white shrimp is the species of choice for commercial shrimp farming. From 1950-1972, shrimp farming research and development in the U.S. were aimed at native *Penaeus* shrimp species found in the coastal waters of the Atlantic Ocean and Gulf of Mexico (Hopkins *et al.*, 1995). These species were Northern white shrimp (*Penaeus setiferus),* Northern pink shrimp (*Penaeus duorarum),* and Northern brown shrimp (*Penaeus aztecus).* However, a study published in 1974 demonstrated successful intensive culture of the non-native, Pacific white shrimp and, since that

time, research and development of intensive shrimp production in the U.S. has focused on Pacific white shrimp (Hopkins *et al.*, 1995) instead of native shrimp species. Since 1990, Pacific white shrimp accounted for all of the aquaculture production of shrimp and prawns in the U.S. (Food and Agriculture Organization, 2013). Farmed Pacific white shrimp production in the U.S. averaged approximately 2,800 metric tons per year from 1990-2010, which is 0.3% of the global average yearly production (Food and Agriculture Organization, 2013). Due to low production in the U.S., the majority of Pacific white shrimp consumed in the U.S. is imported.

Conventional aquaculture systems include ponds and cages, or net pen systems, which are open-air and open-water environments. These systems are geographically limited to locations that have climates suitable for growing shrimp, require large land areas, utilize large volumes of water to maintain water quality, and are vulnerable to disease. Since the farmer cannot control water temperature in conventional culture systems, pond and cage systems must be located in a water body that has the appropriate temperature for shrimp growth. Aquaculture systems can be extensive, semi-intensive, or intensive depending on the density of cultured organisms. The division between these intensity levels are not rigidly defined, but can be classified as less than or equal to one shrimp per m^2 for extensive, three to ten shrimp per m^2 for semi-intensive, 15 to 40 shrimp per m^2 for intensive. A density greater than 100 shrimp per m^2 is considered ultra-intensive (Fast & Lester, 1992). In extensive systems, farmers rely on natural production, semi-intensive systems use fertilizers to promote the growth of algae as feed for shrimp, and intensive systems rely on feed pellets manufactured from fish meal, plants, nutritional supplements, and a binder to feed shrimp (Boyd & Clay, 1998, Diana, 2012). Conventional systems tend to be semi-intensive or intensive because water quality control is difficult, which limits the number of organisms that can be grown at a given time. They require a large land area to produce a large crop. To maintain water quality, excess nutrients from aquaculture ponds are discharged during water exchange. Water exchange is necessary due to oxygen depletion by the growing shrimp and microorganisms, the accumulation of organic matter (from uneaten feed and feces), and the accumulation of inorganic nitrogen compounds (van Rijn, 1996). Shrimp and other aquatic organisms do not efficiently extract nitrogen from their feed and around 75% of nitrogen provided in the feed is excreted as waste (Piedrahita, 2003, Gutierrez-Wing & Malone, 2006). Most ponds are operated with high rates of water exchange and approximately 40% of pond volume is replaced daily (Samocha *et al.*, 2002). For flow-through systems, the water is

continually discharged. Conventional aquaculture systems are vulnerable to disease because of their open-air, open-water environment. Diseases are transferred by direct water contact with diseased organisms.

As with all food production systems, conventional aquaculture systems affect the environment. Pond and cage culture has documented negative environmental impacts, which include destruction of coastal mangrove forests or wetlands, use of fish meal or fish oil in manufactured feed, accidental release of non-native species, and discharge of excess nutrients causing eutrophication, or low dissolved oxygen, in receiving water bodies (Goldburg & Triplett, 1997, Boyd & Clay, 1998, Naylor *et al.*, 2000). Coastal mangrove forests and wetlands were converted to pond systems for shrimp farming in tropical areas including Ecuador and Thailand (Boyd & Clay, 1998, Naylor *et al.*, 2000). In Thailand, an estimated 161,000 acres (65,000 hectares) of mangrove forests were converted to shrimp farms between 1961 and 1993 (Menasveta, 1997). Cage farming in bays, rivers, lakes, and other coastal areas involved the use of intensive stocking and feeding, which resulted in increased oxygen demand and nitrogen loading (Pillay, 1992). Manufactured feeds contained 30% fish meal and 2% fish oil from wild-caught fish in 1997 (Naylor *et al.*, 2000). Large-scale aquaculture has both negative and positive environmental impacts (Diana, 2009). Positive environmental impacts include supplementing reproduction in natural populations, improving the quality of natural waters through filtering or consuming wastes by cultured organisms, and reducing pressure on fisheries by providing alternative sources in the market (Diana, 2012).

There is potential for the aquaculture industry to expand in the U.S., but it must be done in a sustainable manner. Assessment tools that have been used to evaluate the environmental impact of aquaculture systems include ecological footprint and life cycle assessment (Samuel-Fitwi *et al.*, 2012). Developing sustainable aquaculture can be done using an ecosystem, or ecological, approach (Costa-Pierce, 2010, Klinger & Naylor, 2012) and an intensive culture approach in technological systems that can treat the associated waste (Timmons & Ebeling, 2007, Klinger & Naylor, 2012). According to Costa-Pierce, ecological aquaculture aims to develop aquatic farming ecosystems that preserve and enhance the form and functions of the natural and social environments in which they are situated. This approach is appropriate for small-scale farms or in locations that have large land areas available for aquaculture. Intensive culture systems are suitable systems when high productivity in a small land area is desired from a

profit perspective. Culture intensity is an important consideration in improving the environmental impacts of aquaculture. A life cycle assessment of intensive and semi-intensive shrimp pond systems in China (Cao *et al.*, 2011) showed that the intensive system had significantly higher environmental impacts per unit of production in global warming, acidification, eutrophication, cumulative energy use, and biotic resource use. The use of fishmeal and fish oil from fisheries in shrimp must also be reduced or eliminated to improve the sustainability of shrimp aquaculture (Duarte *et al.*, 2009, Klinger & Naylor, 2012). Current approaches to reduce fishmeal and fish oil in feeds include substituting plant proteins, terrestrial animal proteins, fish processing waste, and using microorganisms as a food source, similar to extensive systems (Klinger & Naylor, 2012).

In the U.S., limitations imposed by land cost and regulation of water quantity/quality and waste discharges are costly to the aquaculture industry (Timmons & Ebeling, 2007). Conventional pond culture must be located on a coastal site, which creates conflict with other uses of coastal areas including residential property and recreation. These issues make indoor operations away from the coast a more viable option than open pond or cage culture systems. However, such a system requires (i) an indoor system with heating and temperature control, (ii) availability of local brackish water sources or artificial preparation of salt water (for marine organisms), and (iii) water treatment to allow recycling of the water and reduction of waste. Using currently developed technologies, the energy and material costs of such a system exceed those of outdoor production facilities in warmer areas with ready access to salt water. An economic feasibility study (Van Wyk, 1999) concluded that shrimp from technologically advanced aquaculture systems cannot directly compete with imported shrimp in the wholesale frozen market. Developing a viable indoor shrimp aquaculture industry in the U.S. will require operations to be highly efficient, predictable, and stable to compete with inexpensive imports.

2.2 Recirculating Aquaculture System (RAS)

Two technologies that may be adopted for shrimp aquaculture operations are the bio-floc system (BFS) and recirculating aquaculture system (RAS). BFS have been under development since the 1990s, while RAS have been under development and refinement since the 1970s. Both systems can be operated at or near zero water exchange, i.e., no water discharge during a

cropping cycle, or the period of time it takes to grow the shrimp from post-larval to harvest size. These systems also rely on microbial resource management (Verstraete *et al.*, 2007), where microbes maintain water quality for production systems, enhance growth, and may serve as supplemental feed source. In BFS, an organic carbon source is added to establish a high carbon to nitrogen ratio to promote growth of heterotrophic bacteria that assimilate ammonium into microbial biomass in the culture tank (Avnimelech *et al.*, 1992, Avnimelech, 1999). On the other hand, RAS consist of an integrated set of processes that are designed to remove the by-products of fish or shrimp metabolism and thus treat water for reuse in the culture tank (Fig. 2.2). The primary treatment unit of RAS is the biofilter, which relies on chemoautotrophic microbial conversion of ammonium to nitrate. Because of the higher water quality associated with RAS, the water can be reused between cropping cycles (van Wyk, 1999). Both systems are attractive in terms of water conservation, reduced land use, and limited impact on receiving water quality when compared to conventional pond culture. Furthermore, these systems are also attractive in terms of disease control: the water used is typically disinfected prior to startup to kill microbial shrimp pathogens, the shrimp used for production can be obtained as certified pathogen-free, and the indoor enclosure reduces crop contamination. In both of these systems, microbes play a central role in maintaining water quality, improving shrimp health, and managing waste. Due to widespread commercial use of RAS, and the potential extensive benefits of RAS for shrimp aquaculture, the focus of this research is on RAS.

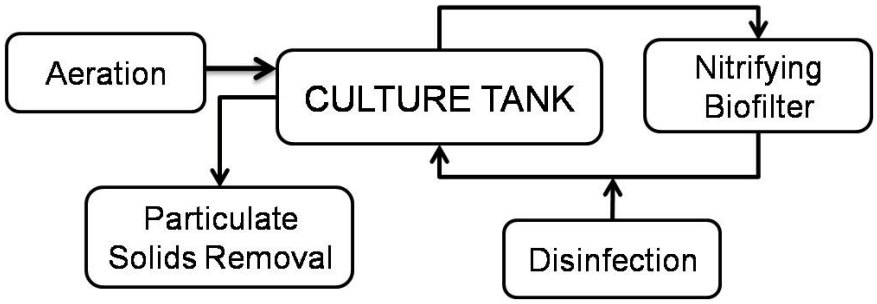

Figure 2.2 Schematic diagram of the unit processes in a recirculating aquaculture system (RAS). Adapted from Ebeling, 2000.

RAS provide benefits such as year-round production of shrimp, disease control, and waste management (Timmons & Ebeling, 2007). They can be operated indoors or outdoors. Both indoor and outdoor RAS can be operated with minimal or no water discharge. However, outdoor systems may have problems with cultured organisms escaping, resulting in the release of non-native species into the environment. Indoor systems allow shrimp farms to be located inland, away from valuable coastal property and close to specialty high-value niche markets, which reduces transportation. Consequently, the focus of this research is on indoor RAS.

RAS are more capital intensive than conventional pond or cage culture. Therefore, they must produce at intensive or ultra-intensive culture levels to be profitable (Timmons & Ebeling, 2007). RAS can support intensive culture conditions better than conventional systems because RAS allow farmers to have a high degree of control over important physical and chemical parameters (e.g., temperature, dissolved oxygen level, pH, and ammonia concentration) in the culture unit. In addition, stress on the shrimp from poor water quality is minimized and shrimp can be produced year-round. Water quality control units play a critical role in RAS operation. These units remove particulate waste solids, oxidize toxic inorganic nitrogen compounds, maintain dissolved oxygen concentrations above 5 mg/L, remove carbon dioxide, and inactivate pathogenic microorganisms (Fig. 2.2, Ebeling, 2000).

The most important process to RAS performance is the nitrifying biofilter(s) in which microorganisms convert ammonium to nitrate. Nitrifying biofilters keep ammonium concentrations below toxic levels through conversion of ammonium to nitrite and then nitrite to nitrate by ammonium-oxidizing and nitrite-oxidizing microbes, respectively (Fig. 2.3). Toxic concentrations of ammonium and nitrite depend on the species being cultured and salinity levels. For Pacific white shrimp, ammonium toxicity levels range from 2.44 to 3.95 mg/l of total ammonium-nitrogen (Lin & Chen, 2001), while nitrite toxicity levels range from 6.1 to 25.7 mg/l of nitrite-nitrogen (Lin & Chen, 2003). Decreases in salinity, in the range of 35 to 15 g/L, result in increased sensitivity to ammonium and nitrite (Lin & Chen 2001, Lin & Chen 2003). RAS utilize various types of biological reactor, or bioreactor, configurations to achieve nitrification. The most commonly used types of bioreactors for nitrification are: upflow or downflow packed bed reactors, trickling filters, floating bead filters, fluidized bed filters, rotating biological contactors (RBC), and moving bed reactors (Timmons & Losordo, 1994, Huguenin & Colt, 2002, Timmons *et al.*, 2002, Timmons & Ebeling, 2007). Parameters important to bioreactor

performance include pH, alkalinity, and concentrations of dissolved oxygen, ammonium-nitrogen, nitrite-nitrogen, and organic matter (particulate and dissolved). Most RAS rapidly remove suspended solids from the culture unit to minimize the growth of heterotrophic bacteria in the biofilter because they out-compete the slow-growing nitrifying bacteria for oxygen and space in the biofilm due to their faster growth rate.

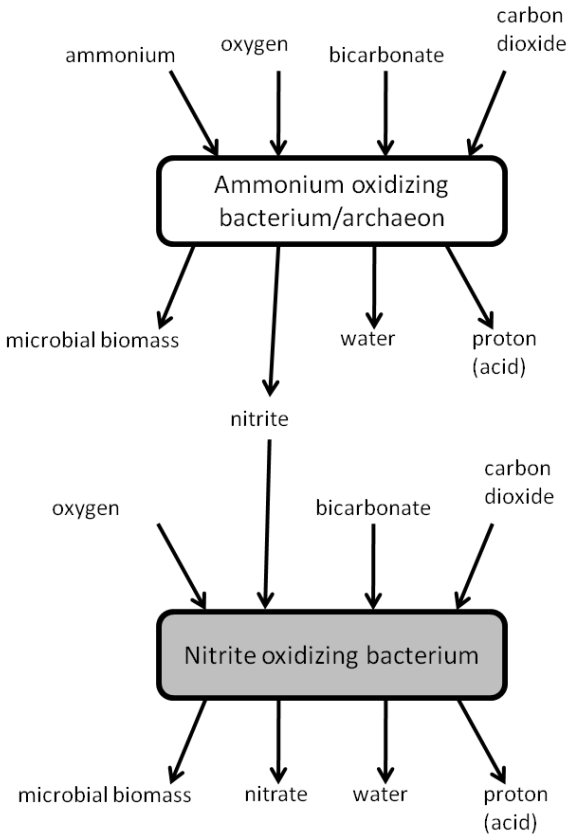

Figure 2.3: Schematic diagram of the nitrification process. The ammonium oxidizing bacterium/archaeon derives energy from the conversion of ammonium to nitrite, while the nitrite oxidizing bacterium derives energy from the conversion of nitrite to nitrate.

2.3 Microbial ecology of RAS biofilters

The activities of various microbes are important to aquaculture in general, but particularly to zero-discharge RAS, in which water quality is maintained solely via biofiltration. To maintain water quality over the long run, and thus reduce the need for water exchange, microbial communities must process excess nitrogen, and the microbes themselves must be controlled such that they do not

accumulate to a density that is detrimental to the operation of the system, e.g. reduce dissolved oxygen concentration below 5 mg/L. Collecting information on the microbial ecology in the water treatment reactors provides insight into the roles of various microbes in the nitrogen transformation processes occurring in the system. This information can be used to enhance the efficiency of microbiological processes through the optimization of operating conditions (e.g., oxygen and nutrient levels) to promote the growth and activity of the desired microbes.

2.3.1 Nitrogen cycle and ammonia removal pathways

The concentrations of various nitrogen species are important in aquaculture because several nitrogen compounds are toxic to aquatic organisms at various concentrations. Un-ionized ammonia is toxic at concentrations of ranging from 0.8 to 2 mg/L ammonia-nitrogen (Timmons & Ebeling, 2007); consequently farmers are advised to maintain total ammonia (sum of un-ionized and ionized ammonia) below 1 mg/L nitrogen (Timmons & Ebeling, 2007). Nitrite above 5 mg/L nitrite-nitrogen changes blood hemoglobin to methemoglobin, which does not carry oxygen and can cause death (Lin & Chen, 2003, Timmons & Ebeling, 2007). Researchers have shown that chronic exposure to nitrate concentrations above 200 mg/L nitrate-nitrogen has a negative impact on growth (Kuhn *et al.*, 2010).

Nitrogen (N) exists in a number of oxidation states in the environment, including zero in nitrogen gas (N_2), negative three in amino groups of proteins, negative three in un-ionized ammonia (NH_3) and ionized ammonia (NH_4^+), positive one in nitric oxide (NO), positive two in nitrous oxide (N_2O), positive three in nitrite (NO_2^-), and positive five in nitrate (NO_3^-). Nitrogen is cycled through these oxidation states via microbially mediated processes (Figure 2.4). Major microbial nitrogen transformations include nitrogen fixation, ammonification, aerobic ammonium oxidation (nitritation) and aerobic nitrite oxidation (collectively referred to as nitrification), denitrification, and anaerobic ammonium oxidation (anammox). Of primary interest to aquaculture systems are ammonia transformation processes which are assimilation, ammonification, nitrification, and anammox. Assimilation is the uptake of ammonia by microbes to create proteins and nucleic acids, and ammonification is the process by which ammonia is released during the decomposition of organic nitrogen compounds such as proteins. Nitrification is the aerobic oxidation of ammonia to nitrite followed by the aerobic oxidation of nitrite to

nitrate. Anammox is the oxidation of ammonium and the reduction of nitrite to form nitrogen gas in environments without oxygen (Strous *et al.*, 1999). Denitrification is an important transformation process in zero-discharge systems that utilize nitrification because in these systems nitrate accumulates to concentrations above 200 mg/L N. The most commonly used ammonia "removal" process in aquaculture and wastewater treatment is nitrification. However, use of anammox has increased in wastewater treatment during the last decade (Kumar & Lin, 2010, Van Hulle *et al.*, 2010, Terada *et al.*, 2011, Bagchi *et al.*, 2012). Since nitrification is the primary ammonia transformation process used in RAS, this review will focus on the microbial ecology of that process.

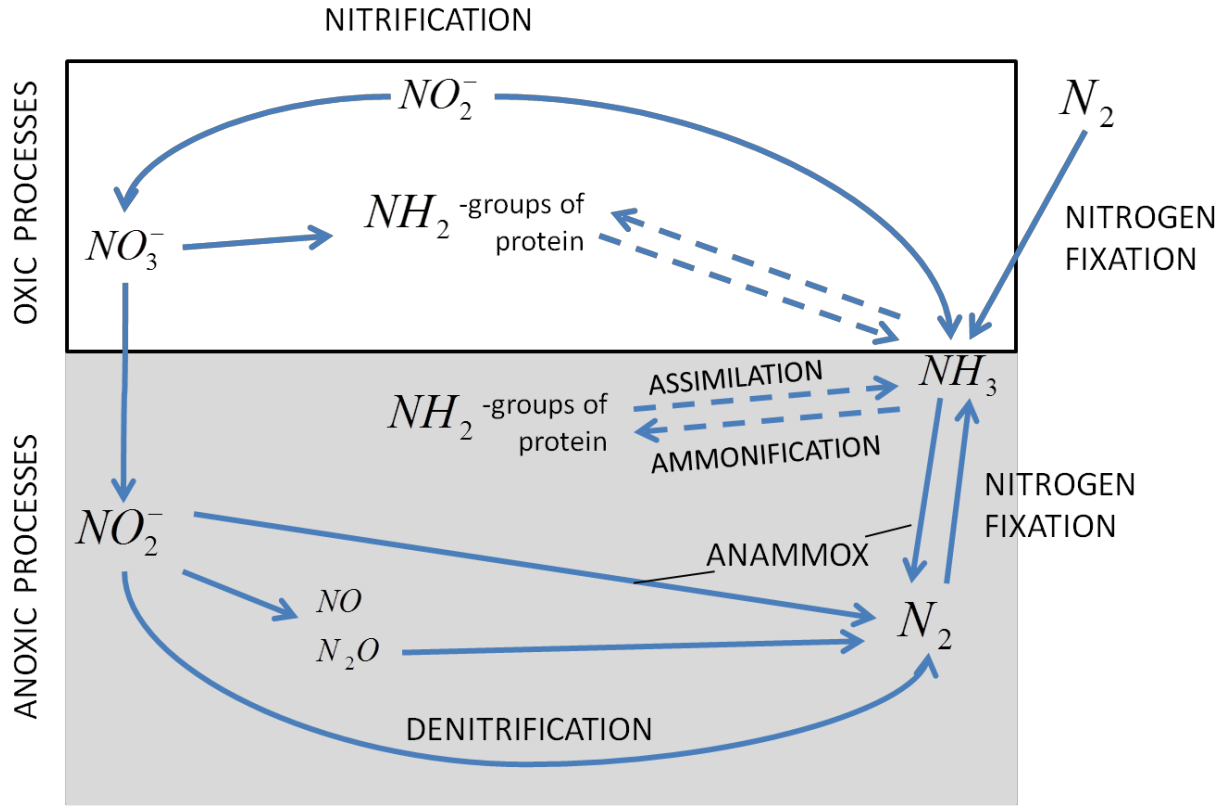

Fig. 2.4 Diagram of the nitrogen cycle (adapted from Madigan *et al.*, 2009). The solid arrows denote oxidation or reduction reactions, and the dashed arrows denote reactions without changes in oxidation state. Anammox reaction is $NH_3 + NO_2^- + H^+ \rightarrow N_2 + 2H_2O$

2.3.2 Aerobic ammonia oxidation

Nitrification is a two step process in which ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB) or ammonia oxidizing archaea (AOA) and nitrite is then oxidized to nitrate by nitrite oxidizing bacteria (NOB) (Figure 2.3, 2.4). The sensitivity of AOB and NOB to a wide variety of environmental factors is well known, so much so that nitrification has been regarded as the "Achilles heel" of wastewater treatment (Daims *et al.*, 2006). In recirculating aquaculture settings, the challenges associated with accumulation of ammonia and nitrite are similar to those in the wastewater treatment field and include problems with low dissolved oxygen levels, pH outside the optimal range for nitrifying microbes (7.5 – 8.6), and accumulation of trace amounts of toxic sulfides due to the activity of sulfate reducing microbes when dissolved oxygen concentrations are low (Joye & Hollibaugh, 1995, Masser *et al.*, 1999, Ling & Chen, 2005). Less is known about the sensitivity of AOA because they have not been under study for as long as the AOB. However, research has shown that AOA have adapted to survive under ammonia limited conditions where AOB cannot grow (Hatzenpichler *et al.*, 2008, Martens-Habbena *et al.*, 2009), AOA utilize a different ammonia oxidation pathway than AOB (Walker *et al.*, 2010), and AOA use a different carbon fixation pathway (Berg, 2011).

AOB mediate the first step in nitrification. They use ammonia as their energy source and carbon dioxide as their carbon source, although some AOB can also use organic carbon as their carbon source (Bock & Wagner, 2013). AOB and were first isolated in the 19th century by Winogradsky from soil (Madigan *et al.*, 2009). Since then, researchers have continued to investigate the diversity of AOB in various terrestrial and aquatic environments. All known AOB share a common ammonia oxidation biochemical pathway, in which ammonia is oxidized to hydroxylamine by an ammonia monoxygenase (AMO) complex and hydroxylamine is oxidized to nitrite by a hydroxylamine oxidoreductase (HAO) complex (Klotz & Stein, 2008, Madigan *et al.*, 2009). These microbes can only respire under aerobic conditions, although some groups may be tolerant of low oxygen or anoxic environments (Schmidt & Bock, 1997). Studies have shown that AOB can reduce nitrite under anoxic conditions to nitrogen gas (Schmidt *et al.*, 2003). The phylogeny of AOB has been inferred from the 16S rRNA gene and the ammonia monooxygenase A (*amoA*) gene. The known AOB belong to two lineages of the *Proteobacteria*, the beta-subclass and the gamma-subclass (Purkhold *et al.*, 2000). The genera *Nitrosomonas, Nitrosospira, Nitrosolobus,* and *Nitrosovibrio* fall within the *Betaproteobacteria*, and the genus *Nitrosococcus*

is affiliated with the *Gammaproteobacteria*. *Betaproteobacteria*-AOB can vary from no salt requirement to obligate halophilic, while *Gammaproteobacteria*-AOB are obligate halophilic (Koops & Pommerening-Roser, 2001). AOB are one of two microbial populations known to oxidize ammonium to nitrite.

AOA is the other microbial population known to oxidize ammonium to nitrite. The first cultivated strain of AOA, *Nitrosopumilus maritimus* strain SCM1, was isolated from a marine aquarium tank in 2005 (Konneke *et al.*, 2005). Reviews of the literature (Prosser & Nicol, 2008, Erguder *et al.*, 2009, You *et al.*, 2009, Hatzenpichler, 2012, Stahl & de la Torre, 2012) indicate that AOA are present in various environments including marine waters, biofilters of aquaria, coral reefs, estuaries, wastewater treatment plants, hot springs, sediments, and soils based on the detection of archaeal *amoA* gene. In most studies in which the abundances of archaeal and bacterial *amoA* gene copies were investigated, the archaeal *amoA* gene copies outnumbered bacterial *amoA* copies (Erguder *et al.*, 2009, Schleper & Nicol, 2010, Hatzenpichler, 2012, Stahl & de la Torre, 2012), which is an indication that AOA are more abundant than AOB in most natural and in some engineered environments. The factors that influence this distribution are still unclear, but researchers have proposed that AOA might be important ammonia oxidizers in low nutrient environments and environments where bicarbonate is the dominant form of inorganic carbon (Erguder *et al.*, 2009, Schleper & Nicol, 2010, Hatzenpichler, 2012). It was originally proposed that the AOA belong to the *Crenarchaeota* kingdom of the domain *Archaea* in the Group I lineage; however, upon further research AOA were suggested to belong to a newly named kingdom "*Thaumarchaeota*" (Brochier-Armanet *et al.*, 2008, Spang *et al.*, 2010, Pester *et al.*, 2011). Several *Thaumarchaeota* strains of AOA exist in enrichment cultures including: a psychrophilic strain *Cenarchaeum symbiosum* (optimal growth at 10°C), which has a symbiotic relationship with a marine sponge (Preston *et al.*, 1996); a moderately thermophilic strain "Candidatus *Nitrososphaera gargensis*," (optimal growth at 46°C), which was enriched from the Siberian Garga hot spring (Hatzenpichler *et al.*, 2008); and a thermophilic strain "Candidatus *Nitrosocaldus yellowstonii*" (optimal growth at 69°C), which was enriched from hot springs in Yellowstone National Park (de la Torre *et al.*, 2008). While some AOA genomes, including the genome of *Nitrosopumilus maritimus SCM1*, suggest mixotrophic growth, to-date experimental results on mixotrophic growth have been mixed (Hatzenpichler, 2012). Organic carbon compounds can promote or inhibit AOA growth depending on the type and concentration. For

example, *Nitrososphaera viennensis*, a soil isolate, grows better in the presence of pyruvate than in purely autotrophic conditions (Tourna *et al.*, 2011). Knowledge on conditions favorable for AOA growth is continually evolving.

The second step of nitrification is mediated by NOB. There are five phylogenetically distinct groups of aerobic NOB, *Nitrobacter, Nitrospina, Nitrococcus, Nitrospira*, and *Nitrotoga*. The genus *Nitrobacter* is part of the *Alphaproteobacteria* and the genera *Nitrospina* and *Nitrococcus* belong to the *Gammaproteobacteria*. The genus *Nitrospira* falls within the separate phylum *Nitrospirae*, which is closely related to the *Deltaproteobacteria* (Koops & Pommerening-Roser, 2001). A cold-adapted betaproteobacterial NOB, Candidatus *Nitrotoga arctica*, was recently cultivated from the Siberian Arctic (Alawi, 2007). Researchers have also identified a nitrite oxidizing bacterium in the phylum *Chloroflexi* (Sorokin *et al.*, 2012). Nitrite oxidizers use the enzyme nitrite oxidoreductase to oxidize nitrite to nitrate (Schmidt *et al.*, 2003, Madigan *et al.*, 2009). For many years, the general consensus was that *Nitrobacter* species were the dominant NOB in most environments because these were isolated most frequently (Abeliovich, 2006). However, since molecular methods have been applied to environmental samples, researchers have found that the dominant nitrite oxidizers in most environments are *Nitrospira* species (Daims *et al.*, 2001, Abeliovich, 2006). Studies have shown that *Nitrospira*-like bacteria can exploit low amounts of nitrite and oxygen more efficiently than *Nitrobacter* (Schramm *et al.*, 1999, Koops & Pommerening-Roser, 2001).

Since nitrifying microbes play a crucial role in the performance of RAS biofilters, researchers have studied these microbes in aquaria, freshwater RAS, and marine RAS. Aquaria and RAS are similar in how they maintain water quality with differences arising from the density and/or variety of animals present. Hovanec and DeLong (1996) indicated that the bacteria thought to be traditionally responsible for nitrification in aquaria, such as *Nitrosomonas europaea* and *Nitrobacter winogradskyi* or close relatives, were not the dominant components of nitrifying freshwater aquaria. On the other hand, *Nitrosomonas europaea* and related species accounted for as much as 20% of the total bacterial rRNA in nitrifying seawater aquaria. Hovanec *et al.* (1998) found that nitrite oxidation in freshwater aquaria was mediated by bacteria closely related to *Nitrospira marina* and *Nitrospira moscoviensis* instead of *Nitrobacter* spp. In a study of the biofilter of a marine RAS culturing shrimp (Brown *et al.*, 2013), the only NOB detected, via 16S rRNA gene targeted clone library and Sanger sequencing and quantitative polymerase chain reaction, were *Nitrospira* spp. Studies evaluating

nitrifier diversity in biofilters of marine aquaria or aquaculture systems have shown that elevated salinity levels select for a less diverse AOB community (Grommen *et al.*, 2005), and various *Nitrosomonas* species including *Nitrosomonas aestuarii* (Itoi *et al.*, 2006), *Nitrosomonas* sp. Nm143 (Itoi *et al.*, 2006), *Nitrosomonas cryotolerans* (Tal *et al.*, 2003), and *Nitrosomonas marina* (Tal *et al.*, 2003, Grommen *et al.*, 2005) have been detected.

2.4 Limitations of Indoor, Zero-Discharge RAS

Indoor, zero-discharge RAS are an alternative technology to pond culture. As with all technologies, RAS have areas that need improvement. These issues include biofilter start-up time, maintaining water quality in a dynamic system, nitrate accumulation, and reliance on commercial feed. It can take several weeks to establish nitrification in a nitrifying biofilter due to the slow growth rate of chemoautotrophic bacteria (Timmons & Ebeling, 2007). One way to solve this issue is to feed the biofilters ammonium once they have been inoculated with biomass from an active nitrifying reactor prior to shrimp addition (Timmons & Ebeling, 2007). The other three concerns listed above are described in more detail below.

2.4.1 Importance of nitrification in zero-discharge RAS

The heart of a zero-discharge RAS facility lies in the biofilters. RAS reliance on chemoautotrophic microorganisms, AOB and NOB, is its weakness, since the microbes that accomplish this task encompass a narrow phylogenetic range, are slow growing, possess limited metabolic versatility, and are sensitive to a variety of environmental factors (Prosser, 1989). Management of microbial communities in zero-discharge RAS is central to process performance, in terms of water quality, shrimp yield, and waste treatment. The presence of AOA, in addition to AOB, provides a more diverse ammonia oxidizing microbial community. Higher diversity should result in biofilters that have a better ability to maintain water quality as concentrations fluctuate over time due to the presence of microbes with similar functions but different niches, such as AOA and AOB. The distribution of AOA and AOB in various environments including in engineered systems is an on-going area of research.

Previous studies have analyzed the nitrifying communities in aquaria and aquaculture biofilms. Researchers have investigated freshwater aquaria (Hovanec & DeLong, 1996, Hovanec *et al.*, 1998,

Burrell *et al.*, 2001, Sauder *et al.*, 2011), marine aquaria (Urakawa *et al.*, 2008), and aquaculture biofilms (Foesel *et al.*, 2008, Keuter *et al.*, 2011, Brown *et al.*, 2013). The work by Hovanec and Delong (1996) indicated that neither AOB nor *Nitrobacter* NOB were present in freshwater aquaria. Hovanec and colleagues (1998) found that nitrite oxidation in freshwater aquaria was mediated by bacteria closely related to nitrite-oxidizing *Nitrospira* instead of *Nitrobacter* species. In several studies, AOA were the dominant ammonia oxidizers (Sauder *et al.*, 2011, Sauder *et al.*, 2012, Brown *et al.*, 2013). Researchers have proposed salinity level (Grommen *et al.*, 2005) and temperature (Urakawa *et al.*, 2008) as factors in determining the niches of ammonium oxidizers. Ammonium concentration has also been suggested as a factor determining the abundance of AOA and AOB (Erguder *et al.*, 2009, Schleper, 2010). In studies of ammonium amended soils, AOB were more abundant than AOA (Taylor *et al.*, 2010, Verhamme *et al.*, 2011). While in studies of freshwater aquarium biofilters (Sauder *et al.*, 2011) and rotating biological contactors treating municipal wastewater (Sauder *et al.*, 2012), AOA were the dominant ammonium oxidizers and their abundance was inversely correlated to ammonium concentration. AOA were also dominant in marine RAS biofilters (Brown *et al.*, 2013) where ammonium concentrations were low. To date no studies have examined the role of ammonium concentration in determining the abundance of ammonia oxidizers in marine RAS.

2.4.2 Nitrate accumulation in zero-discharge RAS

Recirculating systems rely on nitrification for the transformation of ammonia-nitrogen to nitrate-nitrogen, which leads to the accumulation of nitrate-nitrogen in the system. The nitrate concentration in RAS depends on the water exchange rate and the extent of nitrification and denitrification. Discharge of water with large nitrate concentrations from RAS can adversely affect the environment. Nitrate concentrations also need to be monitored from a fish/shrimp growth perspective because nitrate can accumulate to levels that are stressful or toxic. Relative to ammonia and nitrite, nitrate is not as toxic to aquatic organisms (van Rijn, 1996). Consequently, most commercial RAS do not include a nitrate removal unit. Nitrate concentrations below 200 mg/L are acceptable for marine organisms (Huguenin & Colt, 2002, Kuhn *et al.*, 2010), but investigators have reported maximum nitrate concentrations in recirculating systems as high as 400 mg/L (Otte & Rosenthal, 1979). As stocking densities increase and water exchange rates are

reduced to zero, the denitrification process will become more important to RAS operation (Ebeling, 2000).

Microorganisms can reduce nitrate concentrations in RAS two ways. Nitrate reduction pathways can be assimilatory, where nitrate is used as the nitrogen source in biosynthesis, or dissimilatory, in which nitrate is used as an electron acceptor in metabolism. If ammonium is present, it is preferentially used as nitrogen source for cell synthesis. However, when ammonium is unavailable, microbes utilize other compounds as a nitrogen source including nitrate. In terms of energy metabolism, nitrate is a common alternative electron acceptor for cellular respiration when oxygen is not present (Madigan *et al.*, 2009). Both of these pathways are able to reduce nitrate concentrations in RAS.

Dissimilatory denitrification has been the focus of nitrate removal in RAS. Research in this area has been on-going since the 1970s. Investigators studied denitrification in packed bed reactors (Balderston & Sieburth, 1976, Abeysinghe *et al.*, 1996, Sauthier *et al.*, 1998), and an aerobic trickling filter followed by an activated sludge denitrification filter (Otte & Rosenthal, 1979). These studies used an external electron donor source. External electron donor addition increases denitrification efficiency, but it increases the cost of the system. Therefore, a few studies utilized the endogenous organic matter (which includes shrimp feces, microbial biomass, and uneaten feed), in the culture tank as the electron donor: in a digestion basin followed by a fluidized bed reactor (Van Rijn & Rivera, 1990, Aboutboul *et al.*, 1995, Arbiv & van Rijn, 1995, Shnel *et al.*, 2002, Gelfand *et al.*, 2003), a packed bed reactor (Phillips & Love, 1998), activated sludge reactor (Klas *et al.*, 2006a, Klas *et al.*, 2006b), and up-flow fixed bed biofilter (Tal *et al.*, 2009). Researchers have also investigated hydrolysis followed by fermentation of particulate organic matter, collected from a commercial RAS farm, to produce volatile fatty acids for use as a carbon source in denitrification (Suhr *et al.*, 2013). Another technology under investigation for application in aquaculture is electrochemical treatment (Mook *et al.*, 2012) where nitrate is reduced to nitrogen gas on the cathode.

There are other pathways for nitrate removal in aquaculture systems. One such pathway is the BFS in which an organic carbon source is added to the culture tank to promote the growth of heterotrophic bacteria that assimilate ammonium into microbial biomass (Avnimelech, 1999, Avnimelech, 2007). In integrated multi-trophic aquaculture systems (IMTA), production of fed fish/shellfish is combined with an economically valuable organism, e.g., plants, filter feeding

fish, oysters, or clams, that can utilize the metabolic byproducts from fish/shellfish for their growth (van Rijn, 2013). Examples of IMTA are aquaponic systems that co-culture plants (Racocy, 2007), high-rate algal ponds (Metaxa *et al.*, 2006), and constructed wetlands (Zhong *et al.*, 2011). There are multiple ways to manage inorganic nitrogen in aquaculture systems and those that reduce waste and/or recycle nutrients into organisms are the best options in terms of improving the environmental sustainability of aquaculture systems.

2.4.3 Microbial biomass from RAS biofilters as supplemental feed for shrimp

In intensive shrimp culture, the nutritional requirements of the shrimp are typically met through the addition of an artificial feed. This commercial food represents a significant production cost for shrimp farmers. The production of commercial food has a negative environmental impact on fisheries due to the inclusion of fish oil and fish meal from wild-caught fishes (Goldburg & Triplett, 1997, Boyd & Clay, 1998, Naylor *et al.*, 2000). Therefore, reduction in the amount of commercial feed that must be given to shrimp reduces production costs for farmers and improves sustainability of the system because it reduces the need for the use fish meal and fish oil from wild-caught fish.

Researchers are searching for a fishmeal replacement in aquaculture feeds. Fishmeal is important because it provides essential nutrients and is palatable (Suarez *et al.*, 2009). Alternatives to fishmeal must supply essential nutrients, be palatable, and preferably would be cheaper than fishmeal. Fishmeal alternatives under investigation include soy protein (Cruz-Suarez *et al.*, 2009, Salze *et al.*, 2010), soybean meal (Alvarez *et al.*, 2007), and microbial floc meal (Kuhn *et al.*, 2009, Kuhn *et al.*, 2010). Soybean meal is deficient in amino acids and has low digestibility (Gatlin *et al.*, 2007), while soy protein, which is derived from soybean meal, has a better amino acid profile and more digestible proteins (Gatlin *et al.*, 2007, Cruz-Suarez *et al.*, 2009). Microbial floc meal can be obtained from farms that use BFS to manage ammonia. Kuhn *et al*. (2010) replaced fishmeal and/or soybean meal at 10%, 15%, and 21% with microbial floc meal and did not observe significant differences between treatments with microbial floc meal and fishmeal with respect to growth and survival. Bauer *et al.* (2012) evaluated replacing fishmeal with a combination of soy protein and microbial floc meal at 0%, 25%, 50%, 75%, and 100% levels. Both the soy protein and microbial floc meals were processed into feed pellets for

this experiment. This study found that key parameters, including specific growth rate, survival, and feed conversion ratio, were not significantly different between treatments, which indicate that the combination of soy meal and microbial floc meal can replace fishmeal. In addition to plant and microbial floc meal, researchers are also considering reducing aquaculture feed use by supplementing directly with microbial biofilms as discussed below.

Researchers have investigated the impact of natural biota, in the form of biofilms growing in the culture cage, pond, or tank as a supplemental food source for shrimp. Phototrophic biofilms grown with the shrimp contributed significantly to the growth of *Farfantepenaeus paulensis* (Thompson *et al.*, 2002, Abreu *et al.*, 2007, Ballester *et al.*, 2007, Fernandes Da Silva *et al.*, 2008), *Litopenaeus vannamei* (Bratvold & Browdy, 2001, Moss & Moss, 2004, Otoshi *et al.*, 2006, Zarain-Herzberg *et al.*, 2006, Lezama-Cervantes & Paniagua-Michel, 2010), *Penaeus esculentus* (Burford *et al.*, 2004), and *Penaeus monodon* (Arnold *et al.*, 2006). In these studies, a biofilm attachment surface was provided to promote the development of a biofilm for the shrimp to graze upon. Researchers (Fernandes de Silva et al., 2008) also examined the contribution of microorganisms to the protein and lipid content of the biofilm nutritional quality. They found that protein content varied from 0.43-1.76 mg protein per cm^2 of biofilm area, while lipid content ranged between 1.21-4.23 mg lipid per cm^2 of biofilm area. The variation in protein content was related to the abundance of unicellular centric diatoms and nematodes, while the variation in lipid content was related to the abundance of heterotrophic bacteria, flagellates, and nematodes. An evaluation of biofilm as a food source using stable isotope, $\delta^{13}C$ and $\delta^{13}N$, analysis (Abreu *et al.*, 2007) showed that biofilm contributed more than 49% of carbon and 70% of nitrogen to shrimp growth. These studies have examined biofilm contribution to shrimp growth in environments where phototrophic microorganisms are a significant part of the microbial community. However, the microbes present in RAS are predominately bacteria under indoor, non-greenhouse culture conditions.

Section 2.3 focused on the microbial ecology of ammonia- and nitrite-oxidizing microbes because of their importance in ammonia removal. Heterotrophic bacteria are also present in the biofilters of RAS. This is due to the presence of organic compounds in the system even when solids, from uneaten feed and shrimp feces, are removed. A solids removal filter, in addition to the nitrifying biofilter, is an ideal environment for heterotrophic bacterial growth. The growth of heterotrophic bacteria requires that filters be backwashed frequently to remove excess microbial

biomass and prevent short circuiting. As indicated above, biofilm growth with shrimp and microbial flocs grown in separate bioreactors have had a positive impact on shrimp performance. Therefore, it is reasonable to suggest that biofilm from RAS biofilters may also have a positive impact on shrimp performance. To date no studies have examined the impact of biofilm from RAS biofilters on shrimp growth.

2.5 Microbial resource management in indoor RAS

Microorganisms play an important role in RAS by maintaining water quality and serving as a food source for shrimp. Consequently, more knowledge of biological nitrogen conversion processes in indoor RAS can provide farmers with the information needed to make operational modifications to promote growth conditions for the desired microbial community (Chapters 3 and 4). In addition to water quality, excess microbial biomass collected from RAS biofilters can serve as a supplemental feed source resulting in the reduction of commercial feed (Chapter 5).

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Chapter 3: Ammonia oxidizing archaea and nitrite oxidizing nitrospiras in the biofilter of a shrimp recirculating aquaculture system

3.1 Introduction

Recirculating aquaculture systems (RAS) for the production of marine shrimp are a potentially sustainable alternative to traditional aquaculture systems because RAS reduce water requirements and limit the concentration of nutrients discharged to receiving waters. RAS require biological filters to oxidize toxic ammonia and nitrite, and aeration of the water to remove carbon dioxide and increase oxygen concentrations (Ebeling, 2000). Nitrifying biofilters keep ammonia and nitrite concentrations below toxic levels. For the white shrimp, *Litopenaeus vannamei* (Boone), ammonia toxicity levels range from 2.44 to 3.95 mg/l of total ammonia nitrogen (Lin and Chen, 2001), while nitrite toxicity levels vary between 6.1 and 25.7 mg/l of nitrite-nitrogen (Lin and Chen, 2003).

Nitrification is a two step process in which ammonia is oxidized to nitrite by ammonia oxidizing bacteria (AOB) or ammonia oxidizing archaea (AOA) and nitrite is oxidized to nitrate by nitrite oxidizing bacteria (NOB). The sensitivity of AOB and NOB to a wide variety of environmental factors is well known, so much so that nitrification has been regarded as the "Achilles heel" of wastewater treatment (Daims *et al.*, 2006). In recirculating aquaculture settings, the challenges associated with accumulation of ammonia and nitrite are similar to those in the wastewater treatment field, and also include problems with low dissolved oxygen levels, pH outside the optimal range for nitrifying microbes (7.5 – 8.6), and accumulation of trace amounts of toxic sulfides (Joye and Hollibaugh, 1995; Masser *et al.*, 1999; Ling and Chen, 2005).

Less is known about the sensitivity of AOA to environmental parameters. Archaeal ammonia monooxygenase subunit A (*amoA*) gene has been found to be ubiquitous in the environment, including in marine waters, biofilters of aquaria, coral reefs, estuaries, wastewater treatment plants, hot springs, sediments, and soils (Prosser and Nicol, 2008; Erguder *et al.*, 2009;

You *et al.*, 2009). In most studies in which the abundances of archaeal and bacterial *amoA* gene copies were investigated, the archaeal *amoA* outnumbered the bacterial *amoA* gene copies (Erguder *et al.*, 2009). The factors that influence this distribution are still unclear, but Erguder and colleagues (2009) proposed that AOA might be important ammonia oxidizers in low nutrient, low pH, and sulfide containing environments. Furthermore, Martens-Habbena *et al.* (2009) have shown that AOA have adapted to survive at low ammonia concentrations, e.g., 0.2 µM, concentrations at which AOB cannot grow.

There are four validly described genera of NOB: *Nitrobacter*, *Nitrospina*, *Nitrococcus*, *Nitrospira*, as well as a newly communicated NOB species, "Candidatus Nitrotoga arctica" (Bartosch *et al.*, 1999, Alawi *et al.*, 2007). *Nitrospira* spp. have been found to be the main nitrite oxidizers in wastewater treatment plants (Juretschko *et al.*, 1998; Burrell *et al.*, 1999, Daims *et al.* , 2000, Daims *et al.*, 2001, Daims *et al.*, 2006) and the biofilters of a marine RAS (Keuter *et al.*, 2011) because they are better scavengers for nitrite and oxygen than *Nitrobacter* spp. (Schramm *et al.*, 1999; Koops and Pommerening-Roser, 2001). *Nitrospira* spp. have also been found in a hot spring (Lebedeva *et al.*, 2011), a marine sponge (Off *et al.*, 2010), and various soils (Bartosch *et al.*, 2002). An analysis of the publicly available sequences of the *Nitrospirae* phylum (Daims *et al.*, 2001) revealed that the genus *Nitrospira* consists of at least four distinct sublineages.

Nitrification is important to aquaculture in general, but particularly to zero discharge RAS, in which water quality (specifically ammonia and nitrite concentrations) is maintained by biofiltration. The success of these systems depends on stable and reliable performance of its biofilters. The long-term goal of this work is to understand the nitrogen cycling processes in zero-discharge RAS. In the present study, we examined the nitrifier community in the biofilter of a marine, zero discharge, indoor, recirculating shrimp maturation system, a type of RAS that was operated for the growth and reproduction of *L. vannamei*. We evaluated the identities and abundances of nitrifying bacteria and archaea and found AOA and nitrospiras to be the dominant nitrifiers. Few studies have characterized both AOA and nitrite oxidizers in marine systems that allow for process control. In addition to their industrial importance, such systems may serve as important model systems to improve our understanding of the ecophysiology of AOA and NOB.

3.2 Materials and Methods

3.2.1 *Sample Collection*

Samples were collected from an indoor, zero discharge, marine RAS shrimp farm in Okemos, MI (Fig. S1). At the time of sampling, the system had been run continuously for three years with minimal water exchange and stable production, although not at intensive levels (>100 shrimp/m^2 culture area; Fast and Lester, 1992). Because the facility is located away from the coast, artificial seawater was prepared from a commercial salt solution to fill the system. Water quality in the culture tank was measured on site as follows: Ammonium, nitrite, and nitrate concentrations were measured using colorimetric assays using Hach kits; dissolved oxygen and temperature were measured with a YSI model 55 DO meter (Yellow Springs, OH), salinity was measured using a YSI model 30 salinity meter (Yellow Springs, OH), and pH was determined with a Mettler-Toledo SevenGo portable pH meter (Schwerzenbach, Switzerland). All samples for biomass analysis were placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI), and three replicate samples were collected from each location. Samples were collected using sterile equipment from four locations: the culture tank, the bioballs compartment, the oyster shell compartment, and sludge from the basin beneath the filter tower. All samples were stored on ice during transport to the lab and processed within 24 hours.

3.2.2 DNA extraction

DNA from two replicate samples collected from the four RAS compartments were extracted using the following procedures: For water samples, each replicate consisted of DNA extracted from the pellet obtained after centrifuging 250 mL tank water at 3,220 *g* for 30 min. For the bioball compartment, each replicate consisted of DNA extracted from the biofilm stripped from two bioballs. Biofilm (average of 260 mg fresh weight) was stripped by immersing each bioball in phosphate buffered saline (PBS; 130 mM NaCl, 10 mM sodium phosphate buffer [pH 7.2]) and manually brushing the surface with an endocervical brush while sonicating for two minutes using a sonicator bath (L&R, Kearny, NJ). Each sludge sample (average of 560 mg fresh weight) was obtained by centrifuging 1 mL of the sample at 5,000 *g* for 10 min. The oyster shells (average of 260 mg fresh weight) were placed directly into bead beating tubes used for DNA extraction. DNA was extracted from all biomass samples using the FastDNA Spin Kit for Soil (MP Biomedicals, Solon, OH) according to the manufacturer's protocol. Extracted DNA in each

sample was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE).

3.2.3 PCR amplification

Duplicate PCR reactions for each sample replicate were run for each primer set. The primers 8F (Lane, 1991) and 1387R (Marchesi, *et al.*, 1998) were used to amplify the bacterial 16S rRNA gene (Briones, *et al.*, 2007). The archaeal 16S rRNA gene was amplified with the primer set 109f/934b (Grosskopf, *et al.*, 1998). PCR reactions were 50 µL and each reaction contained 5 µL of 10X buffer, 200 µM of each dNTP, 2 mM $MgCl_2$, 0.2 µM of each primer, 1.25 units of *Taq* Polymerase (ExTaq DNA polymerase, Takara Bio USA, Clontech Laboratories Inc., Madison, WI) and 1 µL template.

Archaeal *amoA* gene fragments were amplified using primers Arch-amoAF (Francis, *et al.*, 2005) and Arch-amoARmod (5'-TTWGACCARGCGGCCATCCA-3'; this work). The PCR mixture was as described above. Thermal cycling consisted of initial denaturation of 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min; final extension was at 72°C for 19 min. Bacterial *amoA* gene fragments were amplified using primers amoA-1F/amoA-2R (Rotthauwe, *et al.*, 1997). The PCR mixture was as described above. Thermal cycling consisted of initial denaturation of 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51.5°C for 30 s, and extension at 72°C for 30 s; final extension was at 72°C for 10 min. All PCR results were confirmed with agarose gel electrophoresis.

3.2.4 Cloning, sequencing, and phylogenetic analysis

Triplicate PCR reactions were first pooled and purified using the QIAquick® PCR Purification Kit (Qiagen, Germantown, MD) and then the appropriate band was gel extracted as follows except for the archaeal 16S rRNA product. The bacterial 16S rRNA (1396 bp), archaeal *amoA* (645 bp) and bacterial *amoA* (491 bp) PCR products were run on 0.8%, 2%, and 2%, respectively, agarose gels. The desired bands were excised and purified using the MinElute Gel Extraction Kit (Qiagen, Germantown, MD). The amplified archaeal 16S rRNA genes were purified using the UltraClean® PCR Clean-Up Kit (MO BIO Laboratories, San Diego, CA). The PCR products for archaeal 16S rRNA, archaeal *amoA*, and bacterial *amoA* genes were pooled before cloning to create each respective clone library for the RAS system. All purified PCR

products were cloned using a TOPO TA cloning kit (Invitrogen Corp., San Diego, CA) according to the manufacturer's protocol. All sequencing was done at the Genome Sequencing Center at Washington University, School of Medicine, except for the AOB *amoA* gene sequencing, which was performed by Agencourt Bioscience Corporation (Beverly, MA).

The archaeal and bacterial 16S rRNA gene sequences were aligned with the NAST alignment tool (DeSantis, *et al.*, 2006a) available at the Greengenes website (greengenes.lbl.gov). The aligned sequences were chimera-checked using the Bellerophon version 3 tool (DeSantis, *et al.*, 2006b) at the Greengenes website. The aligned, non-chimera sequences were classified using the classification tool at the Greengenes website. The archaeal and bacterial *amoA* and nitrite oxidizing bacteria (NOB) 16S rRNA gene sequences were aligned using MEGA version 4 (Tamura, *et al.*, 2007). From the alignment, phylogenetic analyses were conducted in MEGA version 4 (Tamura, *et al.*, 2007).

Sequences obtained in this study have been deposited in GenBank under the following Accession Numbers: HM345608-HM345611 (archaeal *amoA* clones), HM345612-HM345622 (bacterial *amoA* clones), and HM345623-HM345625 (*Nitrospira* spp. clones).

3.2.5 Quantitative PCR

Quantitative PCR (qPCR) was used to quantify the AOA and AOB *amoA* gene abundance as well as *Nitrospira* 16S rRNA gene abundance. All sample and standard reactions were carried out in triplicate using SYBR green chemistry on a Mastercycler ep realplex (Eppendorf, North America, Hauppauge, NY) qPCR machine. Standard curves were generated for all experiments from plasmids containing cloned *amoA* or 16S rRNA gene PCR amplicons previously sequenced to verify identity. Samples were diluted to contain 10 ng per µL DNA. All qPCR assays were carried out in 25 µL reactions consisting of 1 µL template DNA, 100 nM (archaeal *amoA*) or 300 nM of each primer (bacterial *amoA* and *Nitrospira* 16S rRNA gene), and 12.5 µL 2x Quantitect MasterMix (Quantitect, Qiagen, Germantown, MD). The specificity of amplification for all qPCR assays was verified via generation of melting curves and agarose gel electrophoresis.

Archaeal *amoA* gene copies were quantified using primers AOA-amoA-fm (5'-TTCTAYACTGACTGGGCYTGGACATC-3') and AOA-amoA-rb (5'-AKGCCGTTTCTAGTGGGTCWGCTA-3'). These primers were modified from the primer set

AOA-amoA-f/AOA-amoA-r (Coolen, *et al.*, 2007) based on the archaeal *amoA* clone sequences obtained in this study. Linear response (R^2 = 0.97) was observed for plasmids containing archaeal *amoA* between 10^1-10^7 gene copies per µl template DNA; PCR efficiency was 1.78. The PCR conditions were as follows: 94°C for 15 min, followed by 43 cycles consisting of 94°C for 15 s, 58.5°C for 30 s, 72°C for 30 s. The one-point calibration method for absolute quantification, as described by Brankatschk, *et al*. (2012), was used to calculate the gene abundance. The LinRegPCR Program (v 2012.0) (Ruijter, *et al.*, 2009) was used to calculate cycle threshold, C_T, and PCR efficiency, E, values for samples and standards from amplification data. The mean E values for samples were 1.34 for tank water, 1.34 for bioballs, 1.29 for oyster shells, and 1.37 for sludge. The mean E value for the standard used in the one-point calibration was 1.40.

Bacterial *amoA* was quantified using the primers amoA-1F/amoA-2R (Rotthauwe, *et al.*, 1997). Linear response (R^2 = 0.99) was observed for plasmids containing bacterial *amoA* between 10^1-10^7 gene copies per µl template DNA and the PCR efficiency was 1.87. The PCR conditions were as follows: 95°C for 15 min, followed by 45 cycles consisting of 95°C for 30 s, 52°C for 30 s, 72°C for 30 s. The standard curve method for absolute quantification was used to calculate gene abundance. NOB 16S rRNA genes were quantified using the primer sets Ntspa4-821f/Ntspa4-1028r and Ntspa2-172f/Ntspa2-311r for Type IV nitrospiras (*N. marina* sublineage) and Type II nitrospiras (*N. moscoviensis* sublineage), respectively. The one-point calibration method for absolute quantification was used to calculate gene abundance as described for AOA *amoA.*

Ntspa4-821f (5'-GGGYACTAAGTGTCGGCGGT-3') and Ntspa4-1028r (5'-RGSTCMTCMCCCTTTCAGGT-3') were designed based on the sequences obtained in this study. Primer design and evaluation of specificity was performed using Primer3 software (Rozen & Skaletsky, 2000) implemented in the Primer-BLAST website (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Optimization of annealing temperatures for each primer pair for real time PCR was conducted using the gradient function of the Mastercycler ep realplex machine. Linear response (R^2 = 0.99) was observed for plasmids containing *N. marina* 16S rRNA between 5 and $1x10^6$ gene copies per µL template DNA; PCR efficiency was 1.90. The PCR conditions were as follows: 95°C for 15 min, followed by 44 cycles consisting of 94°C for 15 s, 57°C for 30 s, 72°C for 30 s. Mean E values for samples were

1.34 for bioballs and 1.38 for oyster shells. Mean E for standard used in one-point calibration was 1.37.

Ntspa2-172f/ (5'-ATACCGCATACGRCTCCTGG-3') and Ntspa2-311r (5'-GCTGATCGTCCTCTCAGACC-3') were designed based on the sequences obtained in this study. Linear response (R^2 = 0.99) was observed for plasmids containing *N. moscoviensis* 16S rRNA between 5 and $1x10^6$ gene copies per µl template DNA; PCR efficiency was 1.77. The PCR conditions were as follows: 95°C for 15 min, followed by 44 cycles consisting of 94°C for 15 s, 61.5°C for 30 s, 72°C for 30 s. The mean E values for samples were 1.25 for bioballs and 1.27 for oyster shells. The mean E value for standard used in the one-point calibration was 1.19.

3.3 Results and Discussion

Samples were collected from an indoor, zero discharge, marine RAS shrimp farm in Okemos, MI. At the time of sampling (May 17, 2007), the system had been run continuously for three years with minimal water exchange and stable production, although not at intensive levels. Because the facility is located away from the coast, artificial seawater was prepared from a commercial salt solution to fill the system. The RAS relies on biofiltration in a multi-stage, nitrifying trickling filter that contains multiple types of biofilm attachment media, including plastic bioballs, plastic corrugated block, and crushed oyster shells (Appendix I, Figure A1.1). The bioballs (polyethylene, Aquatic Eco-System Inc., Apopka, FL) had a diameter of 3.5 cm with a specific surface area of 525 m^2/m^3. The crushed oyster shells are used as a supplement for chicken feed and were obtained from an animal feed store. Stocking densities for this maturation system are 6-9 shrimp per square meter of tank area (personal communication with farmer), which is considerably less than typical stocking densities of ultra-intensive production systems (>100 shrimp per square meter of tank area (Fast, 1991)). In this system, water from the culture tank is pumped and filtered by gravity through the different biofilm attachment media. Beneath the filter tower is a basin to collect water and settled particles (sludge) before the water is pumped back into the culture tank.

To analyze the composition of the microbial community in this RAS, we constructed 16S rRNA gene clone libraries of *Archaea* (Appendix I, Figure A1.2), *Bacteria* (Table A1.1), and *Planctomycetes* (data not shown). Bacterial clone libraries were generated using biomass

collected from each sampling location in the RAS (tank water, bioballs, oyster shells, and sludge; Figure A1.1). However, the archaeal and *Planctomycetes* libraries were generated using pooled PCR products obtained from DNA extracted separately from biomass samples obtained from each of the four sampling locations. Analysis of the archaeal 16S rRNA clone library (Figure A1.2) revealed that 17% of clones were *Nitrosopumilus*-type, a group related to AOA (Könneke *et al.*, 2005). Analysis of the bacterial clone libraries failed to detect any representatives of the proteobacterial AOB and NOB (Table S1). However, NOB from the genus *Nitrospira* were detected (Table A1.1). No sequences closely related to known anaerobic ammonium-oxidizing bacteria were detected in the *Planctomycetes* clone library (data not shown).

To confirm the presence of AOA and to determine their distribution within the RAS, a PCR assay was used to detect archaeal *amoA* genes in biomass samples collected from tank water, bioballs, oyster shells, and sludge (data not shown). The initial PCR result indicated the presence of AOA *amoA* genes, and revealed the highest abundance in the samples obtained from oyster shells (data not shown). These results were confirmed by a quantitative PCR (qPCR) assay targeting archaeal *amoA* (Figure 3.1). The abundance of archaeal *amoA* in the biomass attached to oyster shells ($7.1 \times 10^4 \pm 2 \times 10^4$ copies archaeal *amoA* per ng DNA) was higher than the abundance of archaeal *amoA* associated with bioballs ($P < 0.5$, two-sample t test) and an order of magnitude higher than the abundance associated with tank water ($P < 0.25$) and sludge ($P < 0.5$). Sequence analysis of the archaeal *amoA* PCR products (Figure 3.2) showed that most of the sequences amplified were related to the previously described *Nitrosopumilus* species (Könneke *et al.*, 2005) confirming our initial identification based on analysis of 16S rRNA genes. The sequences were obtained from two replicate samples pooled together to generate one clone library. A total of 24 clones related to *Nitrosopumilus* sequences were obtained, of which 16, five, two, and one clones clustered within groups SF_AOA_A07, SF_AOA_A10, SF_AOA_C12, and SF_AOA_H10, respectively.

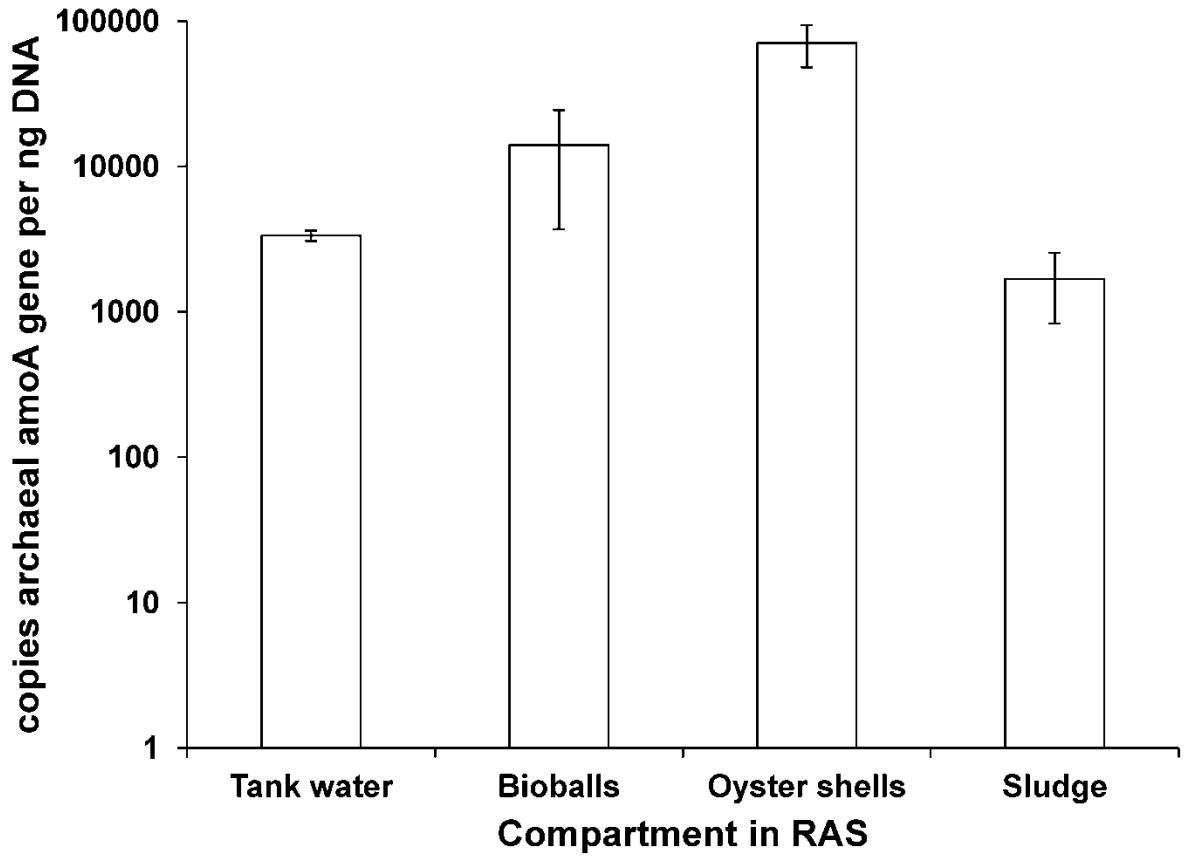

Figure 3.1**:** Archaeal *amoA* gene abundance in four different RAS samples (Figure A1.1), as measured by qPCR. Error bars show standard deviation.

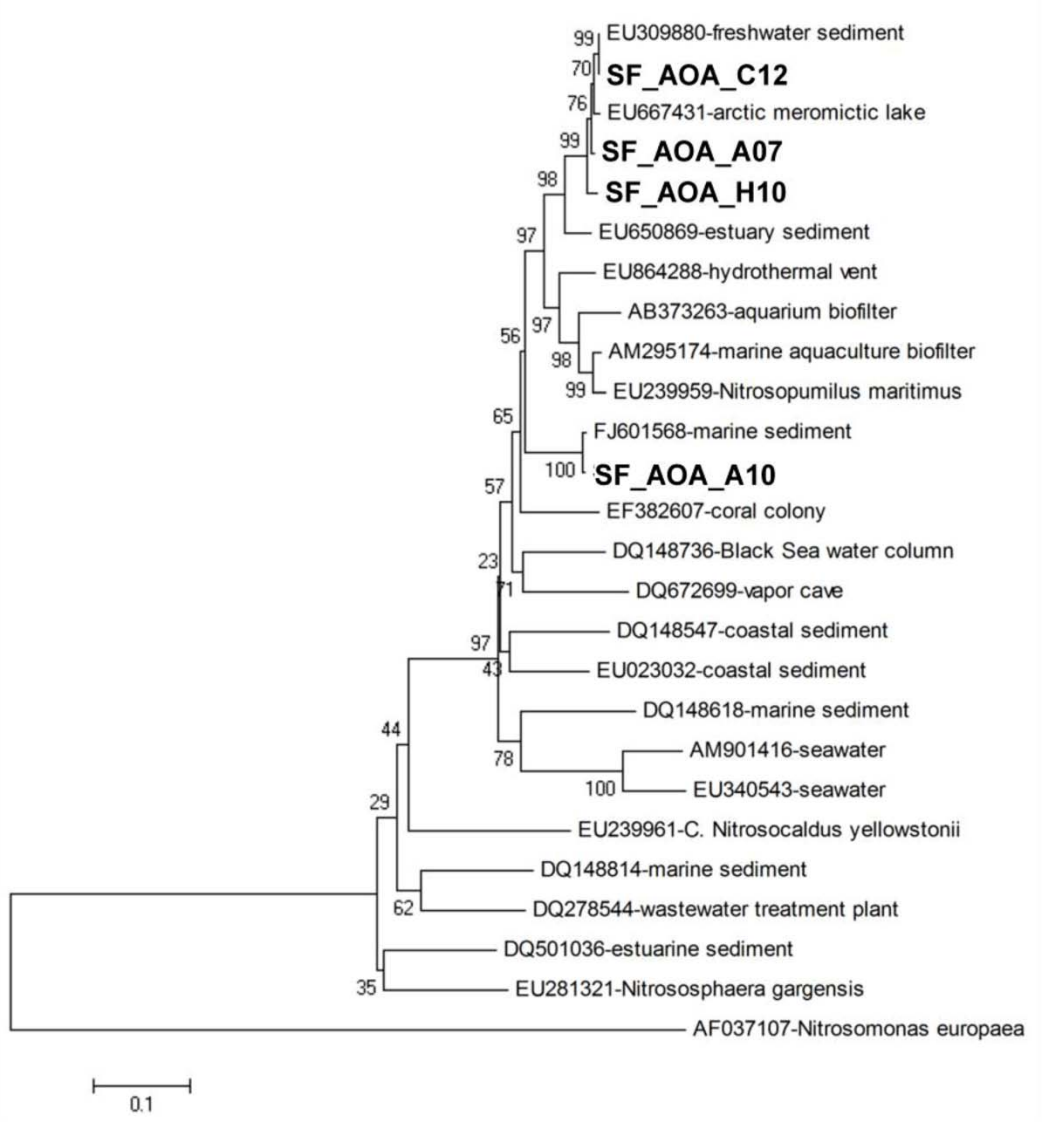

Figure 3.2: Phylogenetic relationships of archaeal *amoA* gene. Sequences obtained in this study are indicated with the prefix "SF_"**.** The tree was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 1,000 replicates (Felsenstein, 1985) is taken to represent the evolutionary history of the sequences analyzed (Felsenstein, 1985). The percentage of replicate trees in which the associated sequences clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site.

The bacterial 16S rRNA gene clone libraries did not detect the presence of AOB. However, using a PCR assay targeting the betaproteobacterial *amoA* gene, AOB were detected, but only in bioball samples (data not shown). These results were confirmed by a qPCR assay targeting bacterial *amoA* (Figure 3.3). The abundance of bacterial *amoA* in the biomass attached

to bioballs (83 ± 15 copies bacterial *amoA* per ng DNA) was approximately an order of magnitude higher than in the biomass associated with tank water, oyster shells, and sludge (Figure 3.3). Sequence analysis of bacterial *amoA* PCR products (Figure 3.4) showed that 98.5% of the sequences amplified were related to obligately halophilic *Nitrosomonas marina*. One clone, designated SF_AOB_C09, was found to be closely related to *Nitrosomonas aestuarii*, which was isolated from brackish water and is closely related to *N. marina* (Purkhold *et al.*, 2000). In summary, the AOB population in the RAS was not abundant and consisted of AOB belonging to the *N. marina*-cluster of betaproteobacterial-AOB (Purkhold *et al.*, 2000).

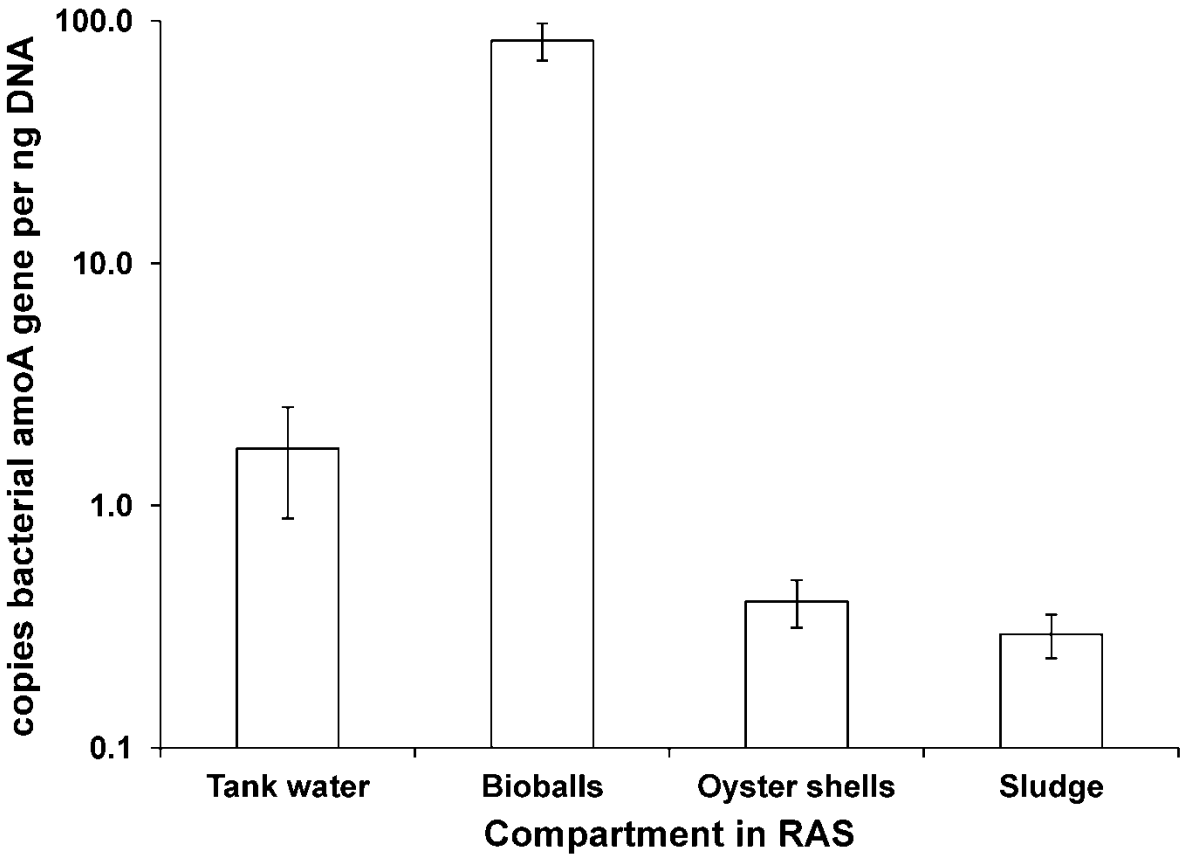

Figure 3.3: Bacterial *amoA* gene abundance in four different RAS samples (Figure A1.1), as measured by qPCR. Error bars show standard deviation.

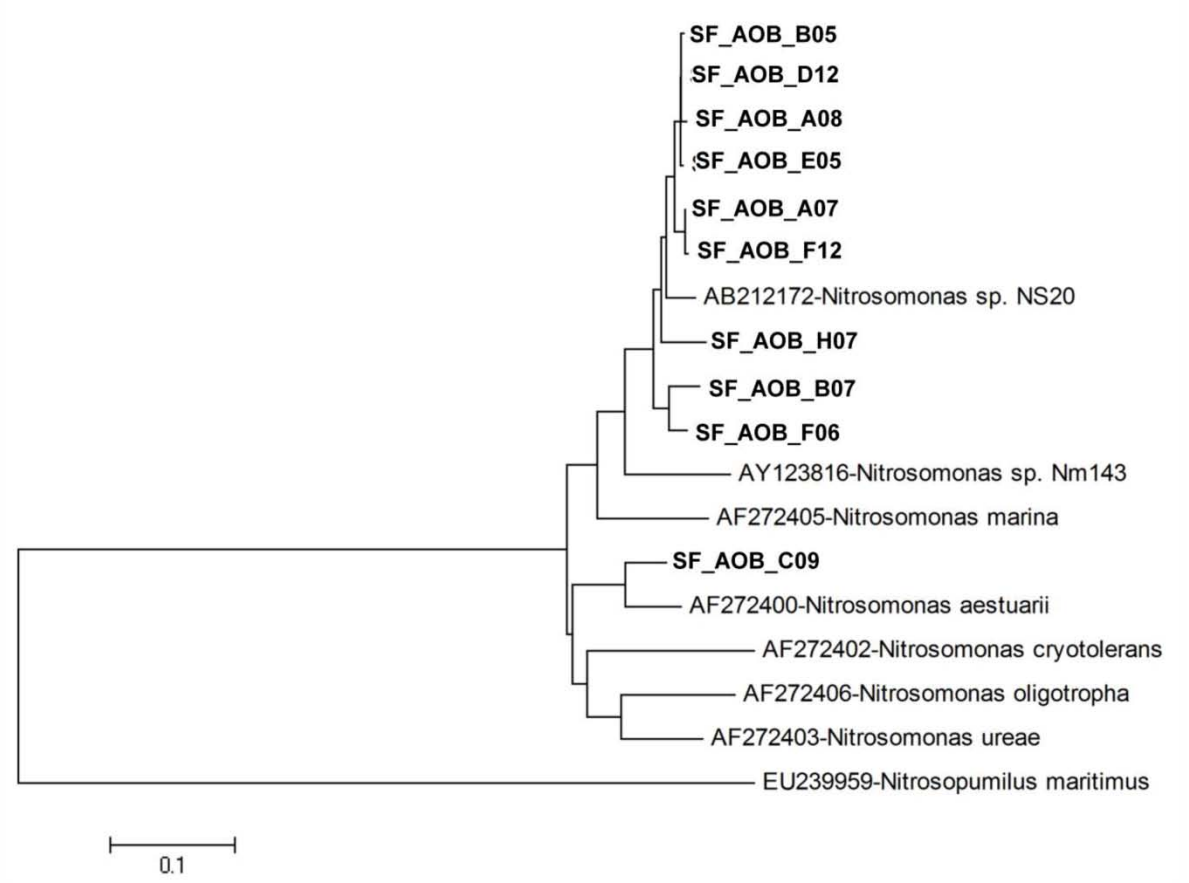

Figure 3.4: Phylogenetic relationships obacterial *amoA* genes. Sequences obtained in this study are indicated with the prefix "SF_"**.** The tree was created as described in the caption for Figure 3.2.

The presence of a functional gene does not necessarily correlate to activity. However, the relatively low abundance of bacterial *amoA* genes in this marine RAS as compared to archaeal *amoA* genes suggests that AOB played a minor role in the function of the biofilter at the time of sampling.

NOB belonging to the phylum *Nitrospirae* were detected in the bacterial 16S rRNA gene clone libraries (supplemental information, Table A1.1) of the bioballs (14.3% of clones) and oyster shells (3.4% of clones). Phylogenetic analysis of these clones revealed that they belong to *Nitrospira* sublineage IV (*N. marina* sublineage) and sublineage II (*N. moscoviensis* sublineage) (Daims *et al.*, 2001), (Figure 3.5). This is consistent with the results from other researchers who analyzed the nitrifier community in freshwater aquarium (Hovanec *et al.*, 1998) and marine aquaculture (Foesel *et al.*, 2008, Keuter *et al.*, 2011) biofilters. A qPCR assay was used to

quantify the abundance of NOB types in the RAS (Figure 3.6). Both *N. marina* and *N. moscoviensis*-like NOB were detected in the bioballs and oyster shell compartments of the biofilter. *N. marina*-like NOB ($3.8x10^4 \pm 2.2x10^4$ copies 16S rRNA gene per ng DNA) were an order of magnitude more abundant than *N. moscoviensis*-like NOB in the biomass obtained from the bioballs, while their abundances were similar in the oyster shell biomass.

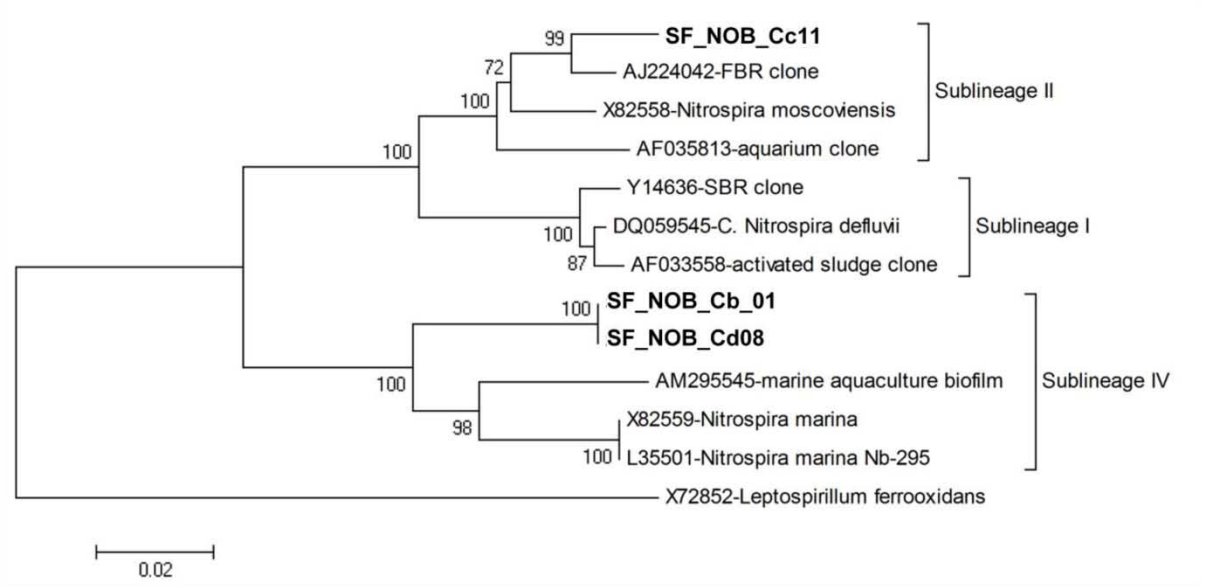

Figure 3.5: Phylogenetic relationships of NOB 16S rRNA genes. The tree was created as described in the caption for Figure 3.2.

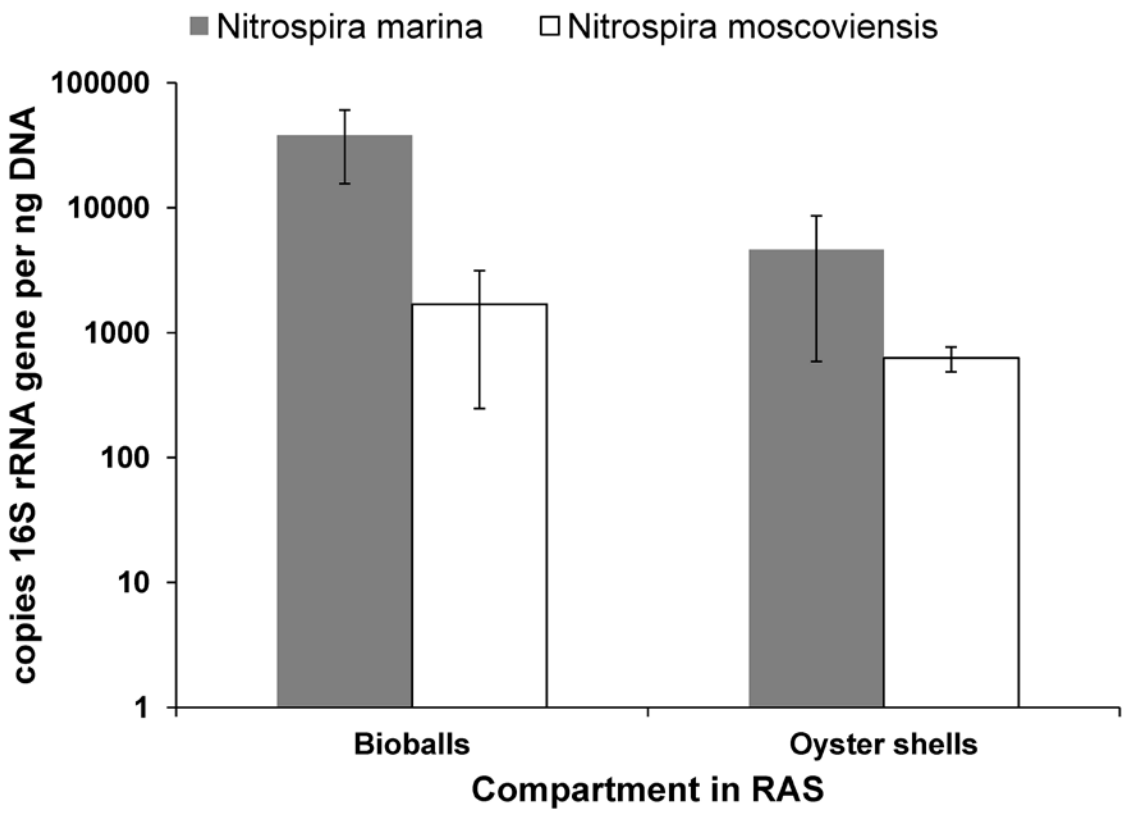

Figure 3.6: NOB 16S rRNA gene abundance in two biofilter compartments (Figure A1.1), as measured by qPCR. Error bars show standard deviation.

The water quality and biofilm attachment media likely play important roles in determining the relative levels of AOA versus AOB and *N. marina*- versus *N. moscoviensis*-type NOB. Low ammonia production rates are expected in the maturation system because of the relatively low shrimp stocking density (6-9 shrimp per square meter of tank area). The water quality data collected for a sample obtained from the culture tank (0.25 mg/L ammonium-N, nitrite-N below detection, 25 mg/L nitrate-N, 6.5 mg/L dissolved oxygen (DO), 26.3 practical salinity units, and pH 8.8) indeed indicated that ammonium and nitrite oxidation in the biofilter were effective in maintaining low levels of ammonium and nitrite in the culture tank, suggesting that AOA and *N. marina*-like NOB were most competitive at low substrate concentrations. Specifically, *Nitrosopumilus*-type AOA were more abundant than *Nsm. marina*-type AOB at the time of sampling. Others have shown that AOA have adapted to survive under ammonia limited conditions where AOB cannot survive (Martens-Habbena *et al.*, 2009). The abundance of AOA

may also be due to mixotrophic or heterotrophic growth of AOA (Prosser and Nicol, 2008). Similarly, *Nitrospira* spp. were the dominant NOB at the time of sampling, which was likely due to the low nitrite concentrations in the biofilter. Schramm *et al.* (1999) proposed that *Nitrospira*-like bacteria are *K*-strategists that grow efficiently at low nitrite and oxygen concentrations. The results obtained by a study characterizing NOB in a nitrifying sequencing batch biofilm reactor with a nitrite concentration gradient ranging from zero to 50 mg nitrite-N per liter confirmed this K/r-hypothesis (Daims *et al.*, 2001). A recent study examining the nitrifying populations of two municipal wastewater treatment plants (Whang *et al.*, 2009) detected the presence of both *N. marina*-like and *N. moscoviensis*-like bacteria. In the current study, *N. marina*-like NOB were significantly more abundant than *N. moscoviensis*-like NOB in the bioballs ($P < 0.01$, two-sample t test) and oyster shells ($P < 0.05$), which was likely due to the advantage that *N. marina* has in halophilic environments. *N. marina* is obligately halophilic (Watson *et al.*, 1986b), whereas *N. moscoviensis* has no salt requirement (Ehrich *et al.*, 1995). In addition to salinity, the metabolic versatility of *N. marina*-like NOB compared to *N. moscoviensis*-like NOB may also impact the abundance. Specifically, the accessible supplies of both organic and inorganic sources of energy in the RAS favor the growth of mixotrophs such as *N. marina* (Watson *et al.*, 1986a), but not of *N. moscoviensis* (Ehrich *et al.*, 1995). To date, studies have not compared sublineage II to sublineage IV nitrite oxidizing nitrospiras in any environment.

The role of the biofilm attachment media in determining the competitiveness of different ammonium and nitrite oxidizing populations needs to be studied further. It is tempting to speculate that crushed oyster shells provide an environment conducive for AOA competiveness, possibly due to the release of alkalinity (carbonates) or trace elements, or due to low DO zones caused by the close packing and aggregation of the oyster shells. The low DO zones combined with the low ammonium levels expected at the bottom of the filter may explain the abundance of AOA in the oyster shells.

3.4 Conclusions

The ammonium- and nitrite-oxidizing populations of a mixed media trickling filter consisting of compartments with plastic bioballs and crushed oyster shells, which was operated to treat water from a marine RAS culturing white shrimp in a maturation system, was dominated

by *Nitrosopumilus*-like AOA and *N. marina*-like bacteria (*Nitrospira* sublineage IV). The AOA were particularly abundant in the biofilter compartment containing oyster shells. The water quality (i.e., low ammonium and nitrite concentrations) and biofilm attachment media played a role in the competitiveness of AOA over AOB and *N. marina*- over *N. moscoviensis*- type NOB, but additional work is needed to elucidate their function and importance in determining the outcome of the competition.

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Chapter 4: Nitrification performance and characterization of nitrifying microbes in a biological aerated filter in an indoor, zero-discharge marine shrimp recirculating aquaculture system

4.1 Introduction[1]

Recirculating aquaculture systems (RAS) rely on nitrification for the transformation of ammonia to nitrate in biofilters to maintain water quality. This is particularly important to zero-discharge RAS where there is no water exchange except to replace water loss due to evaporation. Nitrification is a two step process in which ammonia[2] is oxidized to nitrate via nitrite. Ammonia-oxidizing bacteria (AOB) or ammonia-oxidizing archaea (AOA) and nitrite-oxidizing bacteria (NOB) mediate this process. Therefore, the activities of various microbes are important to recirculating systems in general. Information on microbial ecology in the water treatment reactors of zero-discharge RAS under various conditions would provide insight into the roles of various microbes in the nitrogen transformation processes occurring in the system. This information could be used to enhance the efficiency of microbiological processes through the optimization of operating conditions (e.g., oxygen and nutrient levels) to induce growth of the desired microbes.

Ammonia, nitrite, and nitrate are toxic to aquatic organisms. Both ammonia and nitrite can cause death. Consequently, farmers are advised to maintain total ammonia (sum of un-ionized and ionized ammonia) below 1 mg/L nitrogen (Timmons & Ebeling, 2007) and to maintain nitrite-nitrogen concentrations below 15 mg/L (Lin & Chen, 2003, Timmons & Ebeling, 2007). Researchers have shown that chronic exposure to nitrate concentrations above 200 mg/L nitrate-nitrogen has a negative impact on shrimp growth (Kuhn *et al.*, 2010). Therefore, farmers should maintain nitrate-nitrogen concentrations below 200 mg/L. In zero-

[1] This is an abbreviated version of Section 2.3. Refer to Section 2.3 for a more detailed discussion of the microbial ecology of RAS biofilters.
[2] In this chapter, ammonia refers to total ammonia, which is the sum of un-ionized and ionized ammonia, unless otherwise noted.

discharge RAS, water quality is maintained by nitrifying biofilters where the ammonia produced by shrimp and uneaten feed is converted to nitrate. Ammonia and nitrite should not accumulate to toxic levels in biofilters that are working correctly; however, nitrate can accumulate to concentrations of concern.

The presence of organic matter, particulate or dissolved, in RAS biofilters has an impact on nitrification. Heterotrophic bacteria utilize organic matter for growth and have a higher growth rate than nitrifying bacteria. Several studies investigating the impact of organic matter on nitrification performance have used sucrose as organic carbon source when simulating shrimp waste (Guerdat *et al.*, Fdz-Polanco *et al.*, 2000, Zhu & Chen, 2001, Ling & Chen, 2005). These studies showed that organic carbon reduced the ammonia-nitrogen removal rate at carbon/nitrogen (C/N) ratios greater than 0.5.

For zero-discharge RAS to be viable, the biofilters must be able to oxidize variable influent ammonium concentrations. Consequently, a robust microbial community that has ammonia and nitrite oxidizers that can thrive at high and low substrate, ammonium or nitrite, concentrations is essential for successful RAS operation. The sensitivity of AOB and NOB to a wide variety of environmental factors is well known. In recirculating aquaculture settings, the challenges associated with accumulation of ammonia and nitrite are similar to those in the wastewater treatment field and include problems with low dissolved oxygen levels, pH outside the optimal range for nitrifying microbes (7.5 – 8.6), and accumulation of trace amounts of toxic sulfides (Joye & Hollibaugh, 1995, Masser *et al.*, 1999, Ling & Chen, 2005) from the reduction of sulfate in marine systems. Less is known about the sensitivity of AOA because they have not been under study for as long as the AOB. Researchers (Martens-Habbena *et al.*, 2009) have shown that marine AOA have an ammonia-nitrogen saturation constant of 0.133 nM (0.00186 mg/L), which is two orders of magnitude lower than the corresponding value for AOB with high ammonium affinity. This suggests that AOA have adapted to survive under ammonium-limited conditions where AOB cannot grow.

Previous studies have analyzed the nitrifying communities in aquarium and aquaculture biofilms. Researchers have investigated freshwater aquaria (Hovanec & DeLong, 1996, Hovanec *et al.*, 1998, Burrell *et al.*, 2001, Sauder *et al.*, 2011), marine aquaria (Urakawa *et al.*, 2008), and aquaculture biofilms (Foesel *et al.*, 2008, Keuter *et al.*, 2011, Brown *et al.*, 2013). The work by Hovanec and Delong (1996) indicated that neither AOB nor *Nitrobacter* NOB were present in

freshwater aquaria. Hovanec and colleagues (1998) found that nitrite oxidation in freshwater aquaria was mediated by bacteria closely related to nitrite-oxidizing *Nitrospira* instead of *Nitrobacter* species. As described below, AOA were the dominant ammonia oxidizers in several studies (Sauder *et al.*, 2011, Sauder *et al.*, 2012, Brown *et al.*, 2013). Elevated salinity levels (Grommen *et al.*, 2005) and low temperature (Urakawa *et al.*, 2008) have been proposed as factors in determining the niches of ammonium oxidizers, in addition to ammonium concentration (Erguder *et al.*, 2009, Schleper, 2010). In studies of ammonium amended soils, AOB were more abundant than AOA (Taylor *et al.*, 2010, Verhamme *et al.*, 2011). While in studies of freshwater aquarium biofilters (Sauder *et al.*, 2011) and rotating biological contactors treating municipal wastewater (Sauder *et al.*, 2012), AOA were the dominant ammonium oxidizers and their abundance was inversely correlated to ammonium concentration. AOA were also dominant in marine RAS biofilters (Chapter 3) where ammonium concentrations were low. To date no studies have examined the role of ammonium concentration in determining the abundance of ammonia oxidizers in marine zero-discharge RAS. The purpose of this study was to examine the correlation between nitrifying populations and ammonium concentration in the biofilters of an indoor, zero-discharge RAS.

3.6 Materials and Methods

3.6.1 Experimental Set-up

The experimental set-up consisted of three RAS, each consisting of a culture tank and a down-flow, nitrifying biological aerated filter with two compartments (Figure 4.1). Reactor start-up is described in Appendix III. The three RAS were operated as replicate reactors. Each biofilter compartment consisted of a column that was 20.32 cm in diameter and 91.44 cm in length. All biofilter compartments contained a mix of 1.6 mm by 3.2 mm oval plastic beads (Aquatic Eco-system, Inc., Apopka, FL) and 4 to 10 mm diameter clay spheres (Aquaclay, Keeton Industries, Wellington, CO) as biofilm attachment medium. The mix was two-thirds plastic beads and one-third clay spheres by volume. The culture tanks were semi-square, 50-gallon polyethylene tanks (Polytank Inc., Litchfield, MN). Refer to Appendix III Table A3.1 for a summary of flow rate and media volume for all RAS.

Figure 4.1. Three replicate RAS. Each reactor consisted of a culture tank and two biological aerated filters. Water was pumped from the tank to the first filter and then flowed by gravity through the biofilters back to the tank.

Water for all RAS was prepared in the laboratory as follows. Each system was filled with 50 liters of distilled deionized water (16.5 cm depth) and a sea salt mix (Instant Ocean, Spectrum Brands Inc, Cincinnati, OH) to establish a salinity of approximately 25 g/L. Actual salinity values were 24.3 ± 1.0 g/L, 24.6 ± 0.9 g/L, and 24.5 ± 1.0 g/L for RAS-1, RAS-2, and RAS-3, respectively. Salinity was calculated from measured conductivity and temperature data. Two 150-watt Stealth submersible aquarium heaters (Marineland, Spectrum Brands Inc., Cincinnati, OH) in each tank were used to maintain the water temperatures at 30°C. Measured temperatures were 29.5 ± 0.6 °C, 29.9 ± 0.9 °C, and 29.5 ± 0.9 °C for RAS-1, RAS-2, and RAS-3, respectively. Air-tubing, located at the bottom of the tank, was used in each system to distribute compressed air. The target dissolved oxygen (DO) concentration was 5 mg/L. Average DO concentrations were 5.96 ± 0.54 mg/L, 6.16 ± 0.43 mg/L, and 6.36 ± 0.57 mg/L for RAS-1, RAS-2, and RAS-3, respectively. Bicarbonate, as ammonium bicarbonate and sodium bicarbonate, was added daily to maintain alkalinity above 100 mg/L as $CaCO_3$. Alkalinity was measured biweekly, while pH was monitored daily. During the experiment, alkalinity measured at 251 ± 11 mg/L as $CaCO_3$, 226 ± 13 mg/L as $CaCO_3$, and 150 ±14 mg/L as $CaCO_3$ in RAS-1, RAS-2, and RAS-3, respectively. The average pH in the tank was 8.28 ± 0.10, 8.34 ± 0.14, and 8.26 ± 0.15 for RAS-1, RAS-2, and RAS-3, respectively.

3.6.2 Simulated shrimp waste

Biofilters were supplied with a simulated shrimp waste via the culture tank at a flow rate of 250 mL/min. The simulated waste consisted of ammonium bicarbonate (NH_4HCO_3, ACS grade, Fisher Scientific) as the nitrogen and pH buffer source and sucrose ($C_{12}H_{22}O_{11}$, ACS grade, Fisher Scientific) as organic carbon source. The daily amount of ammonium-nitrogen added was determined by an estimate of the ammonium produced by 100 shrimp weighing 5 g, 10 g, 15 g, and 20 g. Ammonium-nitrogen production was estimated using this equation:

$$P_{TAN} = F * PC * 0.092 \quad \text{(Ebeling } et\ al.\text{, 2006)}$$

where P_{TAN} is the production rate of total ammonium nitrogen (g/day),
F is the feed rate (g/day), and
PC is the protein fraction of the feed (0.4).
The constant is the product of the fraction of feed protein that is nitrogen (0.16), the fraction of nitrogen assimilated (0.8), the fraction of assimilated nitrogen that is excreted (0.8), and the fraction of nitrogen excreted that is total ammonium nitrogen (0.9).

For 100 shrimp weighing 5, 10, 15, and 20 g, the daily ammonium-nitrogen concentration estimated was 552 mg (11 mg/L), 1104 mg (22 mg/L), 1656 mg (33 mg/L), and 2208 mg (44 mg/L), respectively. The ammonium-N dose was adjusted every 14 days starting at 552 mg (11 mg/L) and increasing to 20 g (44 mg/L); the ammonium-N does was added to the tank once per day. Since shrimp waste contains organic carbon in addition to ammonium-nitrogen. Zhu and Chen (2001) estimated a BOD_5/TAN ratio of 4 for aquaculture waste. While a C/N ratio of 5 was measured in actual shrimp waste from Waddell Mariculture Center in South Carolina (Roy *et al.*, 2010). Sucrose was used to maintain a carbon to nitrogen (C/N) ratio of 0.5, by weight, in the simulated waste. Sucrose has been used in other studies investigating impact of C/N ratio on RAS biofilters (Guerdat *et al.*, Fdz-Polanco *et al.*, 2000, Zhu & Chen, 2001). A C/N ratio of 0.5 was selected in this study to account for the affect of organic carbon on nitrification performance, reduced nitrification rate, but not disrupt the stability of the nitrifying microbes.

Researchers have shown that C/N ratios greater than one can reduce nitrification rate by 70% in submerged filters (Zhu & Chen, 2001). Furthermore, other nutrients for growth, which include phosphorus and trace metals, were supplied through the Instant Ocean Salt Mix (Spectrum Brands Inc, Cincinnati, OH) used to prepare the artificial seawater.

3.6.3 Water quality analyses

The experimental period was eight weeks and the biofilters were not backwashed during this period. Parameters such as salinity, temperature, pH, and dissolved oxygen in the culture tanks were monitored with probes. Salinity and temperature were measured with an Orion Model 1230 meter (Thermo Scientific, Waltham, MA) and pH with a Mettler Toledo sevenEasy meter (Columbus, OH). Grab samples were collected 24-hours after ammonium-N addition from the tank and biofilter effluent and depth profile samples were collected biweekly to monitor total ammonium-nitrogen (TAN), nitrite-nitrogen, and nitrate-nitrogen concentrations according to standard methods (APHA, 1998). Other water quality parameters that were measured biweekly included alkalinity, total suspended solids (TSS), and volatile suspended solids (VSS) according to standard methods (APHA, 1998). All analyses were performed in triplicate for each sample collected. Each RAS has eleven sample locations (Figure 4.2).

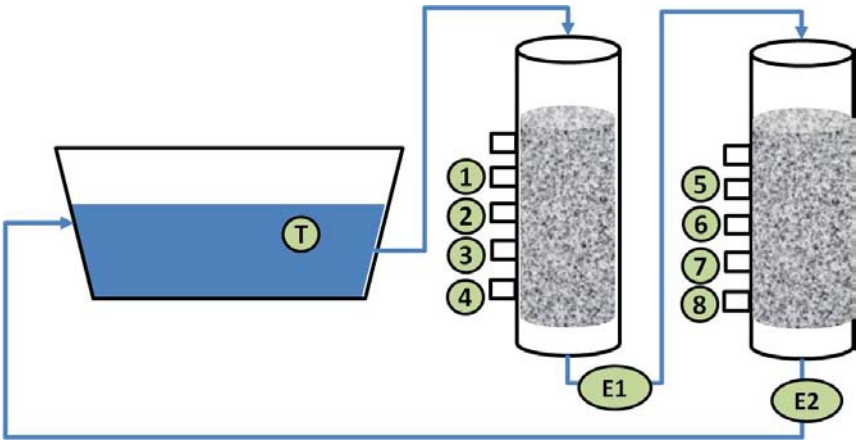

Figure 4.2. Schematic diagram of RAS. Sampling locations are identified by green circles. T is the tank water, E1 is the effluent from biofilter 1, and E2 is the effluent from biofilter 2. The numbers denote ports on the biofilter; ports one through 4 are on biofilter 1 and ports 5 through 8 are on biofilter 2.

3.6.4 Microbial sample collection and DNA extraction

Each RAS had eight sample locations along the height of the column (Figure 4.2). Media samples were collected biweekly, once at each loading condition (Day 14, 28, 42, and 56). A horizontal core was collected from each sample port before ammonium-N addition using a 1-inch diameter, 18-inch long brass cylinder. The media samples were transferred to 50 mL centrifuge tubes and then split into another 50 mL centrifuge tube to create biological replicates. The wet mass of the sample was recorded. Sterile saline water was added to all tubes, which were then vigorously vortexed for one hour at 4°C to detach biomass from media. Biomass was then filtered onto sterile 0.2 µm polycarbonate membrane filter. Filters were folded and put into 2 mL screw cap tubes for DNA extraction. Samples were stored at -80°C until further processing. DNA was extracted from samples using a phenol extraction method described by Griffiths *et al.*, 2000), with modifications (Duangmanee *et al.*, 2007). Extracted DNA in each sample was quantified using a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). Samples were diluted with TE buffer to contain 25 ng per µL genomic DNA.

3.6.5 PCR Amplification for 454-Sequencing and Data Processing

PCR amplification was conducted in triplicate for each sample using Roche 454 titanium compatible primers targeting bacterial 16S rRNA genes as described elsewhere (Pinto & Raskin, 2012). Triplicate PCR products were pooled and purfied using a QiaQuick PCR purification kit (Qiagen Inc.,Valencia, CA). The amount of PCR product from each sample was quantified in triplicate using a Quant-iT dsDNA assay kit (Invitrogen, Carlsbad, CA) on a Nanodrop 3300 (ThermoScientific, Wilmington, DE). All samples were pooled based on DNA concentration and purified using AMPure XP beads to ensure that DNA was uniform in length prior to sequencing. The pooled, purified PCR products were sequenced at the University of Illinois Biotechnology Center (Urbana, IL) on half of a pico-titer plate (56 samples unrelated to the current study were also included).

All data were processed and analyzed using mothur v.1.29.2 (Schloss *et al.*, 2009). A total of 1,606 sequences were obtained for the 16 samples sequenced for this study. The sequences were trimmed to remove barcodes and primers, filtered for quality reads, and checked for chimeras as previously described (Schloss *et al.*, 2011, 454 SOP was accessed March 5, 2013), resulting in a total of 1,592 sequences in thefinal data library. The numbers of quality-filtered, chimera-free reads in each sample are provided in Appendix III Table A3.2. Due to the variation in sequence number, a subset of 23 sequences was randomly selected based on the sample with the lowest sequence number prior to statistical analyses.

3.6.6 PCR

For population specific PCR, only samples collected on Day 14 and Day 56 were analyzed. Triplicate PCR reactions for each sample were run. PCR reactions were 25 µL and each reaction contained 500 nM of each primer, 22.5 µL Platinum PCR Supermix (Invitrogen, Life Technologies Corporation, Carlsbad, CA) and 1 µL template.

Gammaproteobacterial-AOB *amoA-amoB* gene fragment was amplified using primers amoA-3F/amoB-4R (Purkhold *et al.*, 2000). PCR was done prior to qPCR to determine if these samples contained the gene of interest. The PCR mixture was as described above. Thermal cycling consisted of initial denaturation of 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 48°C for 30 seconds, and extension at 72°C for

30 seconds; final extension was at 72°C for 10 minutes. All PCR results were confirmed with agarose gel electrophoresis.

3.6.7 Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was used to quantify the AOA and *Betaproteobacterial*-AOB *amoA* gene abundance as well as *Nitrobacter* and *Nitrospira* NOB 16S rRNA gene abundance. Only samples collected on Day 14 and Day 56 were analyzed. All sample and standard reactions were carried out in triplicate using SYBR green chemistry on a Mastercycler ep realplex (Eppendorf, North America, Hauppauge, NY) qPCR machine. Standard curves were generated for all experiments from plasmids containing cloned *amoA* or 16S rRNA gene PCR amplicons. Samples were diluted to contain 25 ng per µL DNA. All qPCR assays were carried out in 10 µL reactions consisting of 1 µL template DNA, 500 nM of each primer, and 5 µL 2x Quantitect MasterMix (Quantitect, Qiagen, Germantown, MD). The specificity of amplification for all qPCR assays was verified via generation of melting curves. The one-point calibration method for absolute quantification, as described by Brankatschk *et al.*, 2012, was used to calculate gene abundance. The LinRegPCR Program, version 2013.0, (Ruijter *et al.*, 2009) was used to calculate cycle threshold, C_T, and PCR efficiency, E, values for samples and standards from amplification data.

Archaeal *amoA* gene copies were quantified using primers crenAMO_F (Hallam *et al.*, 2006)/Arch-amoAR (Francis *et al.*, 2005). The PCR conditions were as follows: 95°C for 15 minutes, followed by 45 cycles consisting of 95°C for 15 seconds, 58.5°C for 30 seconds, 72°C for 30 seconds. A plasmid containing *Nitrosopumilus maritimus amoA* gene was used as standard. The mean E value for the standard used in the one-point calibration was 1.76 for both days. The mean E value for samples was 1.51 for Day 14 and 1.50 for Day 56.

Betaproteobacterial-AOB *amoA* was quantified using the primers amoA-1F/amoA-2R (Rotthauwe *et al.*, 1997). The PCR conditions were as follows: 95°C for 15 minutes, followed by 45 cycles consisting of 95°C for 30 seconds, 52°C for 30 seconds, 72°C for 30 seconds. A plasmid containing *Nitrosomonas europaea amoA* gene was used as standard. The mean E value for the standard used in the one-point calibration was 1.74 for Day 14 and 1.75 for Day 56. The mean E value for samples was 1.76 for Day 14 and 1.74 for Day 56.

Nitrospira NOB 16S rRNA genes were quantified using the primer set NTSPAf/NTSPAr (Nakamura *et al.*, 2006). The PCR conditions were as follows: 95°C for 15 minutes, followed by 45 cycles consisting of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 60 seconds. A plasmid containing *Nitrospira defluvii* 16S rRNA gene was used as standard. The mean E for the standard used in one-point calibration was 1.81 for both days. The mean E value for samples was 1.25 for Day 14 and 1.19 for Day 56.

Nitrobacter NOB 16S rRNA genes were quantified using the primer set Nitro-1198f/Nitro1423r (Graham *et al.*, 2007). The PCR conditions were as follows: 95°C for 15 minutes, followed by 45 cycles consisting of 95°C for 15 seconds, 58°C for 60 seconds, 72°C for 40 seconds. A plasmid containing *Nitrobacter winogradskyi* 16S rRNA gene was used as standard. The mean E value for the standard used in the one-point calibration was 168 for Day 14 and 1.65 for Day 56. The mean E value for samples was 1.70 for Day 14 and 1.62 for Day 56.

4.3 Results and Discussion

4.3.1 Inorganic nitrogen

Samples were collected 24-hours after ammonium-N addition to monitor inorganic nitrogen. These samples were analyzed for ammonium-N (Table 4.1), nitrite-N (Table 4.1), and nitrate-N (Figure 4.3) for all RAS. The purpose of these samples was to monitor the 24-hour accumulation of inorganic N after ammonium addition. It is important to maintain less than 1 mg/L as ammonium-N and less than 15 mg/L as nitrite-N because higher concentrations are toxic to shrimp (Timmons & Ebeling, 2007). These results show that there was no ammonium or nitrite accumulation within a 24-hour period after the ammonium spike. However, nitrate accumulated in the system as expected.

Because no nitrate removal was performed during the course of this study, nitrate-N accumulated in the system. Concentrations were as high as 700 mg/L as nitrate-N (Figure 4.3), which would likely have negative impacts on the shrimp. A recent study (Kuhn *et al.*, 2010) investigating the chronic toxicity of nitrate on Pacific white shrimp in a RAS, showed negative impacts on shrimp biomass and antennae length at 435 mg/L nitrate-N and negative impacts on growth, survival, total mass, and antennae length at 910 mg/L nitrate-N.

Table 4.1: Average concentrations of ammonium-N and nitrite-N over experimental period in tank

Reactor	Ammonium-N mg/L	Nitrite-N mg/L
RAS-1	0.08 ± 0.37	0.28 ± 0.26
RAS-2	0.07 ± 0.28	0.18 ± 0.22
RAS-3	0.04 ± 0.12	0.12 ± 0.19

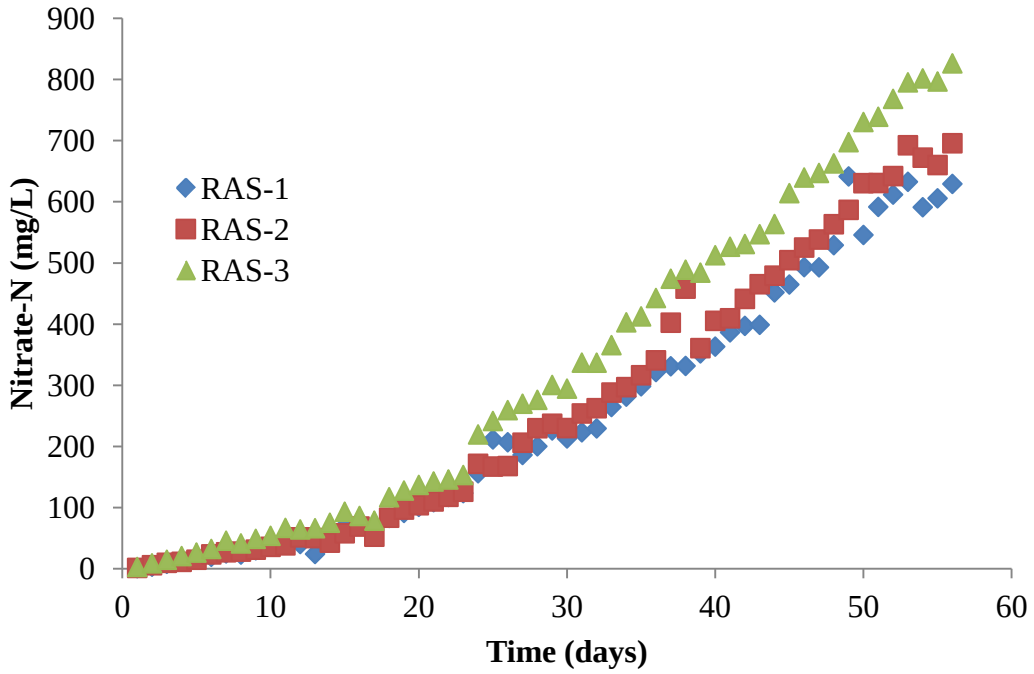

Figure 4.3: Nitrate-nitrogen in RAS tanks.

The purpose of the depth profile was to observe spatial concentration gradients within the biofilters. Depth profile samples were collected 8 days into each 14-day ammonium loading period. Samples were collected 30 minutes after influent addition and were analyzed for ammonium-N (Figure 4.4), nitrite-N (Figure 4.5), and nitrate-N (Figure 4.6). An ammonium-N

concentration gradient developed across the reactor for all time points. This suggests that the highest abundance of ammonium oxidizing microbes was in the first biofilter (Ports 1 – BF1 effluent, Figure 4.2) because the majority of the ammonium was oxidized in the first biofilter based on difference between influent and effluent concentrations. Nitrite-N concentrations generally increased from Ports 1-3 in the first biofilter (Figure 4.2) and then decreased through the second biofilter. This suggests that the highest abundance of nitrite oxidizing bacteria occurred between Ports 4-6. Since the reactors were not operated to remove nitrate, Concentrations of nitrate-N showed little variation across the biofilters (Figure 4.6) due to the short time scale of the sampling; nitrate-N does accumulate over experimental period (Figure 4.3).

Although this system was not designed for denitrification, it could be occurring in the biofilters. A theoretical nitrogen balance over the entire RAS indicates that denitrification is occurring in all RAS and there is more denitrification in RAS-1 and RAS-2 than in RAS-3. Refer to the nitrogen balance section in Appendix III (pages 129 - 134). Based on the mass of ammonium-N input to the system, 77 g, there should be 70 g of nitrate-N. However, the highest observed mass of nitrate-N was 39 g in RAS-3, 33 g in RAS-2, and 30 g in RAS-1. Heterotrophic and autotrophic bacterial uptake of ammonium for assimilation, or cell growth, does not account for the missing nitrate. These processes would remove 7 g nitrate-N when ammonium is used as the nitrogen source. Sucrose was the carbon source for heterotrophic bacteria and typical parameter values (Henze *et al.*, 2000) were used to calculate the mass of nitrogen needed for assimilation. A similar process was used to calculate the nitrogen requirement for autotrophic (ammonium and nitrite oxidizers). Consequently, it is reasonable to infer that denitrification occurred in all RAS. The lower nitrate-N concentrations in RAS-1 and RAS-2 indicate that there was more denitrification in these reactors than in RAS-3 since all RAS received the same mass of ammonium.

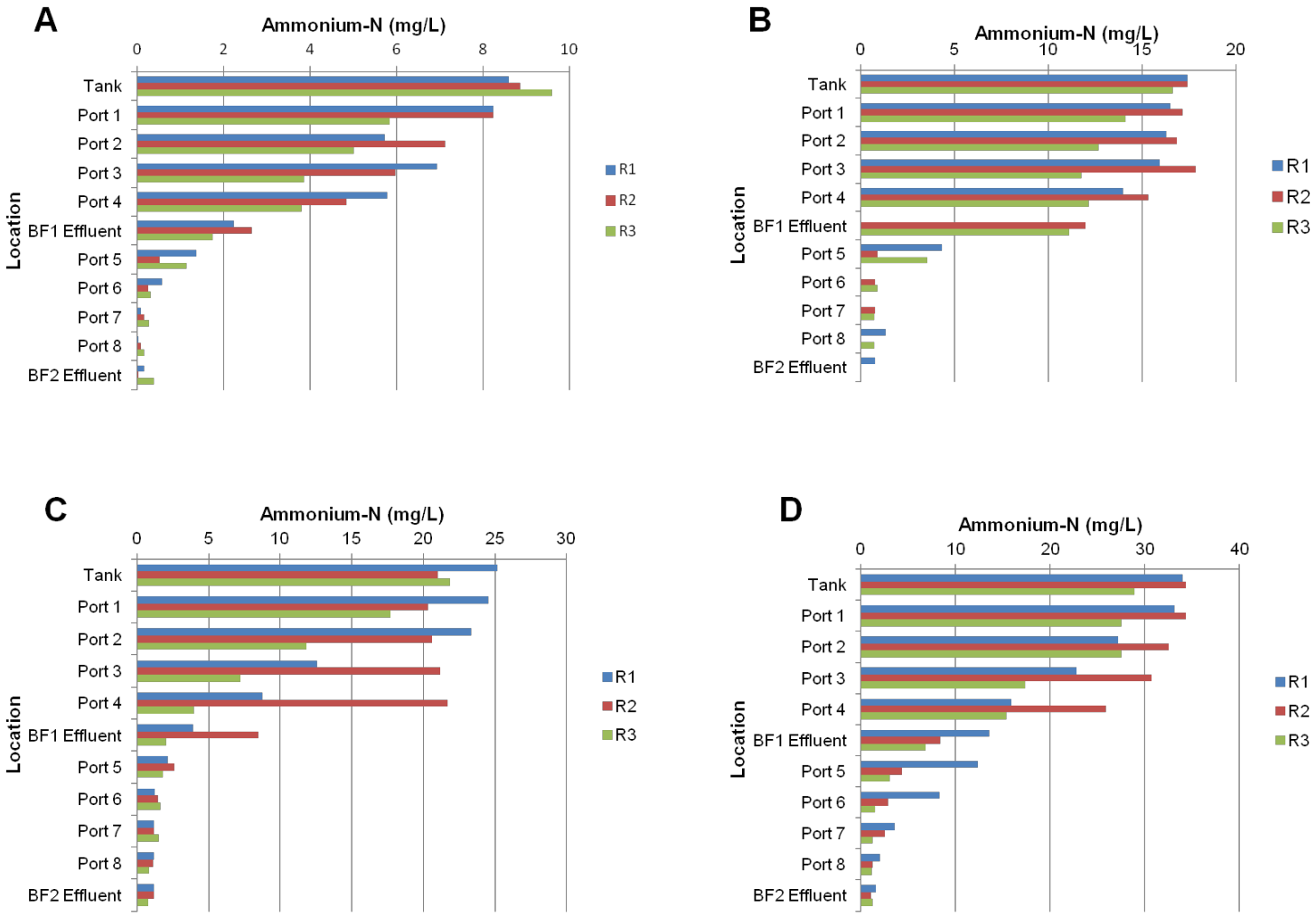

Figure 4.4: Ammonium-N depth profile in each RAS (R1, R2, and R3): (A) 6/15/12 (Day 8), influent ammonium-N concentration was 11 mg/L; (B) 6/29/12 (Day 22), influent ammonium-N concentration was 22 mg/L; (C) 7/13/12 (Day 36), influent ammonium-N concentration was 33 mg/L; and (D) 7/27/12 (Day 50), influent ammonium-N concentration was 44 mg/L. Samples for depth profile were collected 30 minutes after influent addition.

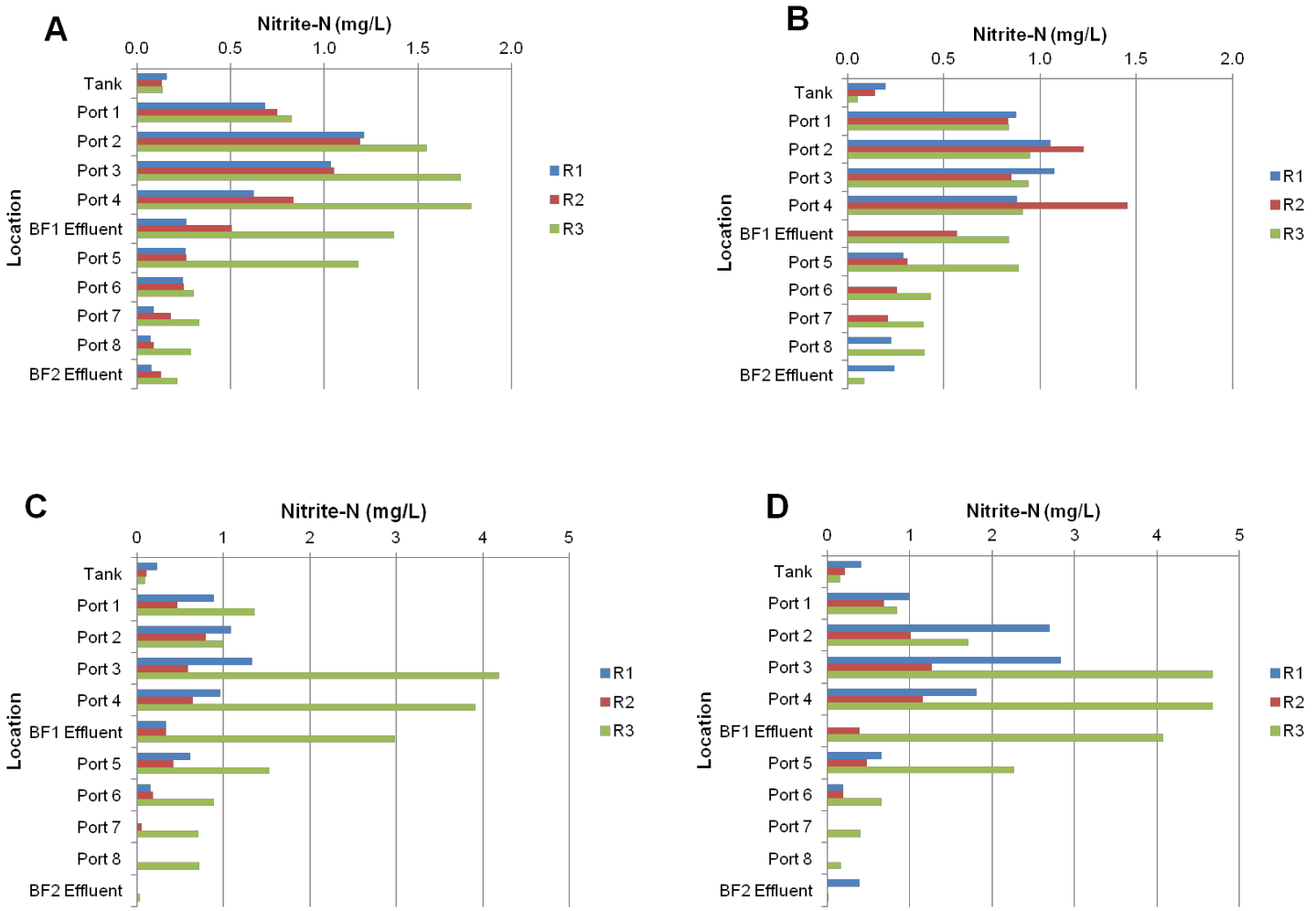

Figure 4.5: Nitrite-N depth profile in each RAS (R1, R2, and R3): (A) 6/15/12 (Day 8), influent ammonium-N concentration was 11 mg/L; (B) 6/29/12 (Day 22), influent ammonium-N concentration was 22 mg/L; (C) 7/13/12 (Day 36), influent ammonium-N concentration was 33 mg/L; and (D) 7/27/12 (Day 50), influent ammonium-N concentration was 44 mg/L. Samples for depth profile were collected 30 minutes after influent addition. Concentrations increased after Port 1, reached a maximum at Port 3 or Port 4, and decreased through Port 8 for each time point.

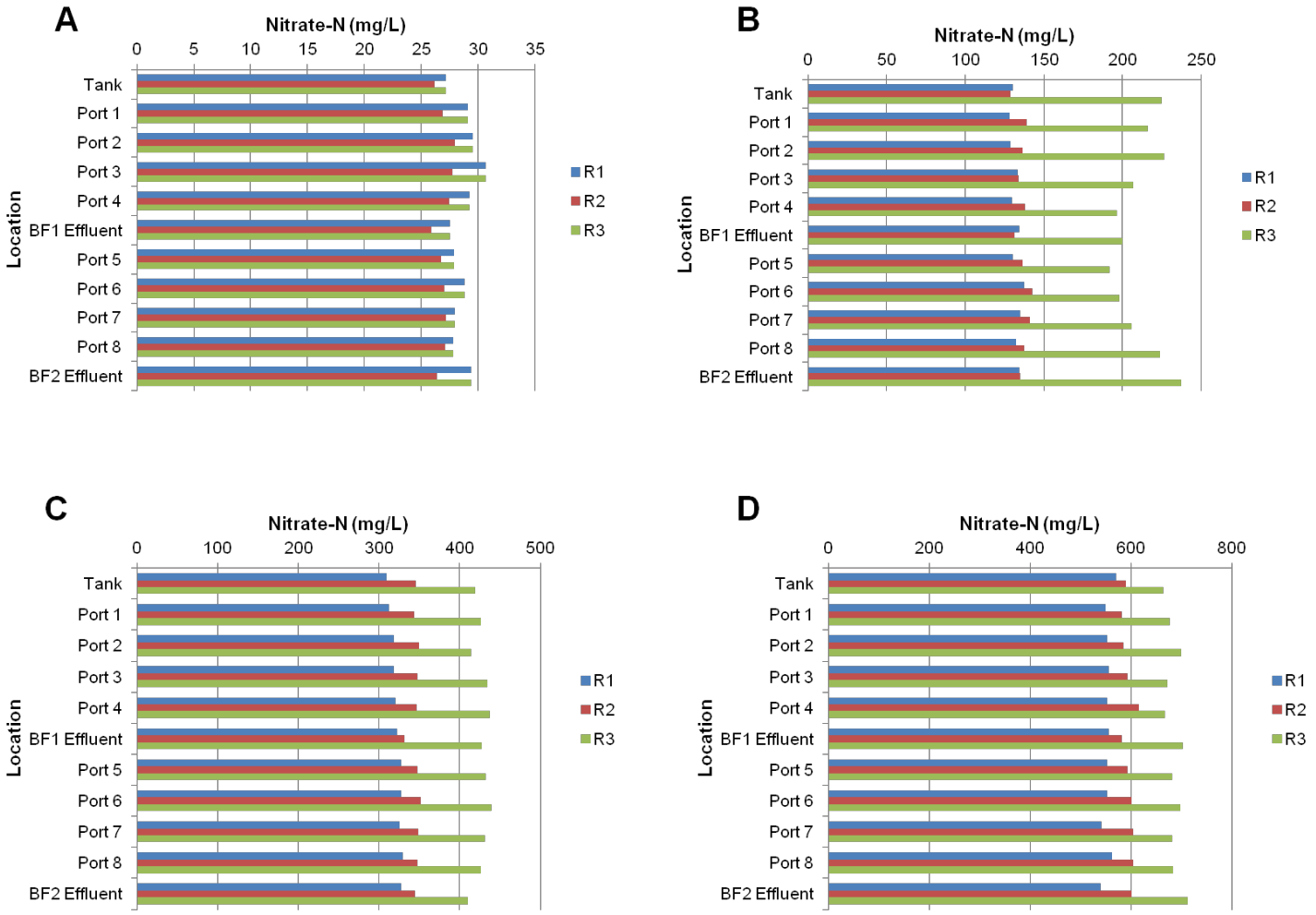

Figure 4.6: Nitrate-N depth profile in each RAS (R1, R2, and R3): (A) 6/15/12 (Day 8), influent ammonium-N concentration was 11 mg/L; (B) 6/29/12 (Day 22), influent ammonium-N concentration was 22 mg/L; (C) 7/13/12 (Day 36), influent ammonium-N concentration was 33 mg/L; and (D) 7/27/12 (Day 50), influent ammonium-N concentration was 44 mg/L. Samples for depth profile were collected 30 minutes after influent addition.

4.3.2 Microbial community analysis of RAS-1

A subset of samples from RAS-1 was analyzed. These were samples from Ports 1, 4, 5, and 8 (Figure 4.2) at all four ammonia loading rates. Refer to Table A3.2 for a summary of the total number of sequences obtained pre- and post-filtering for quality. Sequences were classified at the phylum level (Figures A3.1 to A3.4). The genus *Nitrobacter* is part of the phylum *Alphaproteobacteria*, and the phylum relative abundance of this phylum suggests that *Nitrobacter* were present. The AOB genus *Nitrosoccocus* is part of the phylum *Gammaproteobacteria*, and the relative abundance of this phylum suggests that *Nitrosococcus* were present. PCR and qPCR analyses on samples collected from all RAS at 11 and 44 mg/L ammonium-N were conducted to confirm and quantify the ammonium- and nitrite-oxidizers.

4.3.3 Ammonia- and nitrite-oxidizing population abundances

Microbial biomass samples were collected along the depth of each biofilter (Figure 4.2). Samples from Port 1 to Port 4 were collected from biofilter #1 while samples from Port 5 to Port 8 were collected from biofilter #2. Samples were collected 14 days into each ammonium-N load, on Day 14 (11 mg/L ammonium-N), Day 28 (22 mg/L ammonium-N), Day 42 (33 mg/L ammonium-N), and Day 56 (44 mg/L ammonium-N).

To analyze the population dynamics of ammonium- and nitrite-oxidizing microbes with increasing ammonia concentration and depth in the biofilter, qPCR was used. Samples collected on Day 14 (lowest ammonium-N load at 11 mg/L) and Day 56 (highest ammonium-N load at 44 mg/L) were analyzed. Microbial populations of interest were AOA, *Betaproteobacteria* AOB (β-AOB), *Gammaproteobacteria* AOB (γ-AOB), nitrite-oxidizing *Nitrospira*, and *Nitrobacter*. Since these were marine systems, it was likely that AOB that require salt, halo-tolerant or halophiles, were present in the biofilters. Salt-tolerant genera of AOB are *Nitrococcus* (γ-AOB) and some species of *Nitrosomonas* (β-AOB) (Koops & Pommerening-Roser, 2001). Therefore, PCR was used to screen all samples for γ-AOB, and γ-AOB were not detected (data not shown). All other populations of interest were detected via qPCR. AOA were more abundant than β-AOB at all depths in the biofilter and at both ammonium concentrations in RAS-1 (Figure 4.7), RAS-2 (Figure 4.8), and RAS-3 (Figure 4.9). This is consistent with results observed in the biofilters of an indoor shrimp farm (Chapter 3). β-AOB abundance increased from Day 14 to Day 56, corresponding to increasing ammonium-N concentrations from 11 to 44 mg/L, while AOA

abundance remained relatively constant over time. AOB *amoA* gene abundance was more correlated to ammonium-N concentration than AOA *amoA* gene abundance (Figures 4.7 – 4.9). This suggests that with increasing ammonium-N concentration, the β-AOB population will become more abundant than the AOA. This follows from observations in ammonia amended soil experiments where AOB were more abundant than AOA at high ammonium concentrations (Taylor *et al.*, 2010, Verhamme *et al.*, 2011).

For NOB, *Nitrospira* were generally more abundant than *Nitrobacter* (Figures 4.10 – 4.12). There was minimal correlation between NOB 16S rRNA gene abundance and nitrite-N concentrations. *Nitrospira* abundance decreased with increased ammonium-N conecentration in all RAS, while *Nitrobacter* abundance remained relatively constant.

The microbial population dynamics were needed to help explain the inorganic nitrogen depth profiles (Figures 4.4 - 4.6). There was minimal variation between the reactors for the ammonium-N profile (Figure 4.4). However, RAS-3 deviated from RAS-1 and RAS-2 in both the nitrite-N (Figure 4.5) and nitrate-N (Figure 4.8) profiles. Where nitrite-N concentrations were highest in RAS-3, both *Nitrospira* and *Nitrobacter* were an order of magnitude less abundant in RAS-3 compared to RAS-1 and RAS-2, which may explain the higher nitrite-N concentrations. In RAS-1 and RAS-2, on the other hand, both NOB population abundances were relatively constant across all ports.

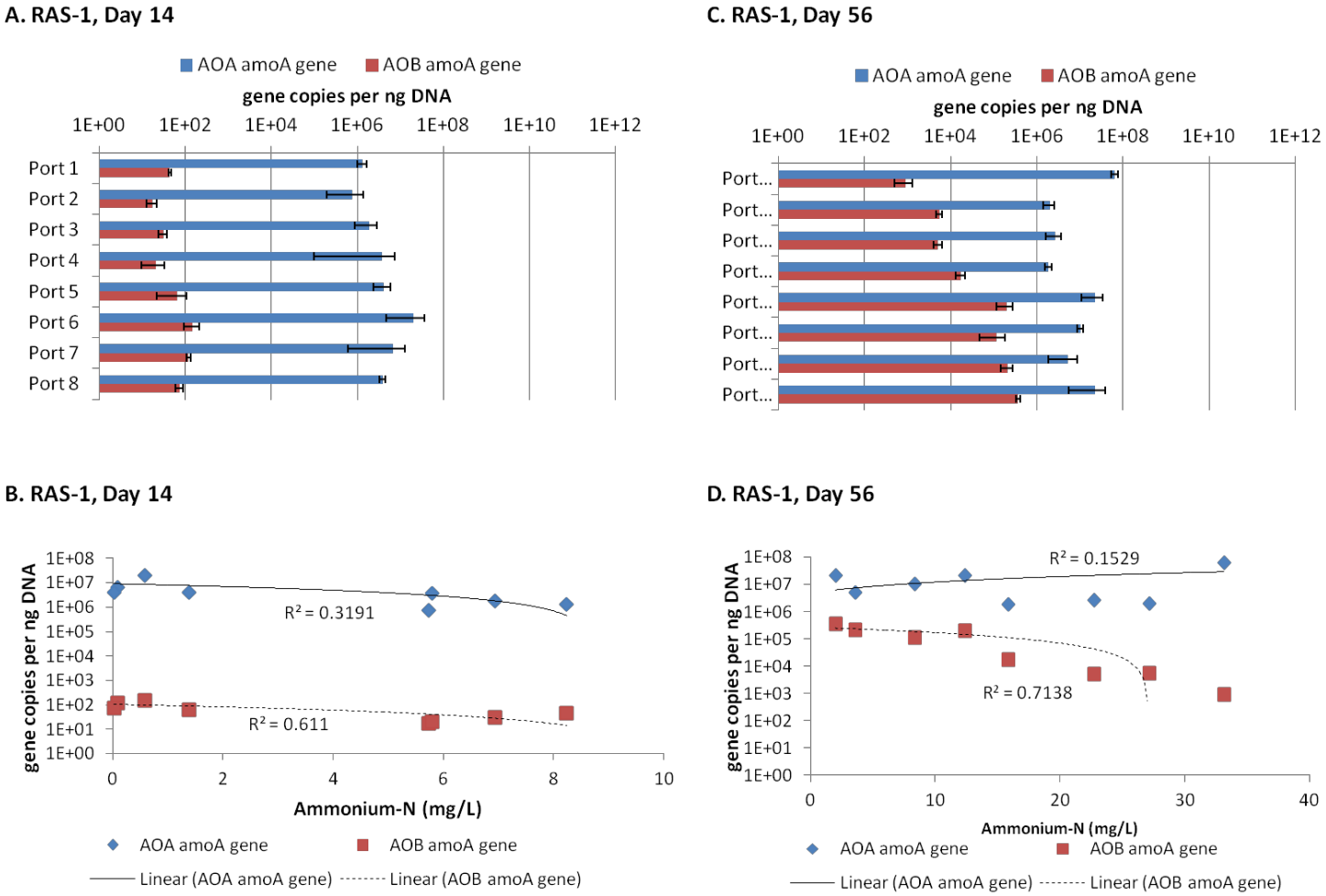

Figure 4.7: Ammonium-oxidizing population abundances in RAS-1. (A) and (B) Day 14, influent ammonia load of 11 mg/L ammonium-N. (C) and (D) Day 56, influent ammonia load of 44 mg/L. AOA were more abundant at all depths in biofilter by at least two orders of magnitude (A). While AOA were still more abundant on Day 56 (C), AOB abundance was higher at this concentration and increased with depth in biofilter. There was higher correlation between AOB *amoA* gene copy number and ammonium-N concentration (B) and (D).

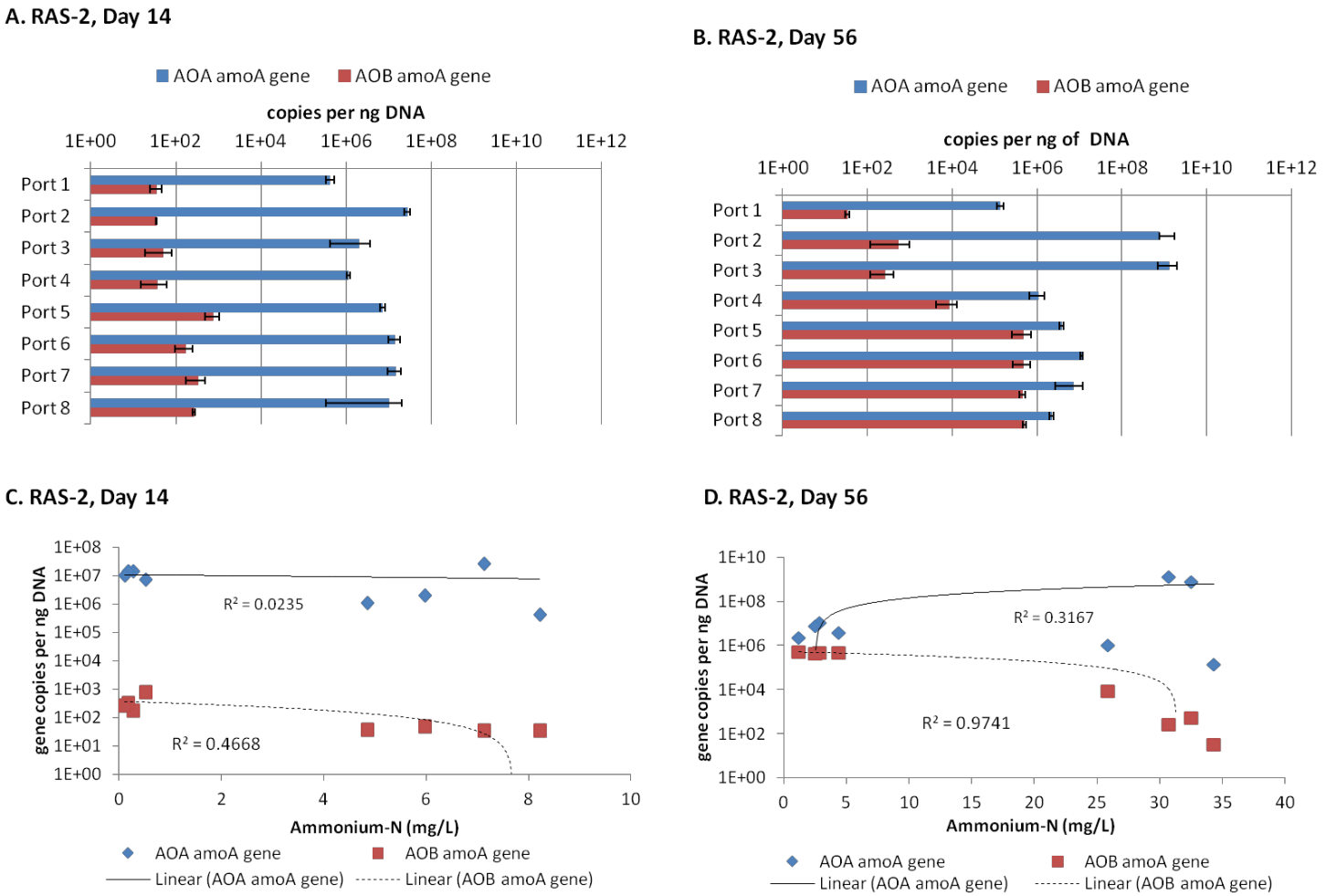

Figure 4.8: Ammonium-oxidizing population abundances in RAS-2. (A) and (B) Day 14, influent ammonia load of 11 mg/L ammonium-N. (C) and (D) Day 56, influent ammonia load of 44 mg/L. AOA were more abundant at all depths in biofilter by at least two orders of magnitude (A). While AOA were still more abundant on Day 56 (C), AOB abundance was higher at this concentration and increased with depth in biofilter. There was higher correlation between AOB *amoA* gene copy number and ammonium-N concentration (B) and (D).

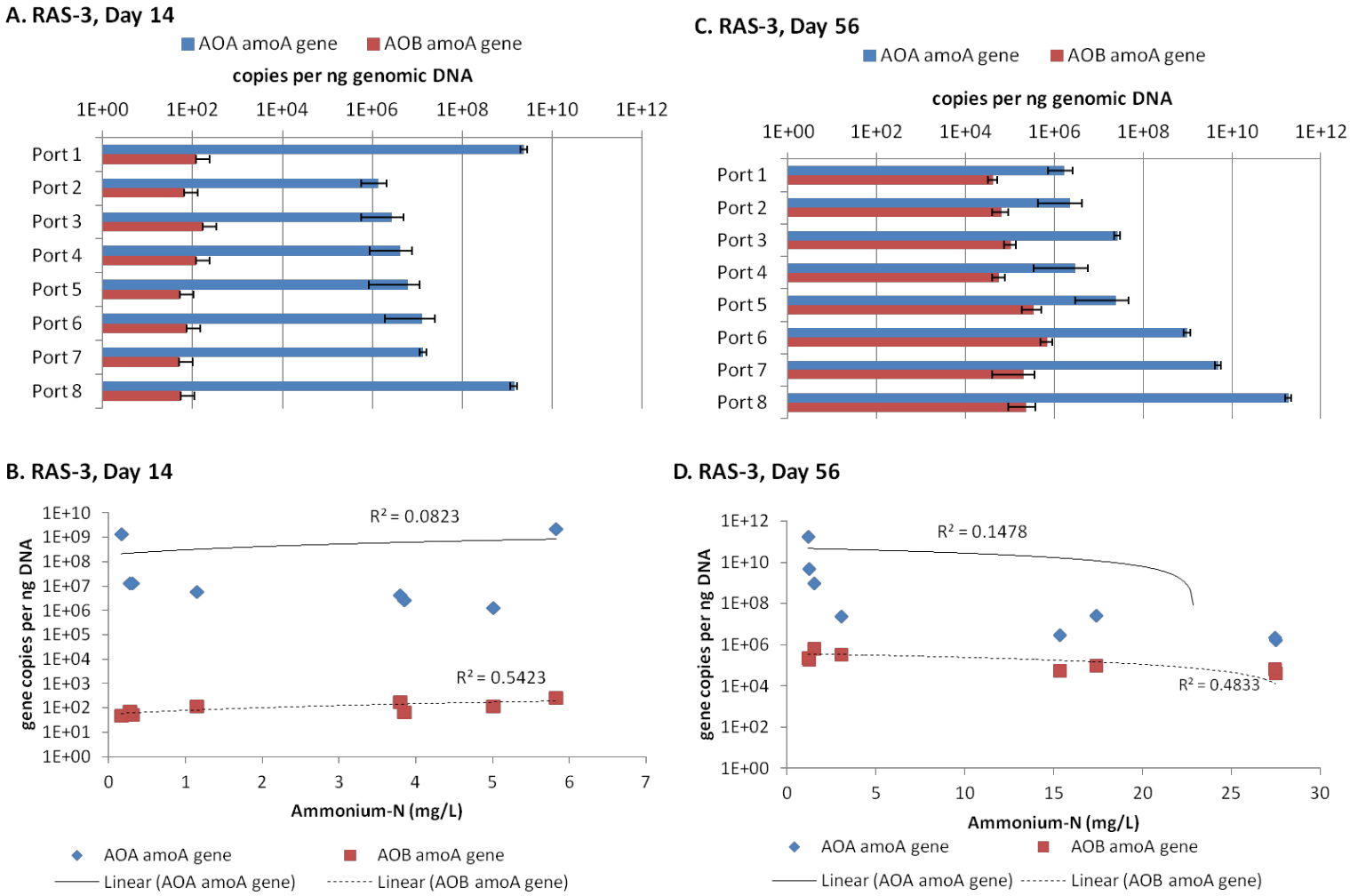

Figure 4.9: Ammonium-oxidizing population abundances in RAS-3. (A) and (B) Day 14, influent ammonia load of 11 mg/L ammonium-N. (C) and (D) Day 56, influent ammonia load of 44 mg/L. AOA were more abundant at all depths in biofilter by at least two orders of magnitude on Day 14 (A). While AOA were still more abundant on Day 56 (C), AOB abundance was higher at this concentration and was relatively constant with depth in biofilter. There was higher correlation between AOB amoA gene copy number and ammonium-N concentration (B) and (D).

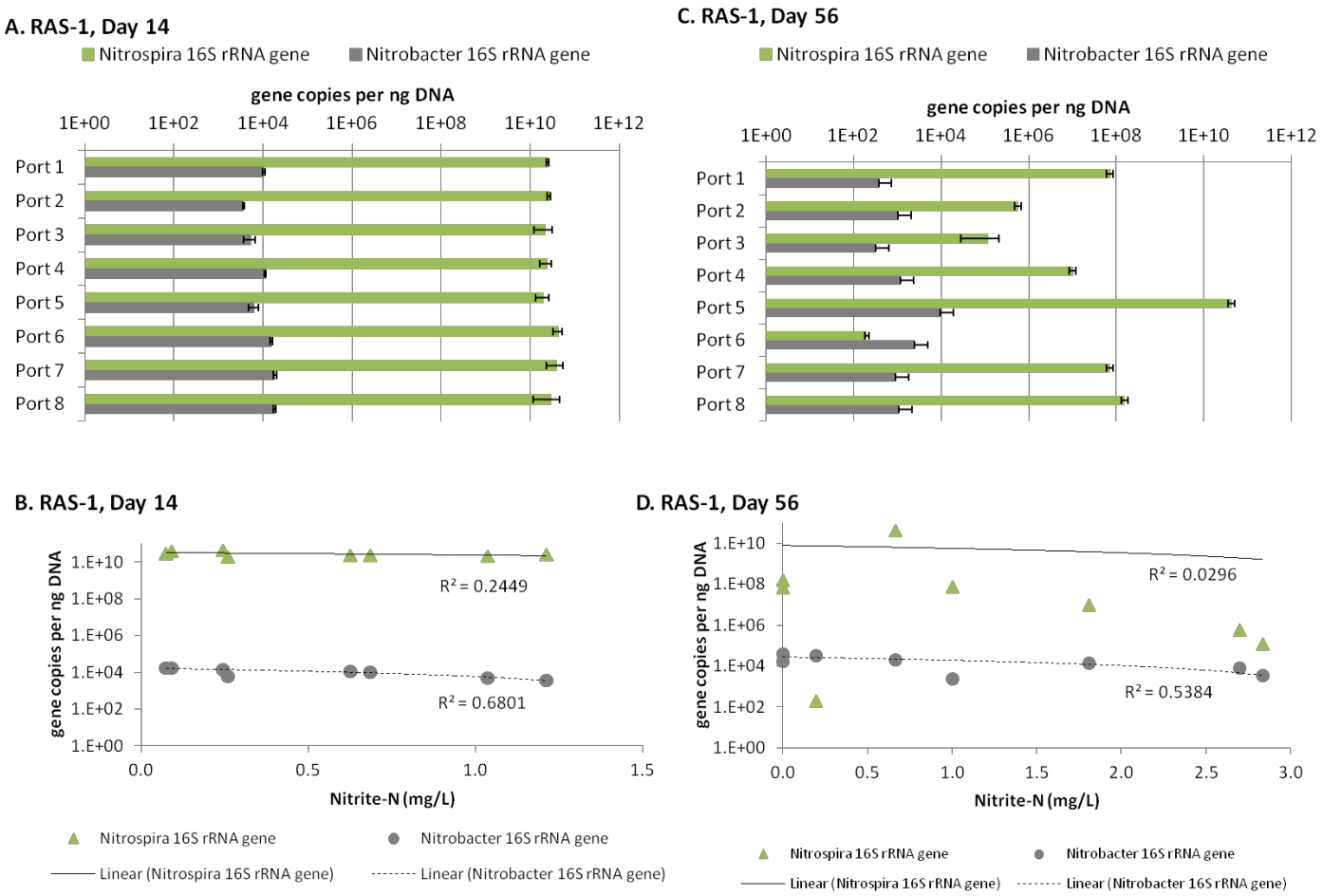

Figure 4.10: Nitrite-oxidizing population abundances in RAS-1. (A) and (B) Day 14, influent ammonia load of 11 mg/L ammonium-N. (C) and (D) Day 56, influent ammonia load of 44 mg/L. *Nitrospira* were more abundant at all depths in biofilter by at least two orders of magnitude on Day 14 (A). While *Nitrospira* were still more abundant on Day 56 (C), their abundance decreased and at some locations was less than *Nitrobacter. Nitrobacter* abundance was relatively constant with depth in the biofilter and with increased ammonium-N concentration. There was higher correlation between *Nitrobacter* 16S rRNA gene copy number and nitrite-N concentration (B) and (D) than *Nitrospira* 16S rRNA gene copy number.

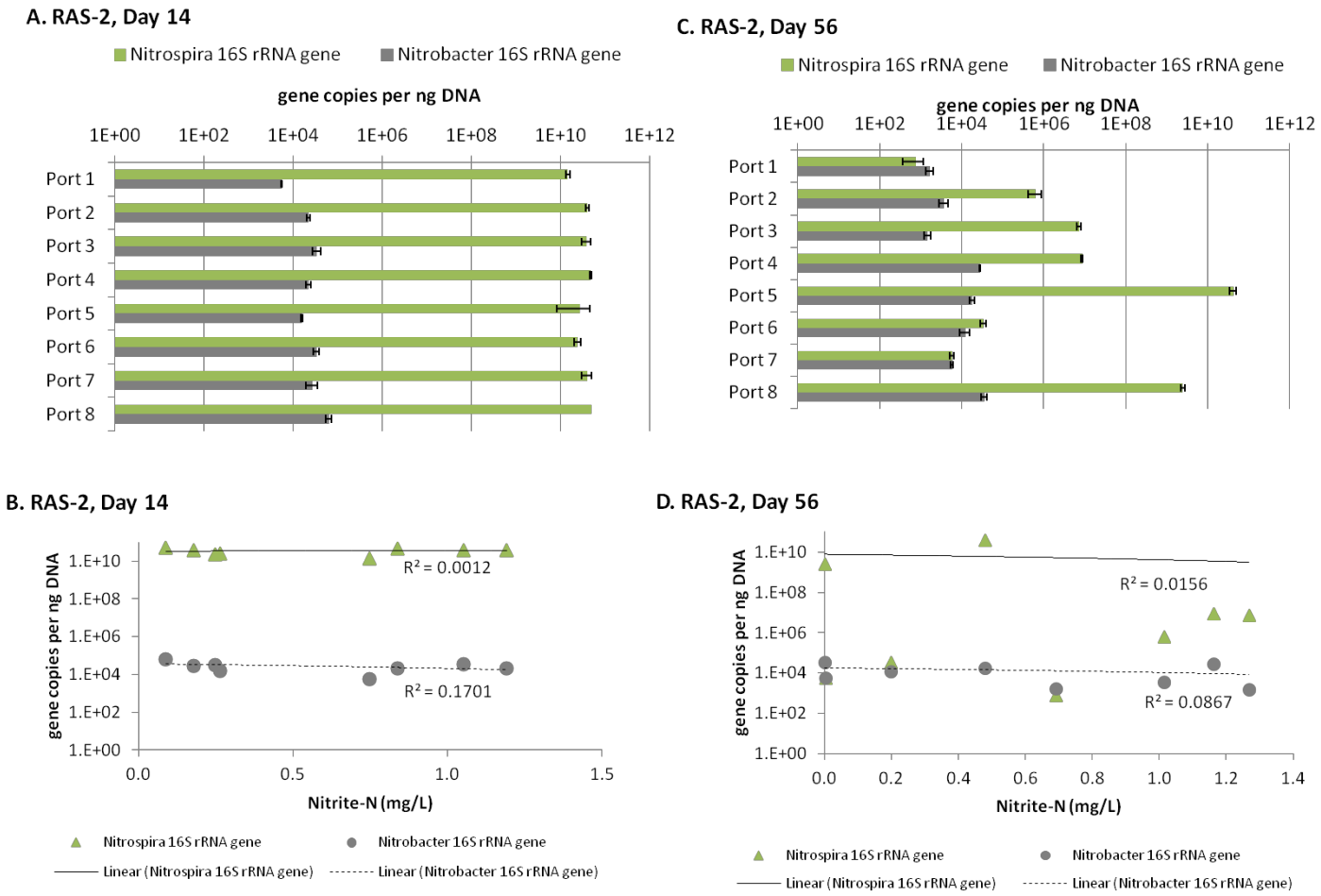

Figure 4.11: Nitrite-oxidizing population abundances in RAS-1. (A) and (B) Day 14, influent ammonia load of 11 mg/L ammonium-N. (C) and (D) Day 56, influent ammonia load of 44 mg/L. *Nitrospira* were more abundant at all depths in biofilter by at least two orders of magnitude on Day 14 (A). While *Nitrospira* were still more abundant on Day 56 (C), their abundance decreased and at some locations was less than *Nitrobacter. Nitrobacter* abundance was relatively constant with depth in the biofilter and with increased ammonium-N concentration. There was minimal correlation between *Nitrobacter* 16S rRNA gene copy number and nitrite-N concentration or between *Nitrospira* 16S rRNA gene copy number.concentration (B) and (D)

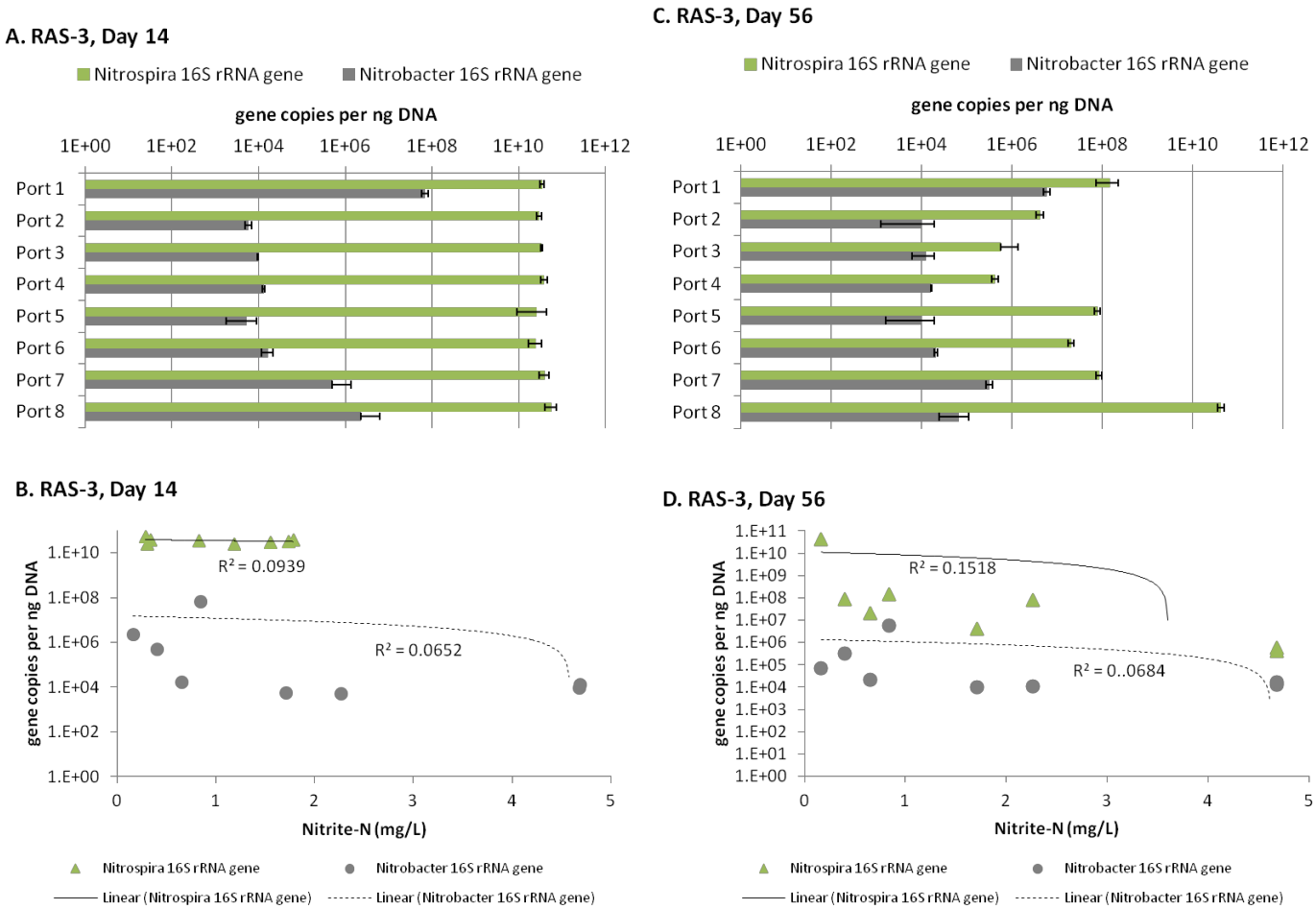

Figure 4.12: Nitrite-oxidizing population abundances in RAS-2. (A) and (B) Day 14, influent ammonia load of 11 mg/L ammonium-N. (C) and (D) Day 56, influent ammonia load of 44 mg/L. *Nitrospira* were more abundant at all depths in biofilter by at least two orders of magnitude on Day 14 (A). While *Nitrospira* were still more abundant on Day 56 (C), their abundance decreased. *Nitrobacter* abundance was relatively constant with depth in the biofilter and with increased ammonium-N concentration. There was minimal correlation between *Nitrobacter* 16S rRNA gene copy number and nitrite-N concentration or between *Nitrospira* 16S rRNA gene copy number.concentration (B) and (D)

The ammonium and nitrite concentrations likely played a role in the relative levels of AOA versus β-AOB (Figures 4.7 – 4.9) and *Nitrospira*-NOB and *Nitrobacter*-NOB (Figures 4.10 – 4.12). These concentrations were dynamic with the highest concentrations, 11 mg/L ammonium-N, occurring after influent addition (Figures 4.4 and 4.5) and the lowest concentrations, below 0.05 mg/L ammonium-N detection limit, observed 23 hours after influent addition (Table 4.1). Such a range of concentrations provided substrate concentrations that were suitable for populations that have high and low substrate affinities. This suggests that the RAS biofilters were robust and able to oxidize both high and low concentrations. AOA were more abundant than AOB at the time of sampling which is consistent with previous aquarium and aquaculture studies (Sauder *et al.*, 2011, Brown *et al.*, 2013). However, AOA abundance was not correlated to decreasing ammonium concentration as previously observed in freshwater aquaria (Sauder *et al.*, 2011) and rotating biological contactors treating municipal wastewater (Sauder *et al.*, 2012). In this study, AOA abundance remained relatively constant while β-AOB abundance increased as the initial ammonium concentration increased. This may be due to the cycling of ammonium levels between values higher than the half-saturation constant of AOB with low ammonium affinity and those lower than the half-saturation of AOB with high ammonium affinity (Limpiyakorn *et al.*, 2013). The half-saturation constants of AOB ranges from 12.3 to 27.4 mg/L ammonium-N (Laanbroek *et al.*, 1994) for AOB with low ammonium affinity to 0.4 to 1.1 mg/L ammonium-N (Bollmann *et al.*, 2002) for AOB with high ammonium affinity. The half-saturation constant for *Nitrosopumilus*-like AOA, on the other hand, is 0.00186 mg/L N (Martens-Habbena *et al.*, 2009), which is two orders of magnitude lower than the lowest measured value for AOB.

Similarly, nitrite-oxidizing *Nitrospira* were the dominant NOB at the time of sampling, which was likely due to low nitrite concentrations in the biofilter. Nitrite-oxidizing *Nitrospira* were also the dominant nitrite oxidizers in other aquaculture biofilters (Foesel *et al.*, 2008, Keuter *et al.*, 2011, Brown *et al.*, 2013). Researchers (Schramm *et al.*, 1999) have proposed that *Nitrospira*-like bacteria are *K*-strategists that grow efficiently at low nitrite and oxygen concentrations. The results obtained by a study characterizing NOB in a nitrifying sequencing batch biofilm reactor with a nitrite concentration gradient ranging from zero to 50 mg nitrite-N per liter confirmed this K/r-hypothesis (Daims *et al.*, 2001). In this study, AOA were the most

abundant ammonia oxidizer while nitrite-oxidizing *Nitrospira* were the most abundant nitrite oxidizer.

4.4 Conclusions

A waste stream simulating the ammonium-N excretion of 100 shrimp weighing an average of 5, 10, 15, and 20 g was used to investigate the response of increasing influent ammonium-N concentration on water quality and the nitrifying microbes in biological aerated filters used to treat wastewater in an indoor shrimp farm. Three replicate reactors were examined, and all reactors performed well with respect to water quality with the removal of ammonium-N to below detection levels and no accumulation of nitrite. Nitrate-N accumulated in the system as expected because the system was not operated for nitrate removal. Future work should investigate nitrate removal because nitrate-N concentrations will reach levels where they have negative impacts on shrimp growth in zero-discharge systems.

The biofilters investigated in this study had a robust nitrifying community. Two populations of both ammonium oxidizers and nitrite oxidizers were detected and quantified via qPCR. AOA were the dominant ammonia oxidizers, while nitrite-oxidizing *Nitrospira* were the more abundant nitrite oxidizers at all influent ammonium-N concentrations, which deviates from previous observations in aquaria studies. However, β-AOB abundance increased as the influent ammonium-N concentration increased. Future work should continue to examine the correlation between influent ammonium concentration and the abundance of ammonia oxidizing populations.

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Chapter 5: Effect of biofilter biofilm as a supplemental food on Pacific white shrimp, *Litopenaeus vannamei*, growth in an indoor, zero-discharge recirculating aquaculture system

5.1 Introduction[3]

In intensive shrimp culture, the nutritional requirements of the shrimp are typically met through the addition of an artificial food source, which is usually purchased from commercial feed suppliers. This commercial food represents a significant production cost for shrimp farmers. The use of commercial food has a negative environmental impact on shrimp production due to the inclusion of fish oil and fish meal from wild-caught fish (Goldburg & Triplett, 1997, Boyd & Clay, 1998, Naylor *et al.*, 2000). Therefore, reducing the amount of commercial food used for shrimp production decreases the production cost for farmers and improves the environmental sustainability of the system.

Researchers have investigated the impact of natural biota, in the form of biofilms growing in the culture cage, pond, or tank as a supplemental food source for shrimp. Studies have suggested phototrophic biofilms, which is a biofilm that includes algae, cyanobacteria, diatoms, flagellates, and/or nematodes in addition to bacteria, grown with the shrimp contribute significantly to the growth of various shrimp species, including *Farfantepenaeus paulensis* (Thompson *et al.*, 2002, Abreu *et al.*, 2007, Ballester *et al.*, 2007, Fernandes Da Silva *et al.*, 2008), *Litopenaeus vannamei* (Bratvold & Browdy, 2001, Moss & Moss, 2004, Otoshi *et al.*, 2006, Zarain-Herzberg *et al.*, 2006, Lezama-Cervantes & Paniagua-Michel, 2010), *Penaeus esculentus* (Burford *et al.*, 2004), and *Penaeus monodon* (Arnold *et al.*, 2006). In these studies, biofilm attachment surfaces were provided to promote the development of biofilms for the

[3] This is an abbreviated version of Section 2.4.3. Refer to Section 2.4.3 for a more detailed discussion on alternatives to fish meal in commercial feed.

shrimp to graze upon. Experiments in these studies had treatments with and without commercial feed. Fernandes Da Silva *et al.*, (2008) also examined the protein and lipid content of the biofilm to evaluate nutritional quality. They found that biofilm protein content varied from 0.43-1.76 mg protein per cm^2 of biofilm area, while lipid content ranged between 1.21-4.23 mg lipid per cm^2 of biofilm area. The variation in protein content was significantly correlated to the abundance of unicellular centric diatoms and nematodes, while the variation in lipid content was correlated to the abundance of heterotrophic bacteria, flagellates, and nematodes. An evaluation of biofilm as a food source using stable isotope analysis (Abreu *et al.*, 2007) showed that biofilm contributed more than 49% of carbon and 70% of nitrogen to shrimp tissue in a net cage experiment where shrimp received commercial feed and biofilm was available for shrimp to graze upon. The studies cited above examined the contribution of biofilm to shrimp growth in environments where phototrophic microorganisms were a significant part of the biofilm microbial communities. However, microbes in RAS biofilters under indoor, non-greenhouse culture conditions will be predominately bacteria.

Recirculating aquaculture systems (RAS) are being promoted as sustainable alternative to pond culture. They consist of a culture tank, biological filters to oxidize toxic ammonia and nitrite, and aeration of the water to remove carbon dioxide and increase oxygen concentrations (Ebeling, 2000). Ammonia- and nitrite-oxidizing bacteria are important to the functioning of biofilters in RAS as ammonia generated through hydrolysis of shrimp waste (urea) is converted to nitrite, which is in turn converted to nitrate. In addition, heterotrophic bacteria are present due to the availability of dissolved organic compounds and residual organic suspended solids in the system even when efforts are made to remove the suspended solids (uneaten feed and shrimp feces) regularly. The filter operated for suspended solids removal, in addition to the nitrifying biofilter, is an ideal environment for heterotrophic bacterial growth. The growth of heterotrophic bacteria in both types of filters requires that filters be backwashed frequently to remove excess microbial biomass and residual suspended solids removed from the culture tank to prevent head loss build up and short circuiting. As indicated above, culturing shrimp in systems with biofilms have had a positive impact on shrimp performance. Therefore, it is reasonable to suggest that biofilm from RAS biofilters and filters operated for suspended solids removal may also have a positive impact on shrimp performance. To date no studies have examined the impact of biofilm from RAS biofilters on shrimp growth. The purpose of this study was to examine the impact of

microbial biomass from the backwash of an indoor, non-greenhouse biological aerated filter as a supplemental feed source based on protein content on shrimp growth and survival.

5.2 Materials and Methods

5.2.1 Experimental design

5.2.1.1 RAS description

The three RAS consisted of a culture tank and two biofilter compartments operated as biological aerated filters (Figure 5.1). Each biofilter compartment consisted of a plexi glass column that was 20.32 cm in diameter and 91.44 cm in length. All biofilter compartments contained a mix of 1.6 mm by 3.2 mm oval plastic beads (Aquatic Eco-system, Inc., Apopka, FL) and 4 to 10 mm diameter clay spheres (Aquaclay, Keeton Industries, Wellington, CO) as biofilm attachment media. The mix was two-thirds plastic beads and one-third clay spheres by volume. The culture tanks were semi-square, 50-gallon polyethylene tanks (Polytank Inc., Litchfield, MN). Table 5.1 provides a summary of flow rate and media volume for all RAS.

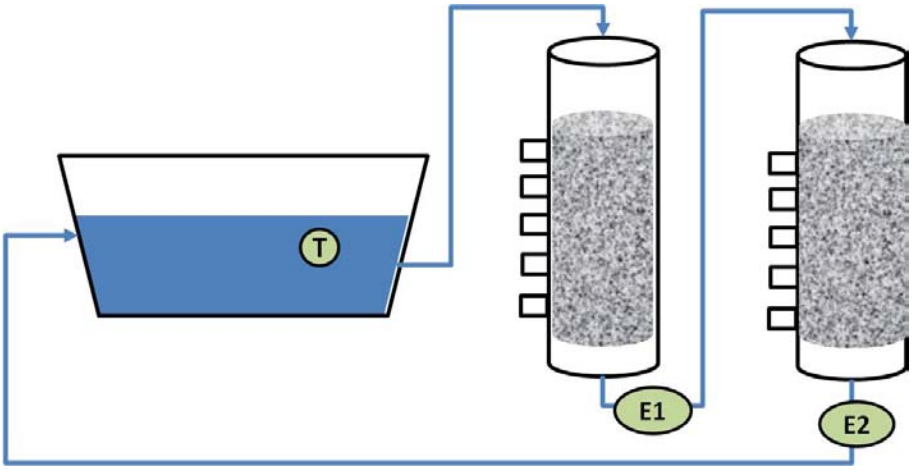

Figure 5.1: Schematic diagram of RAS. Sampling locations are identified by green circles. T is the tank water, E1 is the effluent from biofilter 1, and E2 is the effluent from biofilter 2.

Table 5.1: RAS hydraulic information

Reactor	Volume of water in RAS (m^3)	Flow rate (m^3/d)	Hydraulic load (m^3/(d-m^2))	Media volume (m^3)	
				Biofilter 1	Biofilter 2
Control	0.05	0.0060	1.88	0.0167	0.0162
Treatment-1	0.05	0.0060	1.88	0.0167	0.0165
Treatment-2	0.05	0.0060	1.88	0.0164	0.0161

Water for all RAS was prepared in the laboratory as follows. Each system was filled with 50 liters of distilled deionized water (16.5 cm depth) and a sea salt mix (Instant Ocean, Spectrum Brands Inc, Cincinnati, OH) to establish a salinity of 25 g/L. Salinity was calculated from measured conductivity and temperature data. A 150-watt Stealth submersible aquarium heater (Marineland, Spectrum Brands Inc., Cincinnati, OH) in each tank was used to maintain water temperatures at 30°C (RAS Feeding Experiment Trial #1) or 25°C (RAS Feeding Experiment Trial #2 and Batch Experiment). Air-tubing, located at the bottom of the tank, was used in each system to aerate the water. The target dissolved oxygen (DO) concentration was 5 mg/L. Bicarbonate, as ammonium bicarbonate and sodium bicarbonate, was added to maintain alkalinity above 100 mg/L as $CaCO_3$. Alkalinity was measured biweekly and pH daily (RAS Feeding Experiment Trial #1) or several times per week (RAS Feeding Experiment Trial #2 and Batch Experiment).

The RAS used in this study had different operational histories. Treatment-2 RAS was started in March 2011, while Treatment-1 RAS was started in March 2012 and Control RAS was started in May 2012. Microbial biomass from an indoor shrimp farm located in Okemos, MI was used to seed the biofilters of Treatment-2 RAS (Brown *et al.*, 2013). Microbial biomass from Treatment-2 RAS was used to seed the biofilters of Treatment-1 RAS and Control RAS. A preliminary shrimp study was conducted in Treatment-2 RAS, while no shrimp were grown in Treatment-1 RAS and Control RAS prior to this study. Furthermore an experiment to evaluate the correlation between increasing ammonia load and ammonia- and nitrite-oxidizing microbes (Chapter 4) was conducted with simulated waste prior to the feeding experiment described in this chapter in all RAS.

5.2.1.2 RAS Feeding Trial #1

Commercial feed was supplemented with biofilter biofilm based on protein content in two treatments: 25% biofilm + 75% commercial feed (treatment-1, T1) and 50% biofilm + 50% commercial feed (treatment-2, T2); the control (C) was 100% commercial feed. The commercial feed used in this experiment contained 40% protein as reported by the manufacturer (Shrimp Grower, Rangen, Buhl, ID). Biofilter biofilm was collected during backwashing prior to the feeding experiment. Backwashing consisted of air scour for 10 minutes followed by air and water scour for 20 minutes. The bed was not fluidized during this process. The backwash effluent was collected and allowed to settle for 48-72 hours at 4°C. The supernatant was returned to the culture tank and the biomass was further concentrated via centrifuging 200 mL bottles at 2800 g for 10 minutes. The concentrated wet biomass was then stored at -20°C in 50 mL centrifuge tubes. Biomass samples were sent to Rtech Laboratories (Arden Hills, MN) for protein analysis using the Kjeldhal protein method. The appropriate amounts of Rangen shrimp feed and backwash biomass, on a nitrogen basis, were mixed, which formed a slurry, and freeze dried (Freezone 6 Freeze Dry System, Labconco, Kansas City, MO) for at least 24 hours. The freeze dried mix was stored at 4°C. Refer to Appendix IV for details on the calculation used to determine how much biomass was needed based on the protein content of the biofilter backwash.

Tanks were stocked with 88, 83, and 80 post-larval Pacific white shrimp for the control, treatment-1, and treatment-2, respectively. This equates to a stocking density of 282, 266, and 256 shrimp/m^2 for the control, treatment-1, and treatment-2, respectively. Shrimp were fed at 6% body weight per day for 29 days. The amount of feed provided was adjusted based on wet weight shrimp measurements assuming 100% survival. The wet weight of 8-10 shrimp was measured at stocking, once per week during the trial, and at the end of the trial. Shrimp measured before the end of the experimental period were returned alive. Survival was monitored by counting the number of shrimp at the beginning and end of the experimental period.

5.2.1.3 RAS Feeding Trial #2

Trial #2 was conducted similarly to Trial #1 with several modifications. These modifications were as follows: (1) Microbial biomass collected from the RAS biofilters was freeze dried after centrifuging and the freeze dried biomass was shipped for protein analysis; (2) a four-day feeding cycle was used that consisted of four days of feeding with commercial feed for the control, three days of commercial feed plus one day of microbial biomass for treatment-1

(75/25), and two days of commercial feed plus two days of microbial biomass for treatment-2 (50/50); (3) feed was given manually once per day; and (4) wet weight of shrimp was measured every 10 days. All tanks were stocked with 100 Pacific white shrimp PLs. This equates to a stocking density of 320 shrimp/m^2. Shrimp were fed at a rate of 6% body weight per day for the first 32 days of the experiment; from Day 33 to the end of the experiment shrimp were fed at 12% body weight per day.

5.2.1.4 Batch Feeding Experiment

The purpose of this experiment was to compare growth rate of shrimp with continuous access to microbial biomass to shrimp fed on microbial biomass at specific intervals. This experiment was conducted concurrently with RAS feeding trial #2. Five-gallon buckets were used as tanks for this experiment with window screen mesh as biofilm attachment media. One week before the start of the experiment, backwash from RAS biofilters was used to establish biofilm in the batch reactors. During this one-week time period, sucrose and ammonia were added to promote the growth of heterotrophic bacteria and establish biofilm on the window screen mesh. Microbial biomass was scraped from the window screen mesh for measurements.

Tanks were stocked with 20 Pacific white shrimp PLs for a stocking density of 270 shrimp/m^2. For the first 32 days shrimp were fed at 6% body weight per day. From Day 33 to Day 68 shrimp were fed at 12% body weight per day. Each treatment (control, treatment-1, and treatment-2) was conducted in duplicate. Feed was given in a four-day cycle as described in RAS Feeding Experiment Trial #2, except that no external microbial biomass was fed on the fourth day (treatment-1) and the third and fourth days (treatment-2) of the feeding cycles. Wet weight of shrimp was measured at the beginning and end of the experiment.

5.2.2 Water quality

Probes were used to monitor several parameters in the tank. Salinity and temperature were measured with a Orion Model 1230 meter (Thermo Scientific, Waltham, MA) and pH with a Mettler Toledo sevenEasy meter (Columbus, OH). Water samples were collected daily (RAS Feeding Trial #1) or three times per week (RAS Feeding Trial #2 and Batch Feeding Experiment) from the tank and biofilter effluents to monitor total ammonium-nitrogen (TAN), nitrite-nitrogen (NO2), and nitrate-nitrogen (NO3) concentrations according to standard methods (APHA, 1998). Other water quality parameters that were measured included alkalinity, total organic carbon (TOC), total suspended solids (TSS), and volatile suspended solids (VSS)

following standard methods (APHA, 1998). All analyses were performed in triplicate for each sample collected.

Samples for TOC analysis were collected several times per week during the experimental period for each treatment. Samples were collected from the tank and each biofilter effluent (Figure 5.1). The purpose of these samples was to monitor organic carbon. Chemical oxygen demand (COD) was not measured due to the high concentration of chloride ion in the water, which interferes with the COD reaction chemistry.

During RAS Feeding Trial #1, TAN, NO2, and NO3 concentrations were measured, in the tank and biofilter effluents (Figure 5.1), once each week at one-hour intervals over twelve hours between feedings to observe variations in concentration not captured by the daily grab sample. Tank samples were also analyzed for TOC.

In the batch experiment, all water quality parameters were measured three times per week except alkalinity, TSS, VSS and TOC which were measured biweekly.

5.3 Results and Discussion

5.3.1 RAS Feeding Experiment Trial #1

In this experiment, water temperature varied between 28.0 and 29.5°C, while salinity ranged from 23.3 to 26.6 (Table 5.2). Bicarbonate was added to maintain alkalinity above 100 mg/L $CaCO_3$, and alkalinity ranged from 243 to 331 mg/L $CaCO_3$ (Table 5.2). Due to the high buffer capacity in each RAS there was minimal variation pH (Table 5.2). Total and volatile suspended solids and total organic carbon were also monitored to assess the amount of organic carbon available to heterotrophic bacteria. Concentrations indicate that organic carbon was relatively low throughout the experiment. For detailed information on TOC refer to Appendix IV, Figures A4.6 and A4.7.

Table 5.2: RAS Feeding Trial #1 Water quality Summary*

Parameter	Control (100)	Treatment 1 (75/25)	Treatment 2 (50/50)
Temperature (°C)	29.2 ± 0.3	29.1 ± 0.3	28.4 ± 0.4
Salinity (psu)	25.1 ± 1.5	24.5 ± 1.2	24.9 ± 1.2
pH	8.34 ± 0.05	8.49 ± 0.04	8.42 ± 0.13
Alkalinity (mg/L $CaCO_3$)	255 ± 12	389 ± 42	368 ± 24
TSS (mg/L)	62.4 ± 7.0	67.2 ± 9.8	65.8 ± 8.4
VSS (mg/L)	16.7 ± 1.7	17.4 ± 3.6	17.2 ± 3.8
TOC (mg/L)	7.5± 3.6	10.4 ± 4.7	7.9 ± 3.1

*numbers in the table are average ± standard deviation

Daily grab samples were collected before the morning feeding and analyzed for ammonium-N (Appendix IV, Figure A4.1), nitrite-N (Figure A4.2), and nitrate-N (Figure A4.3). The purpose of these samples was to monitor the 24-hour accumulation of inorganic nitrogen. It is important to maintain less than 1 mg/L as ammonium-N and less than 15 mg/L as nitrite-N because higher concentrations are toxic to shrimp (Timmons & Ebeling, 2007). These results show that there was no ammonium or nitrite accumulation within a 24-hour period. However, nitrate-N concentrations increased in the system, which indicates that ammonium was produced by the shrimp and converted to nitrate by nitrification since measured concentrations of ammonium were low. Because the system was not operated to promote nitrate removal, nitrate-N accumulated. The highest concentrations of nitrate-N (120 mg/L) were observed in the control (Figure A4.3). These levels should not have had negative impacts on the shrimp based on information from the literature. A study (Kuhn *et al.*, 2010) investigating the chronic toxicity of nitrate on Pacific white shrimp in a RAS at 9, 220, 435, and 910 mg/L nitrate-N, showed negative impacts on shrimp biomass and antennae length at 435 mg/L nitrate-N and negative impacts on growth, survival, total mass, and antennae length at 910 mg/L nitrate-N. The concentrations observed in this study were below these values. Grab samples only capture the concentration in the system at the time of sampling. Consequently, the grab sample at the end of the feeding cycle did not capture potentially higher concentrations that might occur immediately after feeding. Therefore, samples were collected in one-hour intervals after feeding, time series samples, to observe concentrations during a feeding cycle that were not captured by the daily

grab samples. For all treatments, ammonium-N and nitrite-N concentrations were below the detection limit of 0.05 mg/L, while nitrate concentrations remained constant (Figures A4.4 and A4.5).

Shrimp were fed at 6% body weight assuming 100% survival for the experimental period. The growth rate for the control was 2.1 times higher than that for treatment-1 and 3.8 times higher than that for treatment-2 (Table 5.3). The growth rates range from 0.0064 to 0.0245 g/day, and similar range of growth rates, 0.00455 to 0.0427 g/day, was observed in a study on the effect of microbial mats on Pacific white shrimp (Lezama-Cervantes & Paniagua-Michel, 2010). In that study microbial mats were used to treat shrimp waste and were also evaluated for their effect on shrimp growth and survival under various conditions during a 33-day experimental period. The lower growth rate observed in the treatments is possibly due to the unavailability of protein in the biofilter biofilm to the shrimp or the biofilter biofilm may not have been presented in a form suitable for consumption. Thompson *et al*. (2002) observed higher final shrimp weight in tanks with biofilm, even though the biofilm had a low protein content of 6% wet weight. The biofilm used in this study had a wet weight protein content that ranged from 0.73% to 4.53%; the variability in the protein values were caused by the water content. The organic carbon associated with these protein values are shown in Table 5.4.

Survival in all treatments was less than 80% (Table 5.3). The low survival was likely due to stress from the overnight transport from Florida to Michigan as well as temperature stress from moving the shrimp from the transport water to the RAS tanks. Furthermore at the end of the experimental period, shrimp were effectively being fed at 12% to 18% body weight per day because of the low survival. This may have contributed to increased growth towards the end of the experiment.

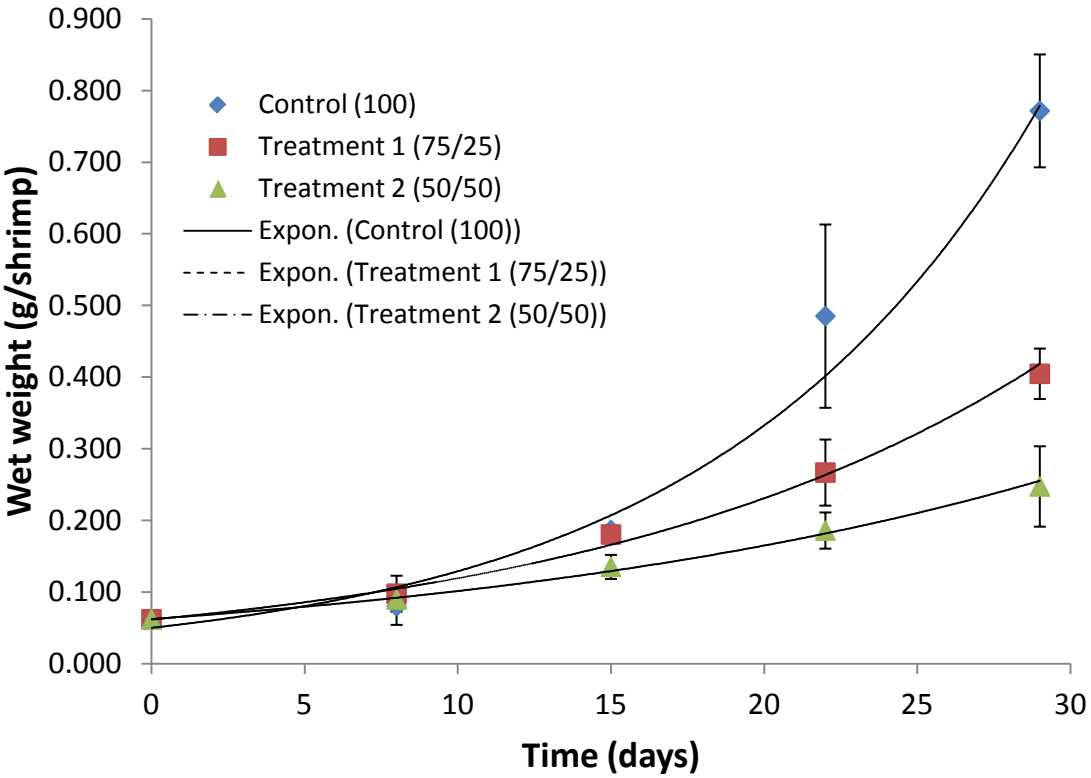

Figure 5.2. Shrimp growth for Trial #1. Error bars show standard deviation. Each curve was fitted with an exponential equation R^2 value greater than 0.9 (data not shown)

Table 5.3: Shrimp growth rate and survival

Treatment	No. shrimp at t = 0	No. shrimp at t = 29 d	Survival (%)	growth rate (g/day)
Control (100)	88	31	35	0.0245
Treatment-1 (75/25)	83	46	55	0.0118
Treatment-2 (50/50)	80	30	38	0.0064

Table 5.4: Protein and carbon content of microbial biomass from six collections of biofilter backwash used in RAS feeding trial #1

Protein content (%)	VSS (mg/L)	COD* (mg/L)	TOC** (mg/L)
4.53	665	944	350
0.73	260	369	137
2.63	284	403	149
2.89	303	430	159
2.51	443	629	233
0.81	248	352	130

*Calculated from 1.42 g COD/g VSS (Rittmann & McCarthy, 2001)

**Calculated from 1 g TOC/2.7 g COD (Henze *et al.*, 2000)

If each growth curve (Figure 5.2) was fitted with an exponential equation, the time to reach an average weight of 20 g/shrimp would take 64 days (control), 88 days (treatment-1), and 119 days (treatment-2). This corresponds to a growth period 27% and 46% longer in treatment-1 and treatment-2, respectively, compared to the control. An exponential equation was selected to describe the data because previous results showed that shrimp growth is exponential (A4.8). There is a tradeoff between reduced commercial feed inputs and time to harvest size. Reducing the amount of commercial feed slows the growth rate which results in a longer growout time. The operational cost, or cost of commercial feed plus cost of electricity needed to operate RAS, is higher for the treatments compared to the control (Figure 5.3). Electricity cost is the highest fraction of the operation cost. Therefore, for the treatments to be cost competitive more work needs to be done to optimize the supplemental level that yields a growth rate similar to that obtained for 100% commercial feed.

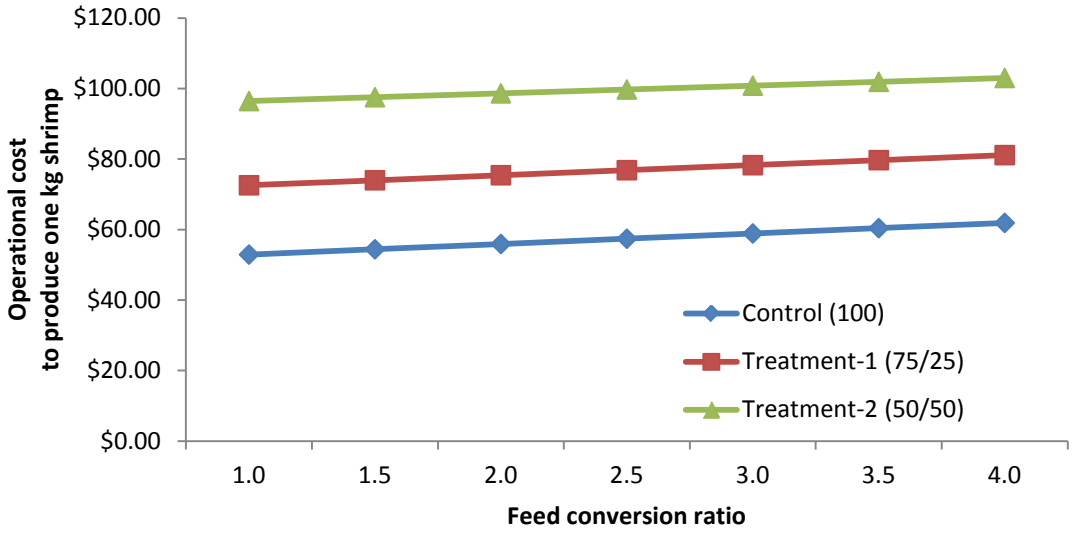

Figure 5.3: Operational cost to produce one kg shrimp for Trial #1 as a function of feed conversion ratio. Operation cost is the cost of commercial feed plus cost of electricity needed to operate RAS. The majority of the operation cost, at least 94%, is due to electricity usage.Refer to Table A4.3 for summary of feed cost and to Tables A4.4., A4.5, and A4.6 for summary of electricity costs.

5.3.2 RAS Feeding Experiment Trial #2

During the study period, the water temperature ranged from 24.4 and 25.4°C, while salinity ranged from 24.4 to 30.6 (Table A4.7). pH, alkalinity, TSS, VSS and TOC values were similar to Trial #1 (refer to Table A4.3 for detailed information on these parameters in Trial #2). Daily samples were collected before feeding and analyzed for ammonium-N (Figure A4.9), nitrite-N, and nitrate-N (Figure A4.10). Ammonia-N concentrations were below 0.2 mg/L, there was no detectable nitrite-N, and nitrate-N accumulated in the system to approximately 43 (Treatment-2), 60 (Treatment-1), and 65 (Control) mg/L nitrate-N.

Modifications were made to the experimental design, as described in the Materials and Methods, to address potential issues with nutrient availability in the microbial biomass and to determine if shrimp were being under fed. The shrimp in the treatments were fed on a four-day cycle and the feeding rate was doubled halfway through the experiment. Shrimp were fed at 6% body weight assuming 100% survival from Day 0 to 32, and then were fed at 12% body weight assuming 100% survival from Day 33 to Day 68. The growth rate for the control was 1.9 times

higher than that for treatment-1 and 2.9 times higher than that for treatment-2 (Table 5.5). There was a significant difference in the growth rate between shrimp fed 6% and 12% body weight per day ($P < 0.1$, t test). The biofilm used in this study had a dry weight, after freeze drying, protein content of 6%, 14%, 20%, and 30%. Survival rate was 80% or better in this trial (Table 5.5). Shrimp were acclimated to the temperature difference between the water they were shipped in and tank water. This, combined with changes in feed delivery, likely played a role in the increased survival rate in Trial #2.

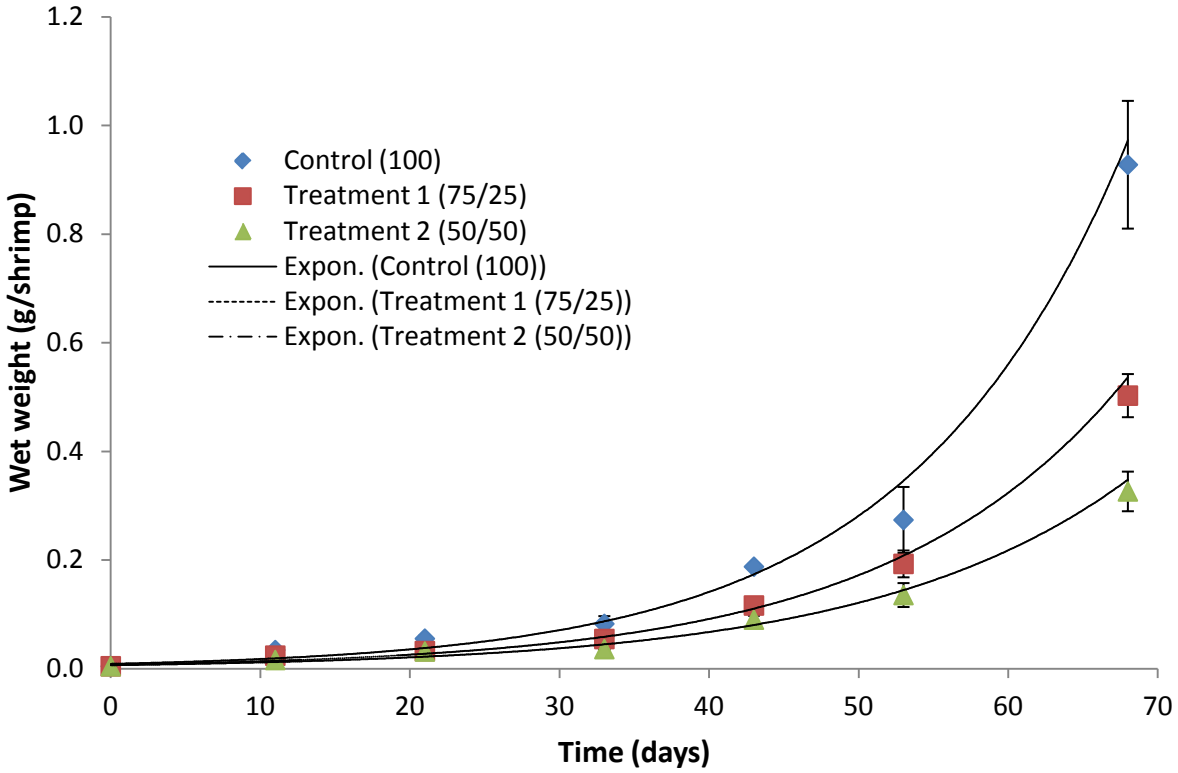

Figure 5.4: Shrimp growth for Trial #2. Error bars show standard deviation. Each curve was fitted with an exponential equation R^2 value greater than 0.9 (data not shown)

Table 5.5: Shrimp growth rate and survival for Trial #2

Treatment	No. shrimp at t = 0	No. shrimp at t = 68 d	% survival	Growth rate (g/day)
Control (100)	100	80	80	0.0136
Treatment 1 (75/50)	100	93	93	0.0073
Treatment 2 (50/50)	100	87	87	0.0047

If each growth curve (Figure 5.4) was fitted with an exponential equation, the time to reach an average weight of 20 g/shrimp would take 112 days (control), 126 days (treatment-1), and 137 days (treatment-2). Similar to the results for Trial #1, the increased time it takes to reach harvest size has increased operational costs (Figure 5.5) in the treatments as compared to the control. This corresponds to a growth period 11% and 18% longer in treatment-1 and treatment-2, respectively, compared to the control. The time needed to reach harvest size was longer in Trial #2 as compared to Trial #1 because the size of the shrimp at the beginning of the experiment was different. The starting average weight in Trial #1 was 0.06 g/shrimp while the initial average weight in Trial #2 was 0.005 g/shrimp. The difference between the growth rates in Trial #1 and Trial #2 were not statistically significant ($P > 0.1$, t test).

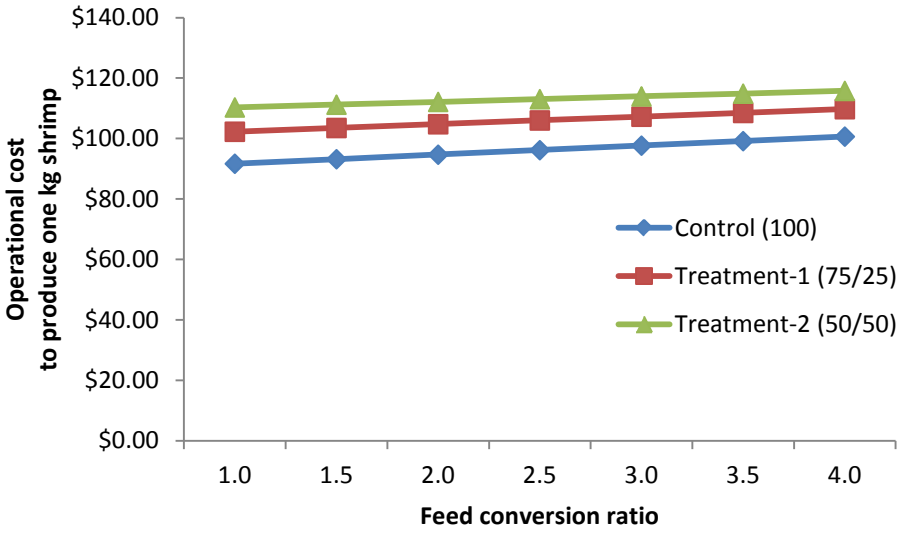

Figure 5.5: Operational cost to produce one kg shrimp as a function of feed conversion ratio for Trial #2. Operational cost is the cost of commercial feed plus cost of electricity needed to operate RAS. The majority of the operation cost, at least 94%, is due to electricity usage. Refer to Table A4.9 for summary of feed cost and to Tables A4.10, A4.11, and A4.12 for summary of electricity costs.

5.3.3 Batch Experiment

The growth rates in this experiment were 0.0192 g/day (Control), 0.0095 g/day (Treatment-1), and 0.0086 g/day (Treatment-2). These results were similar to those observed in RAS feeding Trial #2. However, the difference in growth rate between the batch experiment and RAS Feeding Trial #2 was not significant ($P > 0.1$, t test). For details on water quality refer to Table A4.13.

5.4 Conclusion

Microbial biomass from RAS biofilters was evaluated as a supplemental feed source based on protein content for Pacific white shrimp. This studied showed that replacing 25% and 50% of commercial feed protein content with microbial biomass from RAS biofilters resulted in a decreased growth rate. However, the longer grow out time increases operational cost, cost of commercial feed plus electricity cost needed to operate RAS. The results of this study can be

refined by using a feeding method that provides sufficient feed for shrimp growth to optimize growth rate. Also, shrimp in this study were not grown to 20 g; consequently, time to grow to 20 g was calculated. Future studies should use longer experimental periods to improve growth projections. Shrimp farmers using RAS can use their biofilter backwash waste and/or solids waste as a supplemental feed source to reduce solid waste and mass of commercial feed in Pacific white shrimp production.

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Chapter 6: Conclusions and Future Work

The long-term goal of the research presented in this dissertation is to improve the environmental sustainability of shrimp recirculating aquaculture systems (RAS) operated indoors. Microorganisms have a critical role in maintaining water quality and solid waste management in RAS. Therefore, a better understanding of the microbially-mediated nitrogen transformation processes in indoor RAS can help improve performance through appropriate operational modifications. Furthermore, a reduction in commercial shrimp feed may be possible through the use of microbial biomass from RAS biofilters as a supplemental feed source. This research began with the analysis of the nitrifying microbial populations in the trickling filter of a local indoor shrimp farm (Shrimp Farm Market, Okemos, MI). This farm utilized a zero-discharge RAS for culturing Pacific white shrimp (*Litopenaeus vannamei*). Laboratory-scale RAS were fabricated and used to investigate effect of ammonium concentration on ammonia-oxidizing and nitrite-oxidizing populations. The lab RAS were also used to evaluate biofilter biofilm as a supplemental feed source on shrimp growth and survival. In the following section I will discuss the important findings from each chapter and provide recommendations for future research.

Chapter 3: AOA and nitrite-oxidizing nitrospiras were the dominant nitrifying microbes in the tricking filter of an indoor, zero-discharge marine recirculating aquaculture system

This study analyzed the nitrifier community in the biofilter of a zero discharge, RAS for the production of marine shrimp in a low density (low ammonium production) system. The system consisted of a culture tank and a nitrifying trickling filter that contained different types of biofilm attachment media: plastic bioballs at the top and crushed oyster shells at the bottom. Water from the culture tank was pumped and filtered by gravity through the different biofilm attachment media and returned to the culture tank. There was a basin beneath the filter tower to collect water and settled particles (sludge) before the water was pumped back into the culture

tank. Using biomass from the nitrifying trickling filter, archaeal 16S rRNA gene clone libraries were constructed and ammonia oxidizing archaea (AOA) related to *Nitrosopumilus maritimus* were detected in these samples. The presence of AOA was confirmed by PCR targeting archaeal ammonia monooxygenase A (*amoA)* genes. qPCR indicated the abundance of archaeal *amoA* in oyster shells was an order of magnitude higher than its abundance in bioballs. Proteobacterial ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) were not detected by bacterial 16S rRNA gene clone library analysis. However, NOB from the genus *Nitrospira* were represented in the library. The abundance of bacterial *amoA* was found to be very low using qPCR, but bioballs contained significantly greater levels of AOB than oyster shells. qPCR revealed that bioballs contained an order of magnitude higher level of *Nitrospira marina*-type NOB than oyster shells, but its abundance relative to total bacteria was higher in oyster shells. The water quality (i.e., low ammonium and nitrite concentrations) and biofilm attachment media played a role in the competitiveness of AOA over AOB and *Nitrospira marina-type NOB* over *Nitrospira moscoviensis*-type NOB, but additional work is needed to elucidate their function and importance in determining the outcome of the competition.

Chapter 4: A correlation between ammonium concentration and niche differences of ammonium oxidizers was not observed but AOB abundance did increase with increasing ammonium concentration in the biological aerated filters of an indoor, zero-discharge RAS

A waste stream simulating the ammonium-N excretion of 100 shrimp weighing an average of 5, 10, 15, and 20 g was used to investigate the response of increasing influent ammonium-N concentration on water quality and the nitrifying microbes in biological aerated filters used to treat wastewater in an indoor shrimp farm. Three replicate reactors were examined, and all reactors performed well with respect to water quality with the removal of ammonium-nitrogen to below detection levels and no accumulation of nitrite. Nitrate-nitrogen accumulated in the system as expected because the system was not operated for nitrate removal; however, there was partial nitrate removal based on nitrogen mass balance calculations. This passive dentirification did not remove nitrate to concentrations that do not have an impact on shrimp growth. Future work should investigate nitrate removal strategies in zero-discharge marine RAS because nitrate-nitrogen concentrations will reach levels where they have negative impacts on shrimp growth.

The biofilters investigated in this study had a robust nitrifying community. Two populations of both ammonium oxidizers and nitrite oxidizers were detected and quantified via qPCR. AOA were the dominant ammonia oxidizers, while nitrite-oxidizing *Nitrospira* were the more abundant nitrite oxidizers at all influent ammonium-nitrogen concentrations. However, *Betaproteobacterial* AOB abundance increased as the influent ammonium-nitrogen concentration increased. Future work should continue to examine the correlation between influent ammonium concentration and the abundance of nitrifying microbes in engineered environments.

Chapter 5: Supplementing commercial shrimp feed with RAS biofilter biofilm is a viable way to reduce feed costs and improve the sustainability of RAS; however, work needs to be done to optimize the supplemental level for increased shrimp growth and to improve cost estimates

Microbial biomass from RAS biofilters was evaluated as a supplemental feed source based on protein content for Pacific white shrimp. The biological aerated filters of an indoor, zero-charge RAS were backwashed to remove the microbial biomass from the biofilters. The resulting slurry of microbial biomass and water was then processed to remove water and concentrate the solids via centrifuging and freeze drying. Protein content of the microbial biomass was measured, and in the first trial microbial biomass was mixed in a slurry with commercial feed prior to feeding, while in the second trial microbial biomass was not mixed with commercial feed. The supplemental levels investigated in this study were 25% and 50% replacement of commercial feed based on protein content. This studied showed that replacing 25% and 50% of commercial feed protein content with microbial biomass from RAS biofilters results in a decreased growth rate. The longer growth time results in higher operational costs, which includes the commercial feed cost plus the cost of the electricity needed to operate the RAS. The results of this study can be refined by optimizing the supplemental level to achieve the highest growth while reducing feed cost and minimizing additional growth time. Also, shrimp in this study were not grown to harvest size so time to grow to 20 g was estimated. Future studies should use longer experimental periods to improve growth projections. Shrimp farmers using RAS can use their biofilter backwash waste and/or solids waste as a supplemental feed source to reduce waste and feeding costs of Pacific white shrimp production.

Appendices

Appendix I: Supplemental Information for Chapter 3

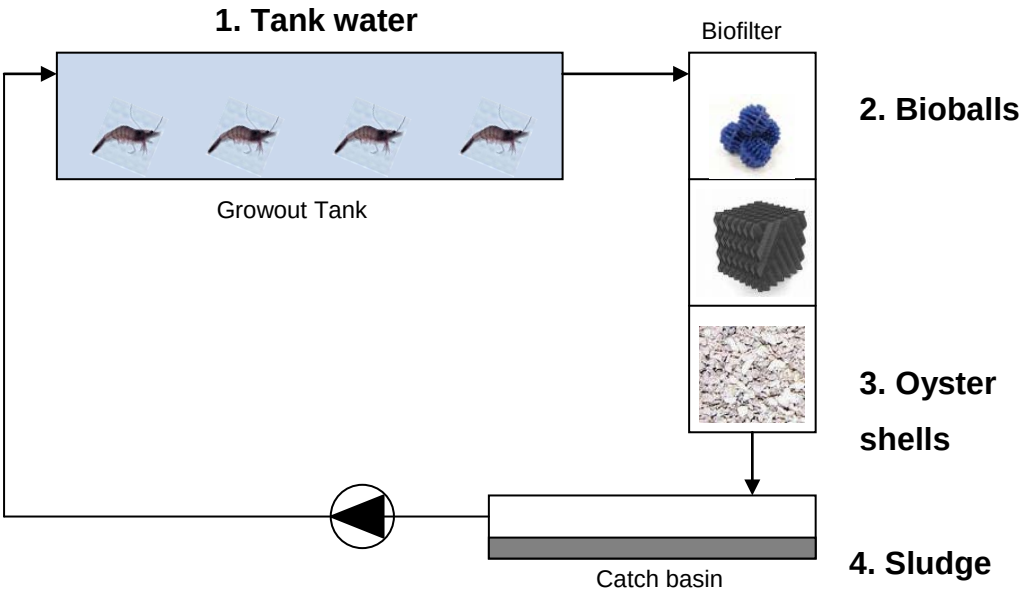


Figure A1.1: Simplified schematic of a zero-exchange RAS for marine shrimp production in Okemos, MI. The system relies on a biofilter containing different biofilm attachment media: plastic bioballs, plastic corrugated block, and crushed oyster shells. In this system, water from the culture tank is pumped and filtered by gravity through the biofilter. The numbers indicate sampling locations.

Table A1.1**:** Phylum level affiliations of bacterial 16S rRNA genes that were PCR amplified, cloned, and sequenced for different RAS sampling locations (Figure A1.1). Values in the table are expressed as a percentage of the total number of clones.

	RAS sampling locations			
Phylum level classifications	Tank water	Bioballs	Oyster shells	Sludge
Bacteroidetes	45.3	2.9	6.8	18.6
Alphaproteobacteria	29.7	28.6	22.7	19.6
Betaproteobacteria	ND*	ND	ND	1.0
Gammaproteobacteria	9.4	17.1	9.1	26.5
Deltaproteobacteria	ND	4.3	5.7	7.8
Planctomycetes	1.6	4.3	25.0	2.0
Firmicutes	ND	1.4	2.3	8.8
Actinobacteria	3.1	4.3	4.5	2.0
Nitrospirae	ND	14.3	3.4	ND
Chloroflexi	ND	2.9	2.3	2.9
Chlorobi	ND	ND	ND	1.0
Verrucomicrobia	ND	ND	2.3	1.0
Gammatimonadetes	ND	ND	2.3	ND
Unclassified	10.9	20.0	13.6	8.8
Number of clones	64	70	88	102

*ND = not detected

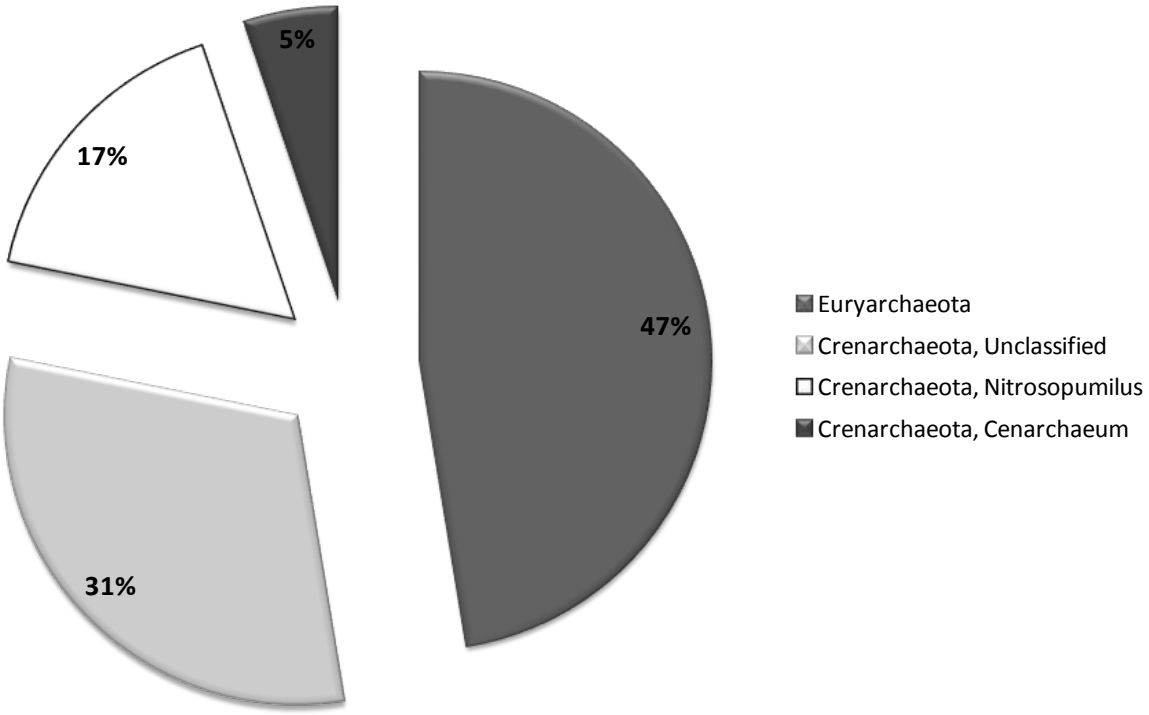


Figure A1.2: Phylum level affiliations of archaeal 16S rRNA genes that were PCR amplified, cloned, and sequenced using samples obtained from the RAS system. This clone library was generated from pooled DNA samples extracted from the different RAS samples (Figure A1.1).

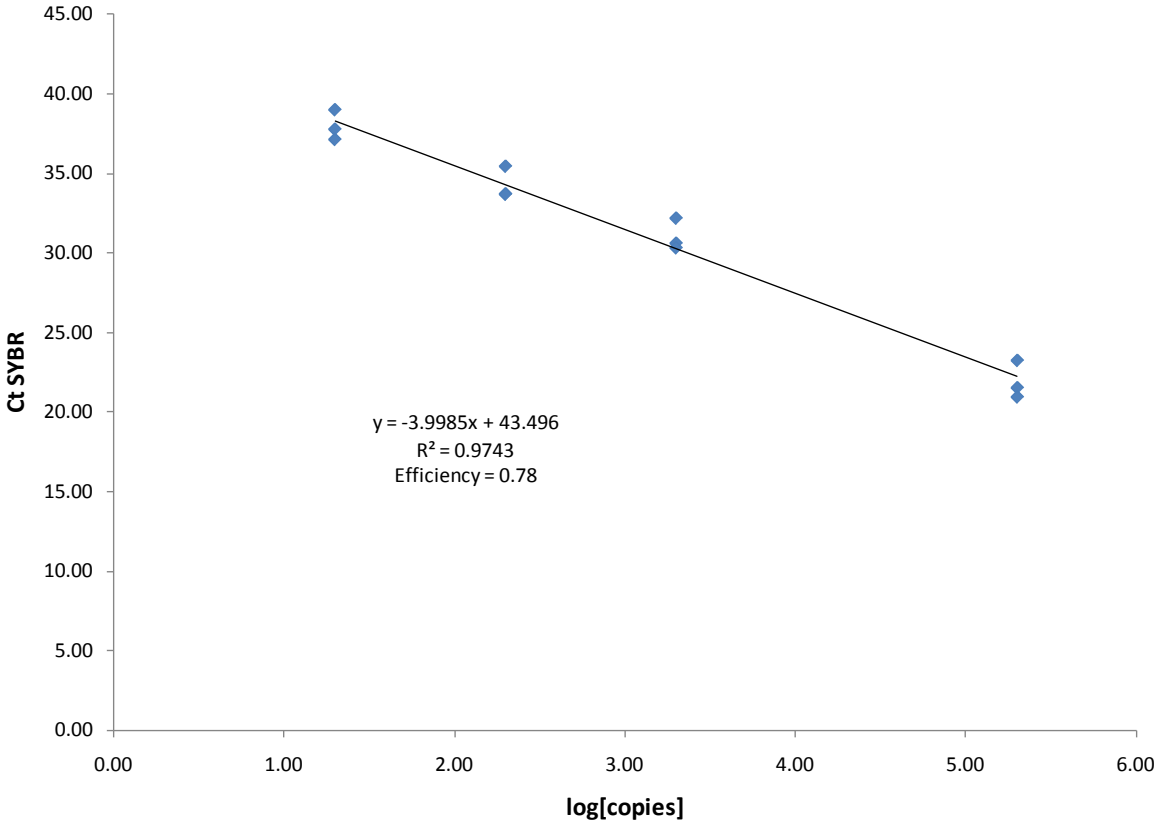


Figure A1.3: Standard curve for archaeal *amoA* gene qPCR. Standard curve was generated from linearized plasmids containing cloned archaeal *amoA* PCR amplicons previously sequenced to verify identity.

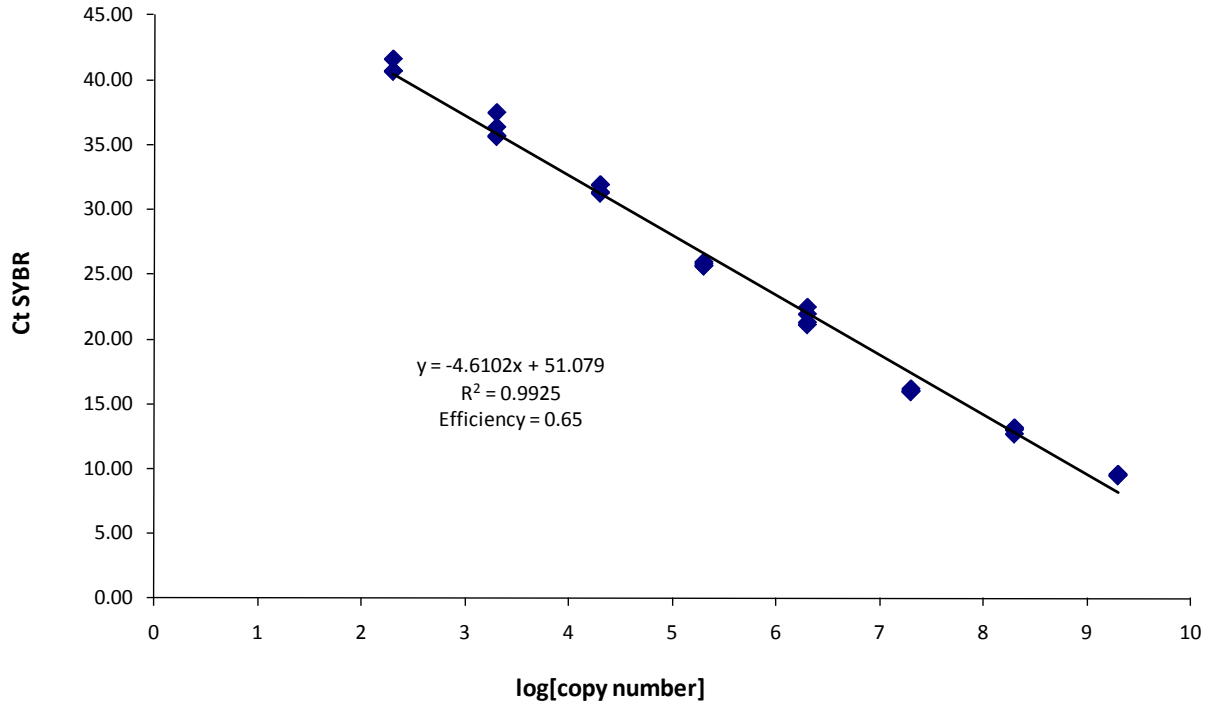


Figure A1.4: Standard curve for archaeal 16S rRNA gene qPCR. Standard curve was generated from linearized plasmids containing cloned archaeal 16S rRNA PCR amplicons previously sequenced to verify identity. Refer to the caption of Figure A1.3 for a discussion of the method used to calculate efficiency.

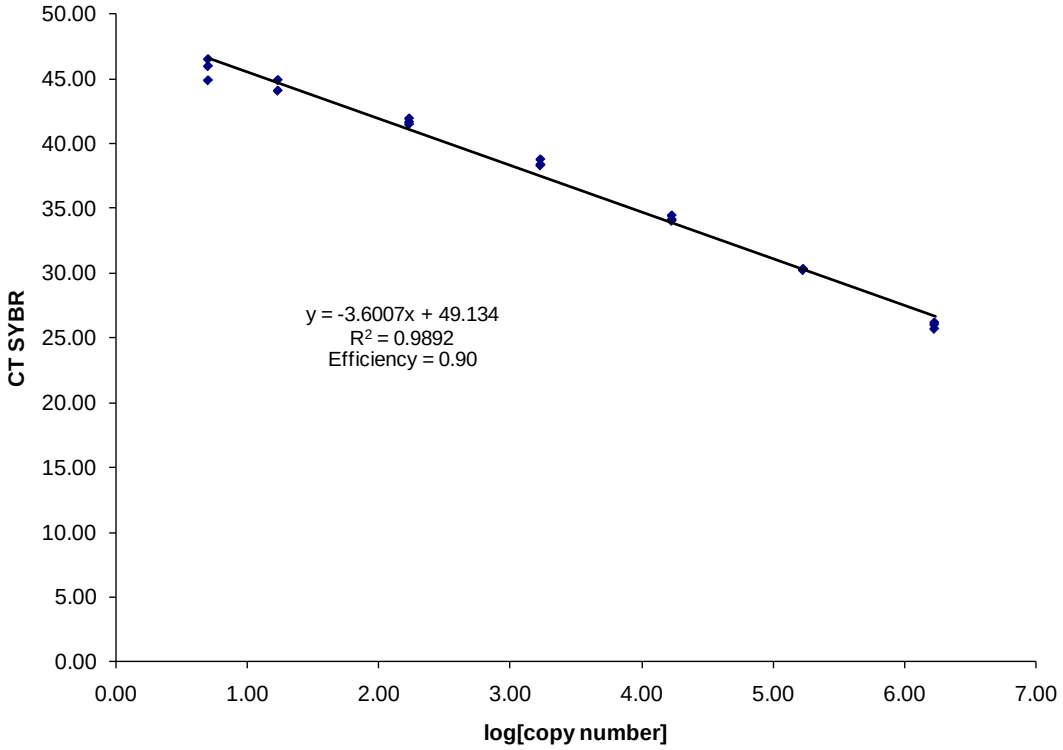


Figure A1.5: Standard curve for the *Nitrospira marina*-type NOB 16S rRNA gene qPCR. Standard curve was generated from linearized plasmids containing cloned bacterial 16S rRNA PCR amplicons previously sequenced to verify identity. Refer to the caption of Figure A1.3 for a discussion of the method used to calculate efficiency.

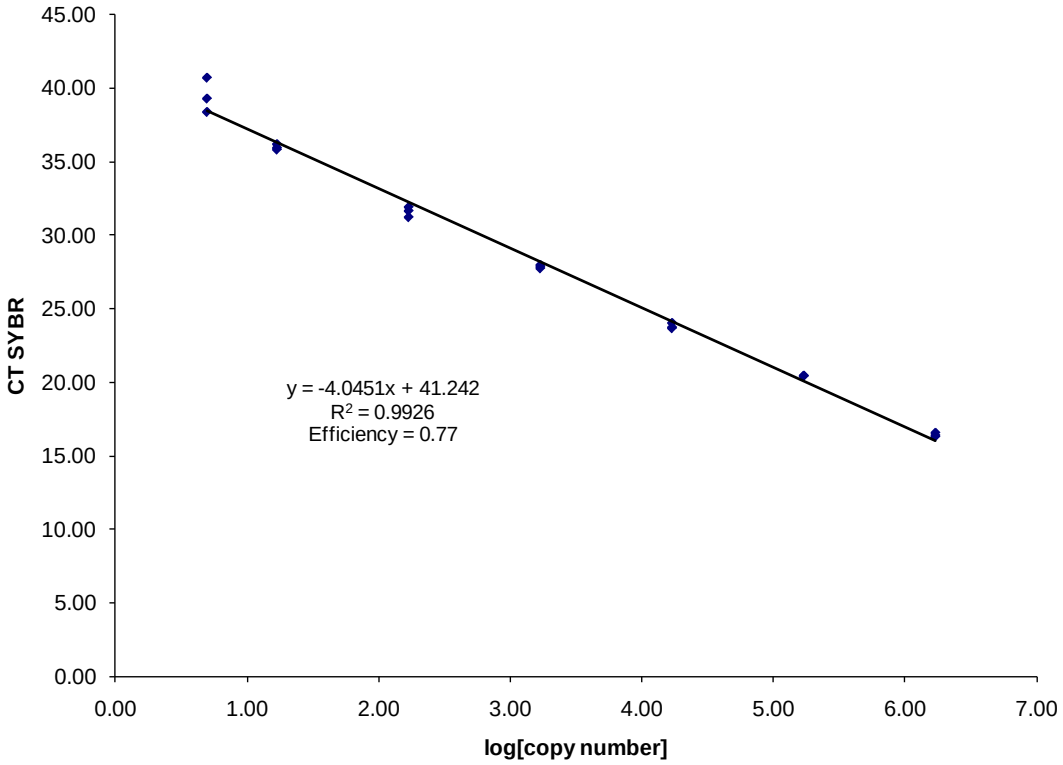


Figure A1.6: Standard curve for *Nitrospira moscoviensis*-type NOB 16S rRNA gene qPCR. Standard curve was generated from linearized plasmids containing cloned bacterial 16S rRNA PCR amplicons previously sequenced to verify identity. Refer to the caption of Figure A1.3 for a discussion of the method used to calculate efficiency.

Appendix II: Residence time distribution analysis to characterize the flow regime in each component of the laboratory recirculating aquaculture systems

Background

Ideal reactors

There are three reference, or ideal, reactor configurations are common in engineered and natural systems. They are the continuously mixed batch reactor (CMBR), continuously stirred tank reactor (CSTR), and the plug flow reactor (PFR). These reactors differ in terms of mixing characteristics and mass flow. The difference between CMBR and CSTR is that the CMBR has no mass transport across the reactor boundary whereas the CSTR has continuous mass flow in and out of the reactor. Neither of these reactors have concentration gradients (Crittenden *et al.*, 2005). The difference between the CSTR and PFR is that the CSTR is completely mixed and has no concentration gradient, while PFRs have continuous mass flow but the elements of fluid parcels move parallel to the reactor axis and do not mix creating a continuous concentration gradient (Crittenden *et al.*, 2005). These basic reactors have more complicated variations which include reactors in series and sequencing batch reactors. Real systems are never completely ideal.

Non-ideal reactors

Non-ideal reactors have flow patterns that deviate from the ideal reactors described above. Deviations from ideal CSTRs are caused by stagnant regions, channeling, and imperfect mixing equipment, while deviations from ideal PFRs are caused by stagnant regions, dispersion, and preferential flow paths. Reactor performance is affected by non-ideal flow (Crittenden *et al.*, 2005). To determine how this flow affects performance, the non-ideal flow has to be described first and then performance can be modeled with the appropriate flow characteristics. Tracers are used to characterize flow in reactors (Crittenden *et al.*, 2005, Danckwerts, 1953).

Materials and Methods

RAS reactor description

There were three RAS in the experimental set-up, each RAS consisted of a culture tank and a down-flow, nitrifying biological aerated filter with two compartments (Figure A2.1). Each biofilter compartment was comprised of a column that was 20.32 cm in diameter and 91.44 cm in length. For the RTD analysis the biofilter compartments in RAS-1 contained 4 to 10 mm diameter clay spheres (Aquaclay, Keeton Industries, Wellington, CO) as biofilm attachment medium. The biofilter compartments in RAS-2 and RAS-3 both contained 1.6 mm by 3.2 mm oval plastic beads (Aquatic Eco-system, Inc., Apopka, FL) as biofilm attachment medium. The culture tank for each RAS was semi-square, 50-gallon polyethylene tanks (Polytank Inc., Litchfield, MN).

Figure A2.1: Three RAS reactors

Tracer test

A tracer test was done for each reactor component to characterize its hydraulic flow. First the flow rate of each reactor component was measured. Chloride was used as the tracer element because it is non-reactive, and the chloride concentration was measured with a conductivity meter during the tracer test. A standard curve was generated to correlate the chloride concentration to the conductivity measurement. The tracer was introduced as a pulse input to the influent of each reactor component, and the conductivity was measured at predetermined time intervals.

Tracer data analysis

The tracer data was analyzed according to the procedure outlined in (Danckwerts, 1953, Crittenden *et al.*, 2005). In summary, the tracer data was normalized with respect to the measured mean residence time and to the total mass concentration of tracer recovered to generate an exit age distribution curve. The tracer results were characterized with hydraulic residence time and the equivalent number of tanks-in-series (Levenspiel & Knovel, 1999). Details of the analysis method are described below.

For a pulse input, the stimulus-response curves are obtained as either C-curves or E-curves depending on how they are normalized. If M_T = mass of tracer injected, V_R = reactor volume, and $C_\Delta = M_T/V_R$, then the C-curve is a plot of time versus C_{out}/C_Δ. The E-curve, or E(t), is a plot of the C-curve values normalized by the area under the C-curve. The E(t) is an exit age distribution function, and the area under the E-curve is one because it is a frequency distribution. The first moment of E(t) about the origin gives the mean value of tracer mass residence time, t_m. The second moment of E(t) about mean mass residence time, t_m, is a measure of the variance of distribution, or extent of spreading of pulse.

Steps to obtain the exit age distribution from a pulse tracer study (Danckwerts, 1953; Crittenden *et al.*, 2005; Levenspiel & Knovel, 1999):

1. Plot the C-Curve and the E-Curve.
2. Determine the tracer mass residence time, t_m.
3. Compare the tracer mass residence time to the hydraulic residence time to determine if there is any short circuiting.

4. Determine the normalization concentration
5. Replot the E-curve as $E(\Theta) = C/C_N$ versus Θ where $\Theta = t/t_m$ and C_N = total mass concentration of tracer recovered

These steps account for the fact that not all of the tracer will be recovered.

A Microsoft Excel spreadsheet was used to analyze tracer data. Details on how to set-up this spreadsheet are provided below. All integrals were calculated using the trapezoid rule.

Column 1. Measured, or raw, time data.

Column 2. Calculated concentration data from chloride to conductivity standard curve.

Column 3. The sum of this column is the mass of tracer recovered. $M_T = \sum Q C_i \Delta t_i$

Column 4. $C_\Delta = M_T/V_R$. The C-Curve is a plot of time versus C/C_Δ.

Column 5. To plot the exit age distribution function, the area under the C-Curve must be calculated. The area under the C-Curve, $A_C = \frac{1}{C_\Delta}\int_0^\infty C(t)dt$

A_C is related to the fractional recovery of tracer mass = QA_C/V_R

Column 6. $E(t) = [C(t)/C_\Delta]/A_C$. E(t) values are obtained by dividing Column 3 values by A_C.

Column 7. The area under the E(t) curve, or the sum of this column, should be one.

Column 8. To calculate the dimensionless exit age distribution, E(Θ), the mean residence time must be calculated. The first moment of E(t) about the origin is the mean tracer mass residence time, t_m. $t_m = \int_0^\infty tE(t)dt$. This column contains the values of *t*E(t)* at each time step.

Column 9. Numerical integration of area under *t*E(t)* function. The sum of this column is t_m.

Column 10. The second moment of E(t) about the mean mass residence time, tm, is a measure of the extent of spreading of the pulse, or the variance of distribution.

$\sigma_t^2 = \int_0^\infty t^2 E(t)dt - t_m^2$. This column contains the values of t^2**E(t)* at each time step.

Column 11. Numerical integration of area under t^2**E(t)* function. This value is used to calculate the variance of distribution

Column 12. C_N is the total mass concentration of tracer recovered. $C_N = \frac{\int_0^\infty C(t)dt}{t_m}$. This column contains the numerical integration of area under the *C(t)* function. These values are summed and divided by t_m.

Column 13. The dimensionless E-curve can be calculated two ways. $E(\Theta) = C/C_N = t_m * E(t)$. This column is C/C_N. The values in this column and Column 15 should be the same.

Column 14. Dimensionless time $\Theta = t/t_m$

Column 15. This column contains values of $E(\Theta)$ calculated as $t_m * E(t)$.

Tanks-in-Series model to describe tracer data

The tanks-in-series (TIS) model was used to model the tracer test results. The exit age distribution for *n* CSTRs in series is given by

$$E(\theta)_n = \frac{n(n\theta)^{n-1}}{(n-1)!} e^{-n\theta}$$

where $E(\Theta)_n$ = exit age distribution for *n* tanks in series and Θ = relative residence time, or dimensionless time t/t_m (Levenspiel & Knovel, 1999, Crittenden *et al.*, 2005).

Results and Discussion

It was expected that the tanks would be described as CSTRS while the biofilters would be described as some number of CSTRs in series instead of as PFRs due to aeration in the biofilters. A tracer study was conducted to obtain the exit age distribution for each RAS component. This data was then fit to the tanks-in-series model to describe the non-ideal flow. The best fit between the tanks-in-series model and the tracer data was determined by the value of *n* tanks that minimized the sum of squared differences between the model and the data. This was done for each tank and biofilter compartment in RAS-1, RAS-2, and RAS-3.

RAS-1

The RAS-1 tank was best described as a CSTR. As shown in Figure A2.2, the tank tracer data was best explained by the one tank-in-series model, which is one CSTR. RAS-1 BF1 was best described as 3 tanks-in-series model (Figure A2.3). The sum of the squared difference between the tracer data and 2 tanks-in-series, 3 tanks-in-series, and 4 tanks-in-series models were 0.911, 0.445, and 0.476, respectively. RAS-1 BF2 tracer data was explained by the 4 CSTRs-in-

series model (Figure A2.4). The sum of the squared difference between the tracer data and 3 tanks-in-series, 4 tanks-in-series, and 5 tanks-in-series models were 0.246, 0.074, and 0.117, respectively.

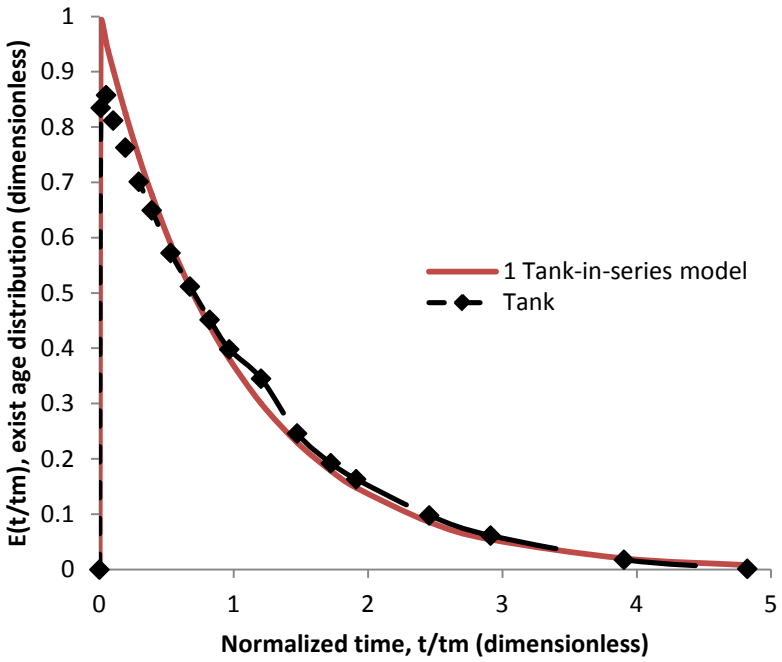


Figure A2.2. Exit age distribution curve, or E-curve, for RAS-1 tank tracer test. The time is normalized by "tm" which is the measured mean residence time. Tank-in-series is a model used to describe non-ideal flow in a reactor (Levenspiel & Knovel, 1999).

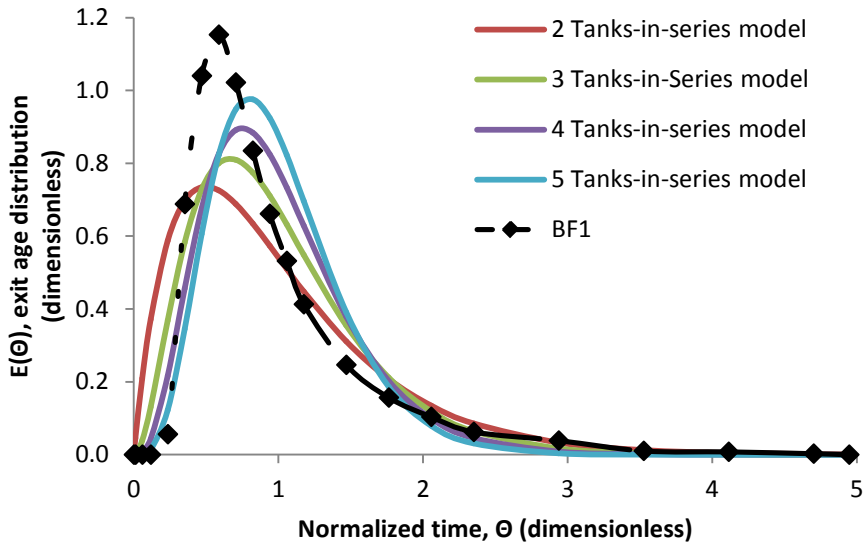


Figure A2.3. Exit age distribution curve, or E-curve, for RAS-1 BF1 tracer test. The time is normalized by "tm" which is the measured mean residence time. Tank-in-series is a model used to describe non-ideal flow in a reactor (Levenspiel & Knovel, 1999).

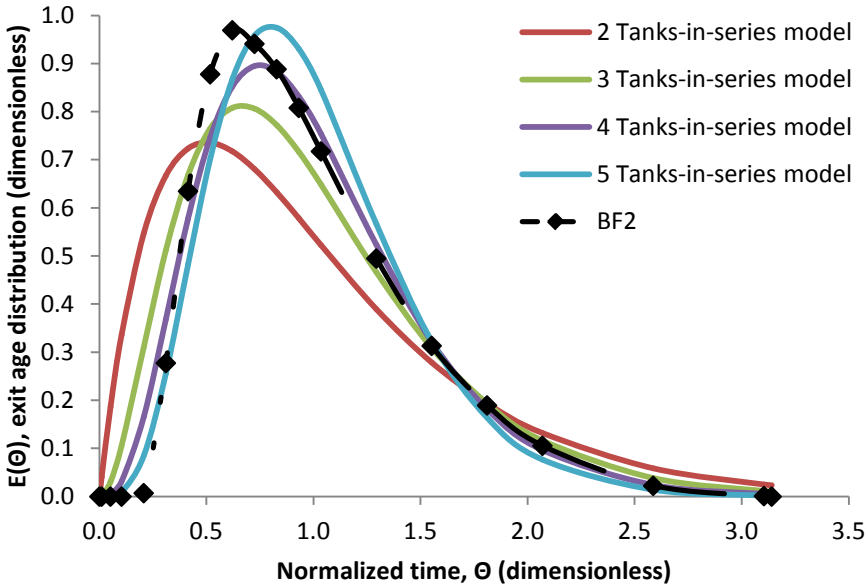


Figure A2.4. Exit age distribution curve, or E-curve, for RAS-1 BF2 tracer test. The time is normalized by "tm" which is the measured mean residence time. Tank-in-series is a model used to describe non-ideal flow in a reactor (Levenspiel & Knovel, 1999).

RAS-2

The RAS-2 tank was best described as a CSTR. As shown in Figure A2.5, the tank tracer data was best explained by the one tank-in-series model, which is one CSTR. RAS-2 BF1 was best described as 3 tanks-in-series model (Figure A2.6). The sum of the squared difference between the tracer data and 2 tanks-in-series, 3 tanks-in-series, and 4 tanks-in-series models were 0.804, 0.136, and 0.354, respectively. RAS-2 BF2 tracer data was explained by the 4 CSTRs-in-series model (Figure A2.7). The sum of the squared difference between the tracer data and 3 tanks-in-series, 4 tanks-in-series, and 5 tanks-in-series models were 0.389, 0.356, and 0.855, respectively.

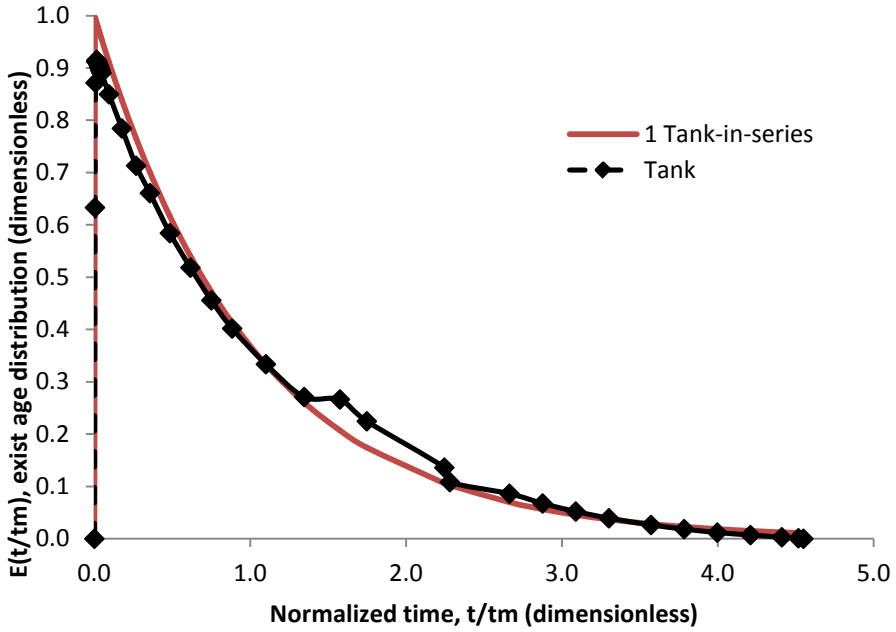


Figure A2.5. Exit age distribution curve, or E-curve, for the RAS-2 tank tracer test. The time is normalized by "tm" which is the measured mean residence time. Tank-in-series is a model used to describe non-ideal flow in a reactor (Levenspiel & Knovel, 1999).

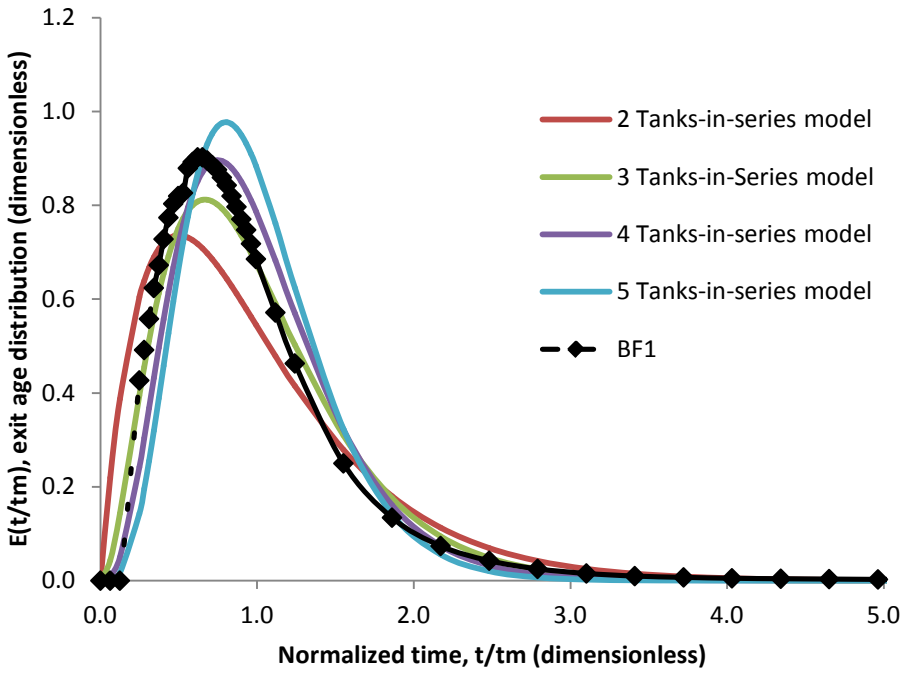


Figure A2.6. Exit age distribution curve, or E-curve, for RAS-2 BF1 tracer test. The time is normalized by "tm" which is the measured mean residence time. Tank-in-series is a model used to describe non-ideal flow in a reactor (Levenspiel & Knovel, 1999).

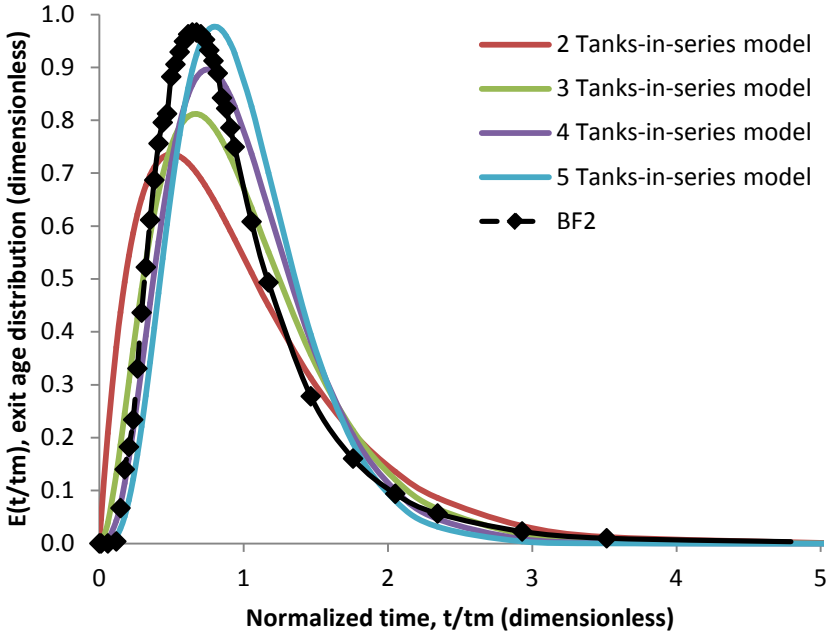


Figure A2.7. Exit age distribution curve, or E-curve, for RAS-2 BF2 tracer test. The time is normalized by "tm" which is the measured mean residence time. Tank-in-series is a model used to describe non-ideal flow in a reactor (Levenspiel & Knovel, 1999).

RAS-3

The RAS-3 tank was best described as a CSTR. As shown in Figure A2.8, the tank tracer data was best explained by the one tank-in-series model, which is one CSTR. RAS-2 BF1 was best described as 3 tanks-in-series model (Figure A2.9). The sum of the squared difference between the tracer data and 2 tanks-in-series, 3 tanks-in-series, and 4 tanks-in-series models were 0.731, 0.379, and 0.952, respectively. RAS-2 BF2 tracer data was explained by the 4 CSTRs-in-series model (Figure A2.10). The sum of the squared difference between the tracer data and 3 tanks-in-series, 4 tanks-in-series, and 5 tanks-in-series models were 0.718, 0.202, and 0.207, respectively.

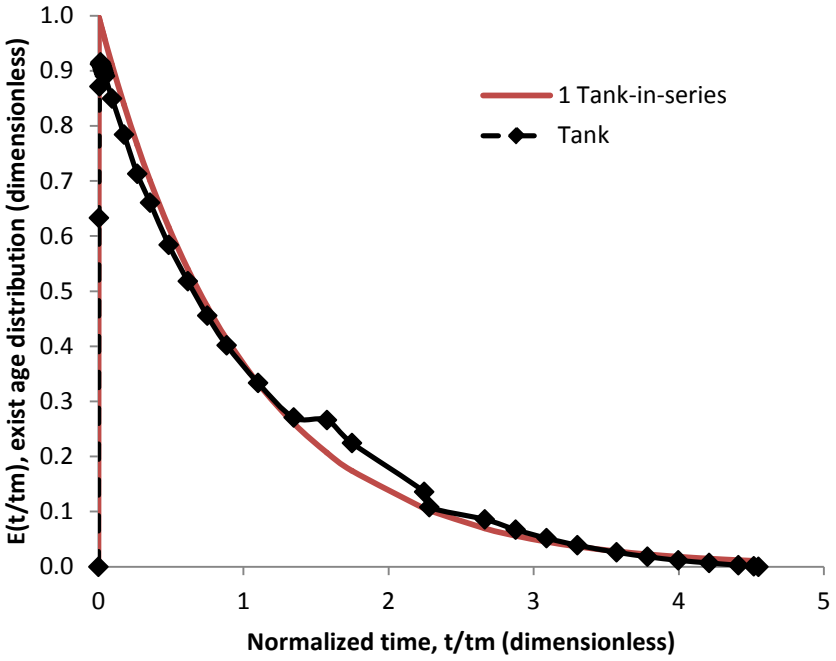


Figure A2.8. Exit age distribution curve, or E-curve, for the tank tracer test. The time is normalized by "tm" which is the measured mean residence time. Tank-in-series is a model used to describe non-ideal flow in a reactor (Levenspiel & Knovel, 1999).

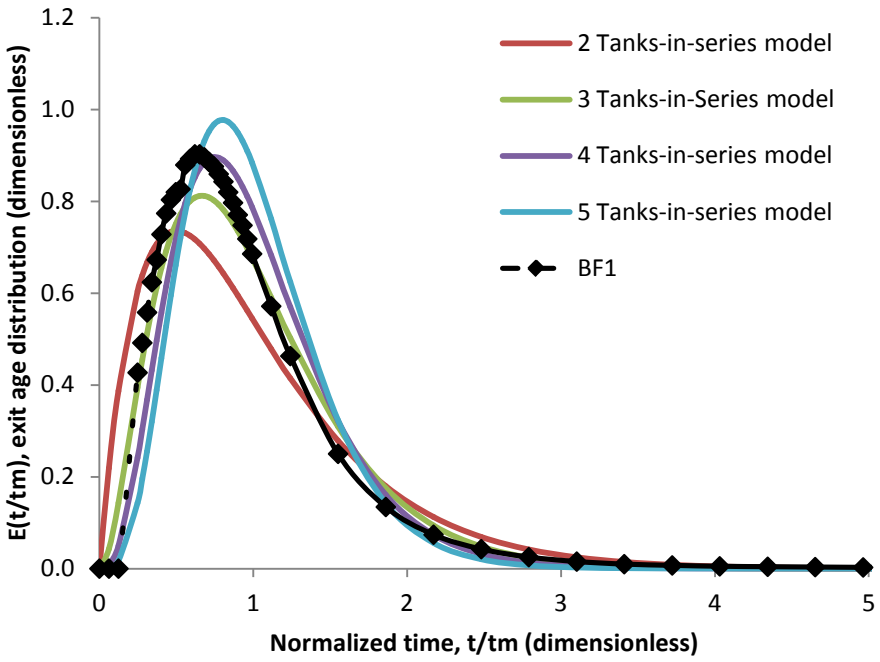


Figure A2.9. Exit age distribution curve, or E-curve, for RAS-3 BF1 tracer test. The time is normalized by "tm" which is the measured mean residence time. Tank-in-series is a model used to describe non-ideal flow in a reactor (Levenspiel & Knovel, 1999).

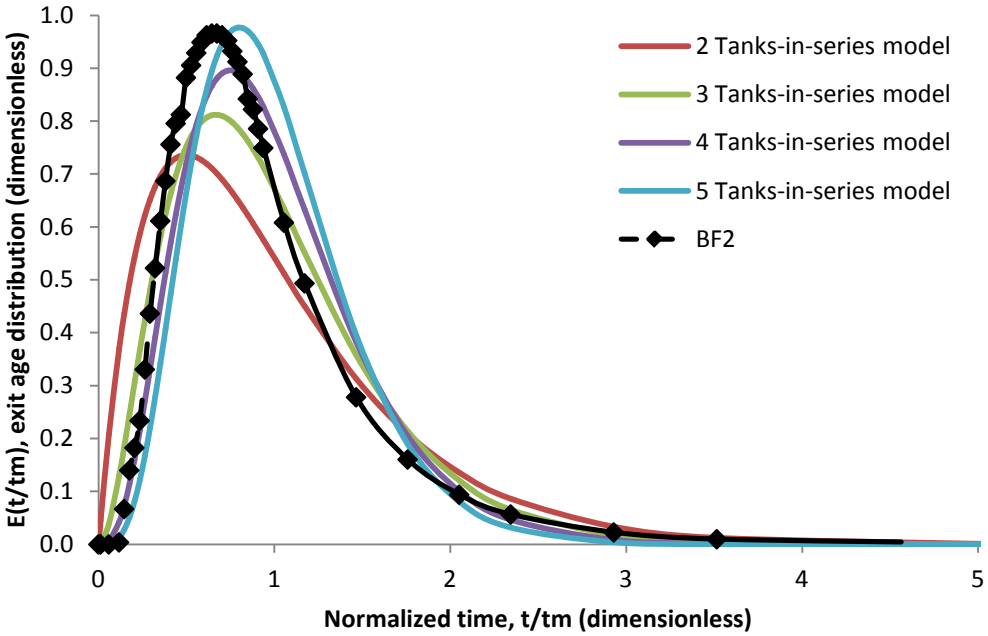


Figure A2.10. Exit age distribution curve, or E-curve, RAS-3 BF2 tracer test. The time is normalized by "tm" which is the measured mean residence time. Tank-in-series is a model used to describe non-ideal flow in a reactor (Levenspiel & Knovel, 1999).

Conclusions

A residence time distribution analysis was conducted on each component of the three RAS reactors. The purpose of the analysis was to describe the non-ideal flow in each reactor. Based on this analysis, all culture tanks were best described as a CSTR. Biofilter #1 was best described as 3 CSTRs-in-series, while biofilter #2 was best described as 4 CSTRs-in-series. The difference between BF1 and BF2 is most likely the placement of the three bendable rubber aeration tubes that span the length of each column.

References

Crittenden JC, Trussell RR, Hand DW, Howe KJ & Tchobanoglous G (2005) *Water Treatment: Principles and Design Second Edition*. John Wiley & Sons, Inc., Hoboken, New Jersey.

Danckwerts PV (1953) Continuous flow systems - distribution of residence times. *Chemical Engineering Science* **2**: 1-13.

Levenspiel O & Knovel (1999) *Chemical reaction engineering*. Wiley, New York.

Appendix III: Supplemental Information for Chapter 4

Reactor Information

RAS-1 was inoculated in April 2011 with biomass from the full-scale RAS used in the preliminary field study (Chapter 3), operated by Shrimp Farm Market, located in Okemos, MI and a salt-tolerant nitrifying activated sludge from a wastewater treatment plant that treats a high salt wastewater (Plum Island Wastewater Treatment Plant, Charleston Water Systems, Charleston, SC). RAS-1 was initially operated by feeding an ammonium chloride/ammonium bicarbonate solution and commercial shrimp feed to ensure that nitrification developed in the system before shrimp were added. Samples were collected regularly for pH, alkalinity, ammonia, nitrite, and nitrate analyses.

RAS-1 was operated with shrimp in the system for 194 days prior to seeding RAS-2. *Litopenaeus vannamei* (Pacific White Shrimp) post larvae (or PLs) were ordered from Miami Aqua-culture, Inc. (www.miami-aquaculture.com). When the shrimp were added to RAS-1, ammonium supplementation was stopped. Shrimp were fed commercial food equal to 8% of their body weight per day and their growth was monitored. Samples were collected regularly for pH, alkalinity, ammonia, nitrite, nitrate analyses, total organic carbon, total suspended solids, and volatile suspended solids according to Standard Methods (APHA 1998). Only one biofilter associated with RAS-1 was periodically backwashed to remove the buildup of particulate matter.

The second RAS (RAS-2) was seeded in March 2012 with inoculum biomass from the biofilter backwash from RAS-1. RAS-2 was initially operated by feeding an ammonium chloride solution for 14 days followed by ammonium bicarbonate and commercial shrimp feed thereafter to ensure that nitrification developed in the system before adding shrimp. Samples were collected regularly for pH, alkalinity, ammonia, nitrite, and nitrate analyses. The third RAS (RAS-3) was started using the same procedure as for RAS-2 in May 2012.

Prior to starting the laboratory experiments, the biofilter attachment media in the biofilters of all three RAS were mixed to remove the variability associated with different RAS

run times. This resulted in biofilter attachment media mix in each compartment of two-thirds plastic beads and one-third clay spheres by volume.

Table A3.1: Reactor information

Reactor	Total volume (L)	Flow rate (m^3/d)	Hydraulic load ($m^3/(d\text{-}m^2)$)	Media volume (m^3)	
				Biofilter 1	Biofilter 2
RAS-1	50	0.0060	1.88	0.0167	0.0165
RAS-2	50	0.0060	1.88	0.0164	0.0161
RAS-3	50	0.0060	1.88	0.0167	0.0162

454-Sequencing

Table A3.2: Number of sequences by sample pre- and post-processing for quality of reads

Concentration	Sample location	Initial number of sequences	Number of sequences after pre-processing	Median read length (bp)
11 mg/L ammonium -N	RAS-1 Port 1 Day 14	60	54	347
	RAS-1 Port 4 Day 14	101	91	345
	RAS-1 Port 5 Day 14	71	64	345
	RAS-1 Port 8 Day 14	156	145	346
22 mg/L ammonium-N	RAS-1 Port 1 Day 28	115	107	347
	RAS-1 Port 4 Day 28	92	89	346
	RAS-1 Port 5 Day 28	101	91	345
	RAS-1 Port 8 Day 28	121	104	346
33 mg/L ammonium-N	RAS-1 Port 1 Day 42	106	91	347
	RAS-1 Port 4 Day 42	142	132	346
	RAS-1 Port 5 Day 42	121	111	346
	RAS-1 Port 8 Day 42	94	84	345
44 mg/L ammonium-N	RAS-1 Port 1 Day 56	27	23	345
	RAS-1 Port 4 Day 56	154	136	346
	RAS-1 Port 5 Day 56	60	52	345
	RAS-1 Port 8 Day 56	85	82	346

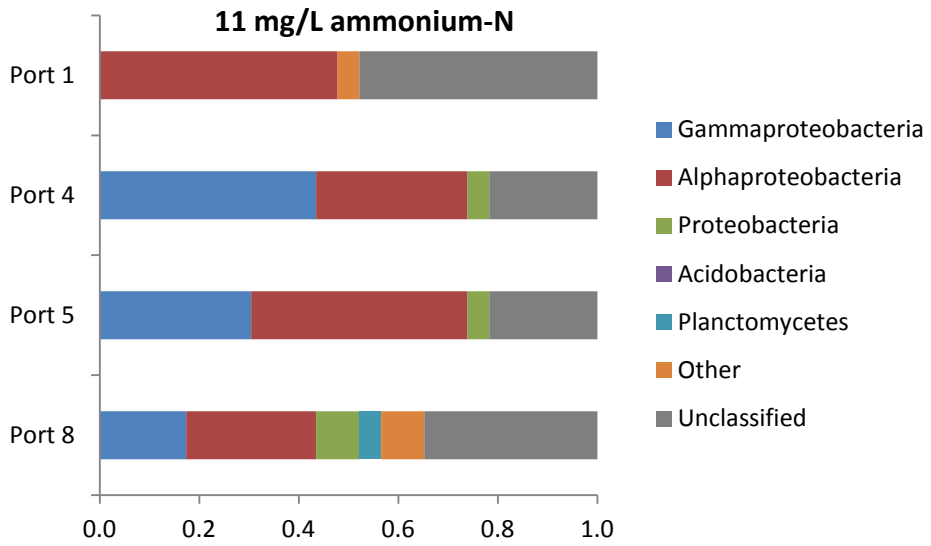


Figure A3.1: Relative abundance of bacterial phyla on Day 14 in RAS-1; influent ammonium-N concentration was 11 mg/L. The detection of *Gammaproteobacteria* suggests the presence of *Gammaproteobacteria* AOB, while the detection of *Alphaproteobacteria* suggests the presence of *Nitrobacter*. Classification was using the RDP training set in mothur (Schloss *et al.*, 2009) at 97% cutoff.

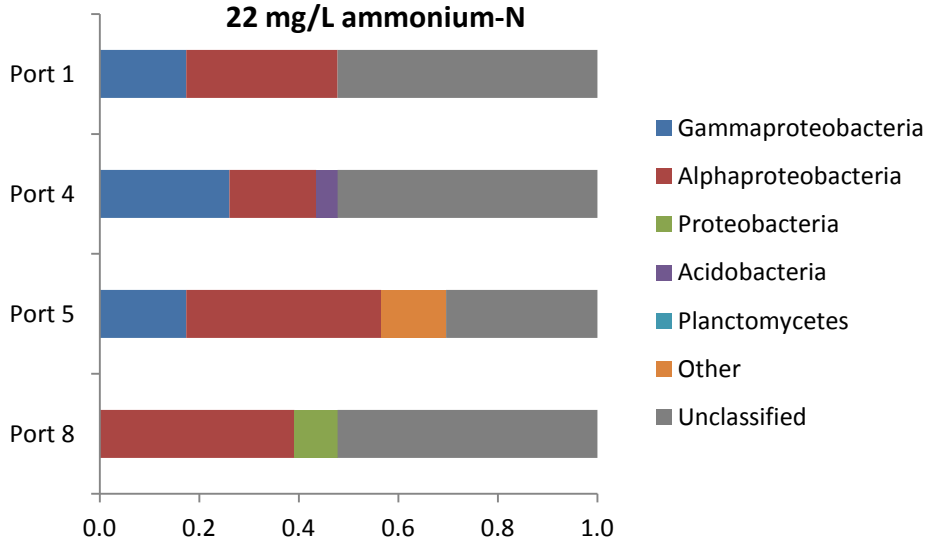


Figure A3.2: Relative abundance of bacterial phyla on Day 28 in RAS-1; influent ammonium-N concentration was 22 mg/L. The detection of *Gammaproteobacteria* indicates the presence of *Gammaproteobacteria* AOB, while the detection of *Alphaproteobacteria* indicates the presence of *Nitrobacter*. Classification was using the RDP training set in mothur (Schloss *et al.*, 2009) at 97% cutoff.

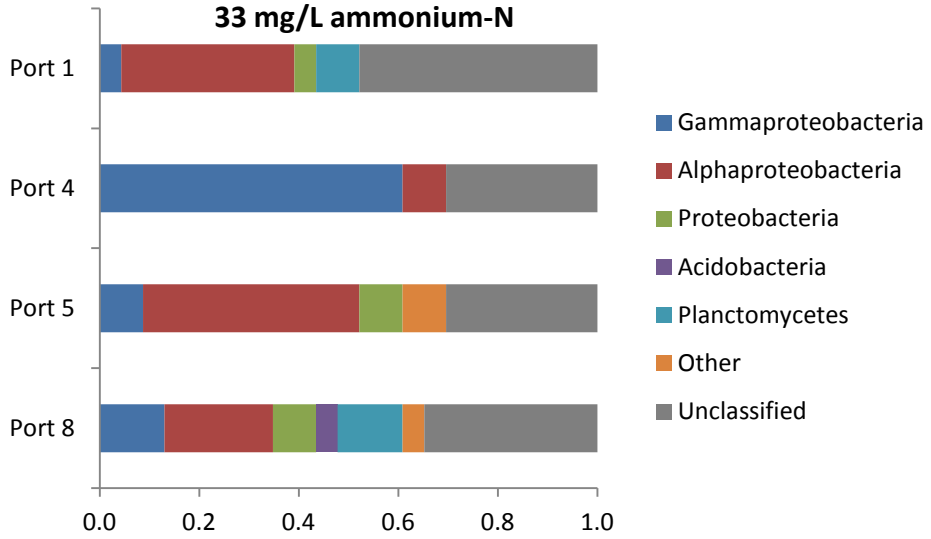


Figure A3.3: Relative abundance of bacterial phyla on Day 42 in RAS-1; influent ammonium-N concentration was 33 mg/L. The detection of *Gammaproteobacteria* indicates the presence of *Gammaproteobacteria* AOB, while the detection of *Alphaproteobacteria* indicates the presence of *Nitrobacter*. Classification was using the RDP training set in mothur (Schloss *et al.*, 2009) at 97% cutoff.

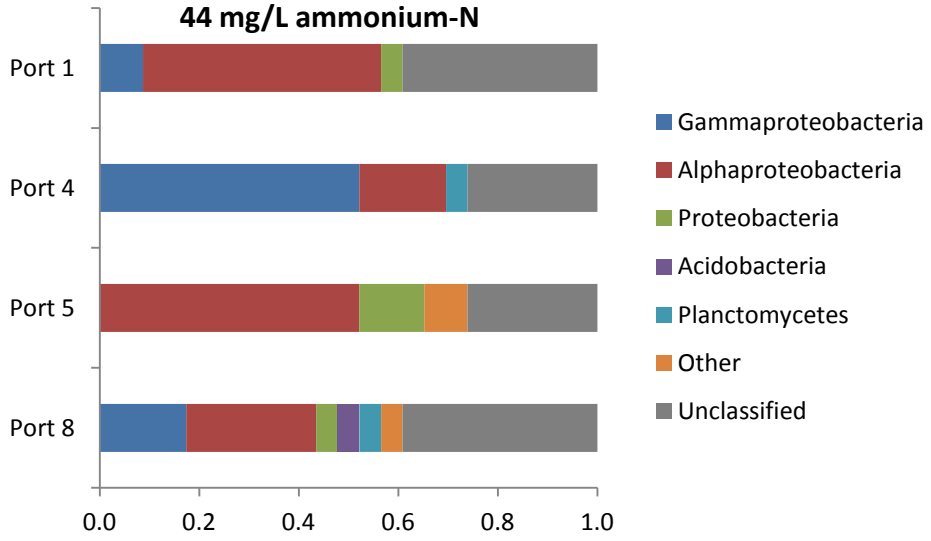
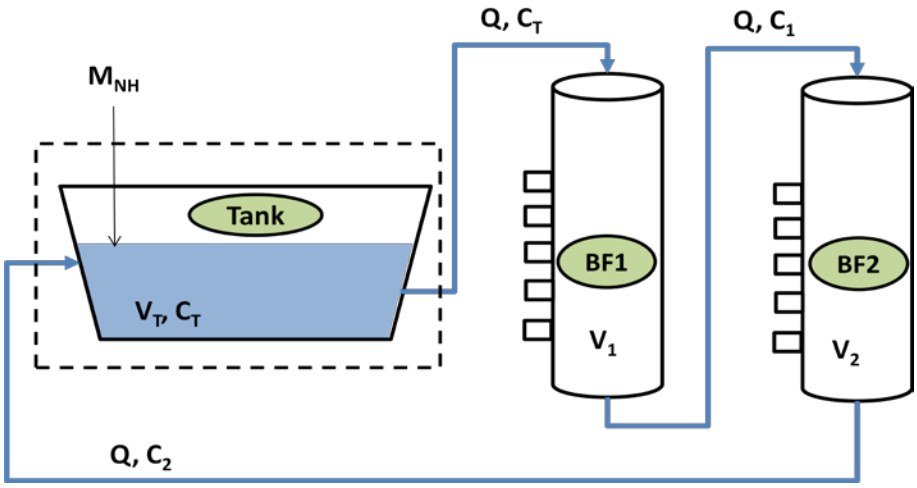


Figure A3.4: Relative abundance of bacterial phyla on Day 56 in RAS-1; influent ammonium-N concentration was 44 mg/L. The detection of *Gammaproteobacteria* indicates the presence of *Gammaproteobacteria* AOB, while the detection of *Alphaproteobacteria* indicates the presence of *Nitrobacter*. Classification was using the RDP training set in mothur (Schloss *et al.*, 2009) at 97% cutoff.

Nitogen Mass Balance
Theoretical
Purpose: to account for mass of nitrate measured in RAS

Schematic diagram of RAS with control volume



M_{NH} = once per day input of ammonium-N (mg/d)

Sucrose-C was also added during this daily mass spike of ammonium-N at a ratio of C/N = 0.5

V_T = 36.4 L
$V_1 = V_2$ = 6.8 L
Q = 250 mL/min = 360 L/d

In Tank, SRT = HRT = V_T/Q = 36.4 L / (360L/d) = 0.1 d

For t = 1 – 14 d, M_{NH} = 552 mg/d NH_4-N; M_C = 276 mg/d sucrose-C
t = 15 – 28 d, M_{NH} = 1104 mg/d NH_4-N; M_C = 552 mg/d sucrose-C
t = 29 – 42 d, M_{NH} = 1656 mg/d NH_4-N; M_C = 828 mg/d sucrose-C
t = 43 – 56 d, M_{NH} = 2208 mg/d NH_4-N; M_C = 1104 mg/d sucrose-C

Total mass of NH_4-N added to RAS =
(552 mg/d)*14 d + (1104 mg/d)*14 d + (1656 mg/d)*14 d + (2208 mg/d)*14 d = 77280 mg NH_4-N

Mass of NH4-N = 77280 mg NH4-N **Box 1**

(E1)

$$\underbrace{V\frac{dC_{NH}}{dt}}_{\text{accumulation}} = \underbrace{M_{NH} + V\frac{dC_{NH,r}}{dt}}_{\text{in}} - \underbrace{V\frac{dC_{NH}}{dt}}_{\text{out}} + \underbrace{rV}_{\text{reaction}}$$

where $C_{NH} = C_T$ and $C_{NH,r} = C_2$

Assumptions

- $M_{NH} >> V(dC_{NH,r}/dt)$, $V(dC_{NH}/dt)$
- dC_{NH}/dt is negligible

$$0 = M_{NH} + rV$$

$$M_{NH} = rV$$

Reactions in the tank

- autotrophic bacterial respiration of ammonium-N (ammonium-N → nitrate-N)
- autotrophic bacterial assimilation of ammonium-N
- heterotrophic bacterial assimilation of ammonium-N

The mass of ammonium-N that is available for respiration is reduced by assimilation reactions

Samples to measure inorganic N were collected prior to water addition; samples were not collected for TOC. Consequently, these concentrations need to be adjusted down by 5% based on dilution. Volume in tank after water addition = 36.4 L, water in tank prior to water addition = 34.6 L

$$((36.4 - 34.6)/36.4)*100 = 5\% \text{ reduction}$$

$$\text{Total mass of nitrate} = M_{NO3,T} + M_{NO3,BF1} + M_{NO3,BF2}$$

Total mass of nitrate-N : RAS-1 at t = 56 d was 30100 mg
RAS-2 at t = 56 d was 33100 mg
RAS-3 at t =56 d was 39300 mg

Box 2

Use the nitrogen requirement (NR) equation (Grady, *et al.* 1999):

(E2) $$NR = \frac{0.087(1+f_D b\theta_C)Y}{1+b\theta_C}$$

Autotrophic bacterial parameters (Henze, *et al.* 2000)

Parameter	Value*
f_D (dimensionless)	0.08
b (1/d)	0.62
Y_A (g biomass COD/ g N oxidized)	0.24

*typical values

Heterotrophic bacterial parameters (Henze, *et al.* 2000)

Parameter	Value*
f_D (dimensionless)	0.08
b (1/d)	0.62
Y_H (g biomass COD/ g COD oxidized)	0.67

*typical values

Nitrogen requirement for assimilation:

$$NR_{Autotrophic\ bacteria} = \frac{0.087\frac{mg\ N}{mg\ biomass\ COD}\left[1+(0.08)\left(\frac{0.62}{d}\right)(0.01d)\right]\left(0.24\frac{mg\ biomass\ COD}{mg\ N\ oxidized}\right)}{1+\left(\frac{0.62}{d}\right)(0.01d)}$$

(E2a)

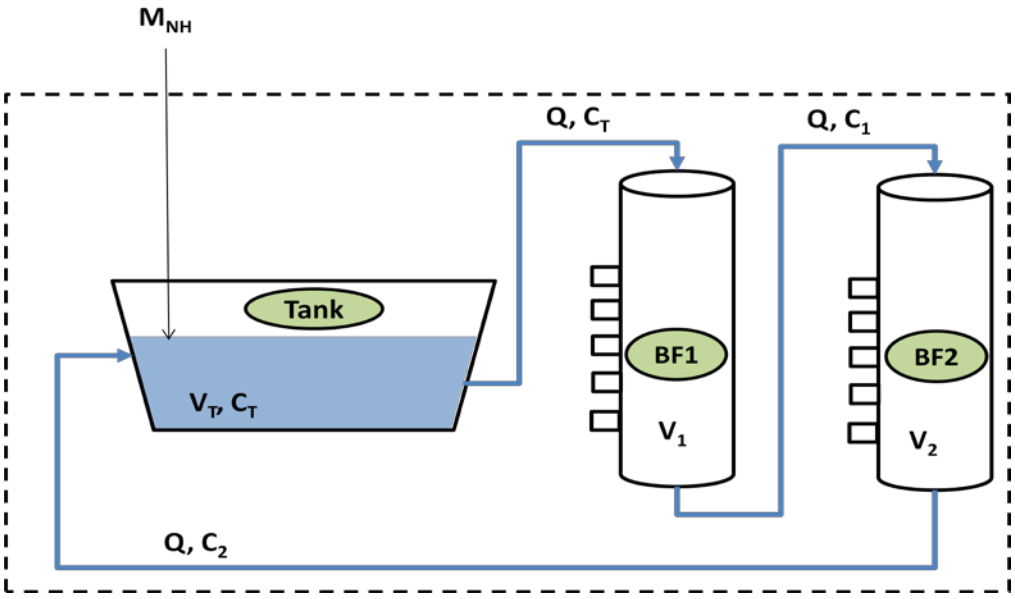
NR_A = 0.0198 mg N assimilated/mg N oxidized

$$NR_{Heterotrophic\ bacteria} = \frac{0.087\frac{mg\ N}{mg\ biomass\ COD}\left[1+(0.08)\left(\frac{0.62}{d}\right)(0.01d)\right]\left(0.67\frac{mg\ biomass\ COD}{mg\ COD\ oxidized}\right)}{1+\left(\frac{0.62}{d}\right)(0.01d)}$$

(E2b)

NR_H = 0.0552 mg N assimilated/mg COD oxidized

A mass balance on each component of the RAS (tank, biofilter 1, and biofilter 2) cannot be calculated because of insufficient TOC data. The TOC data is needed to account for ammonium-N assimilated by heterotrophic bacteria. Therefore, from this point forward the entire RAS is used as the control volume (refer to schematic below).



The mass of ammonium-N available for respiration is reduced by the mass of ammonium-N used for assimilation by autotrophs and heterotrophs.

Based on stoichiometry: $NH_4^+ + 2O_2 \rightarrow NO_3^- + 2H^+ + H_2O$

1 mg nitrate-N is produced from 1 mg ammonium-N oxidized

$$\frac{1mgNO_3^- - N}{1mgNH_4^+ - N}$$ **Box 3**

From Equation E2a, the nitrogen requirement for autotrophs = 0.02 mg NH4-N assimilated per mg NH4-N oxidized

Mass of ammonia-N assimilated by autotrophs =

(0.02 mg NH4-N assim per mg NH4-N oxid)(77280 mg NH4-N_ = 1560 mg

1560 mg NH4-N assimilated by autotrophs	**Box 4**

From Equation E2b, the nitrogen requirement for heterotrophs = 0.0552 mg NH4-N per mg COD oxidized

Mass of ammonium-N assimilated by heterotrophs

A. Determine mass of COD oxidized

Assumption: all influent sucrose-C oxidized; ignore soluble microbial products and particulate matter

Mass sucrose-C =

(14d)*(276 mg/d + 552 mg/d + 828 mg/d + 1104 mg/d) = 38640 mg sucrose-C

B. Calculate the COD mass equivalent of sucrose-C

$$C_{12}H_{22}O_{11} + 12O_2 \rightarrow 12CO_2 + 11H_2O$$

[(12 mol O2)(32g/mol O2)] /[(1 mol sucrose)(12 mol sucrose-C/mol sucrose)(12 g/mol sucrose-C)] =

2.67 g COD/g sucrose-C

Mass of NH4-N assimilated by heterotrophs

=(0.0552 mg NH4-N/mg COD)(38640 mg sucrose-C)(2.67 mg COD/mg sucrose-C) = 5690 mg NH4-N

5690 mg NH4-N assimilated by heterotrophs	**Box 5**

Mass of ammonium-N available for respiration by autotrophs =

Box 1 – Box 4 – Box 5 = 77280 mg – 1560 mg – 5690 mg = 700000 mg NH4-N

70000 mg NH4-N available for respiration	**Box 6**

Each RAS received the same mass of ammonium-N and sucrose-C.

From mass balance calculation there should be 70000 mg nitrate-N if all of the available ammonium was converted to nitrate-N in each RAS. However, the measured mass of nitrate-N on Day 56 was 30100 mg (RAS-1), 33100 mg (RAS-2), and 39300 mg (RAS-3). This suggests that 44 to 57% of the ammonium-N was denitrified.

References

Grady CPL, Daigger GT & Lim HC (1999) *Biological wastewater treatment*. Marcel Dekker, New York.

Henze M, Gujer W, Mino T & van Loosdrecht M (2000) Activated sludge models ASM1, ASM2, ASM2d, and ASM3. IWA Publishing, London, UK.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ & Weber CF (2009) Introducing mothur: Open-Source, Platform-Independent, Community-Supported Software for Describing and Comparing Microbial Communities. *Applied and Environmental Microbiology* **75**: 7537-7541.

Appendix IV: Supplemental Information for Chapter 5

Commercial food and biofilter biomass for calculation for experiment

The commercial feed used in this study was Shrimp Grower (Rangen, Inc., Buhl, ID). According to the manufacturer it contains 40% protein, 9% fat, 4% fiber, 15 % ash, and 10% moisture.

To determine the amount of biofilter biofilm needed, the amount commercial feed needed per day was converted to protein content expressed as nitrogen and then converted to weight of biofilter biofilm needed. This is described in the following equation:

(weight of biofilter biofilm, g) = (weight of shrimp, g)*0.06*0.4*0.16 / 0.025

where 0.06 is the feeding rate
0.4 is protein content of commercial feed
0.16 is percentage of protein that is nitrogen
0.025 is the percentage of protein in biofilter biofilm

Either 25% or 50% of the protein content expressed as nitrogen was replaced with biofilter biofilm. To determine the amount of biofilter biofilm needed, the weight of biofilter biofilm as calculated above was multiplied by 0.25 or 0.5 depending on the treatment. Refer to Table A4.1 for estimation of commercial feed and biofilter biofilm based on 80 shrimp with a starting weight of 0.02 g/shrimp, a growth rate of 2.1 g per week, and 100% survival.

Table A4.1: Estimate of mass of commercial food and biofilter biofilm needed during trial

		Control	Treatment 1 (75/25)		Treatment 2 (50/50)	
Time (day)	Mass of shrimp (g)	Commercial food (g/day)	Commercial food (g/day)	Biofilter biofilm (g wet weight/day)	Commercial food (g/day)	Biofilter biofilm (g wet weight/day)
1	1.60	0.096	0.072	0.384	0.048	0.768
2	1.60	0.096	0.072	0.384	0.048	0.768
3	1.60	0.096	0.072	0.384	0.048	0.768
4	1.60	0.096	0.072	0.384	0.048	0.768
5	1.60	0.096	0.072	0.384	0.048	0.768
6	1.60	0.096	0.072	0.384	0.048	0.768
7	1.60	0.096	0.072	0.384	0.048	0.768
8	3.70	0.222	0.167	0.888	0.111	1.776
9	3.70	0.222	0.167	0.888	0.111	1.776
10	3.70	0.222	0.167	0.888	0.111	1.776
11	3.70	0.222	0.167	0.888	0.111	1.776
12	3.70	0.222	0.167	0.888	0.111	1.776
13	3.70	0.222	0.167	0.888	0.111	1.776
14	3.70	0.222	0.167	0.888	0.111	1.776
15	5.80	0.348	0.261	1.392	0.174	2.784
16	5.80	0.348	0.261	1.392	0.174	2.784
17	5.80	0.348	0.261	1.392	0.174	2.784
18	5.80	0.348	0.261	1.392	0.174	2.784
19	5.80	0.348	0.261	1.392	0.174	2.784
20	5.80	0.348	0.261	1.392	0.174	2.784
21	5.80	0.348	0.261	1.392	0.174	2.784
22	7.90	0.474	0.356	1.896	0.237	3.792
23	7.90	0.474	0.356	1.896	0.237	3.792
24	7.90	0.474	0.356	1.896	0.237	3.792
25	7.90	0.474	0.356	1.896	0.237	3.792
26	7.90	0.474	0.356	1.896	0.237	3.792
27	7.90	0.474	0.356	1.896	0.237	3.792
28	7.90	0.474	0.356	1.896	0.237	3.792
		Totals	5.985	31.920	3.990	63.840

RAS Feeding Experiment Trial #1

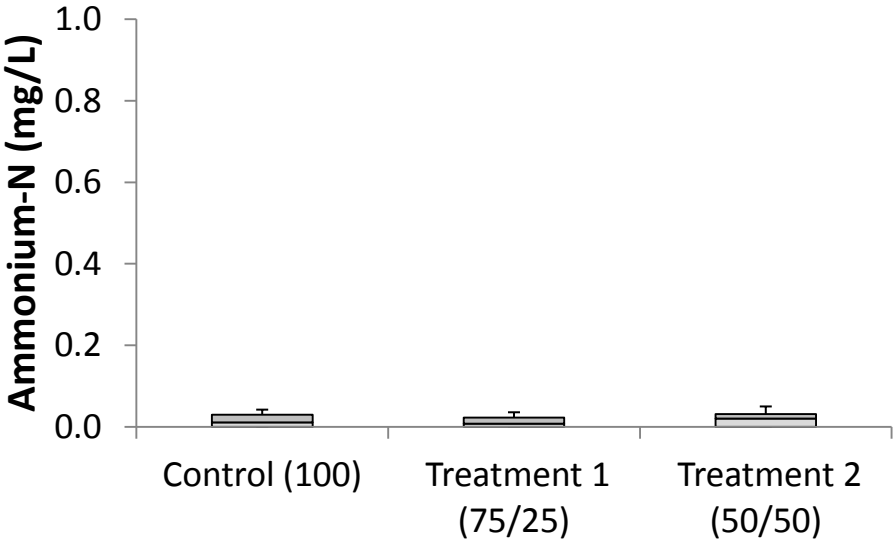


Figure A4.1: Box plot of ammonia-N concentrations in daily, or 24-hour, grab samples from RAS Feeding Experiment Trial #1. Data shown is from the tanks

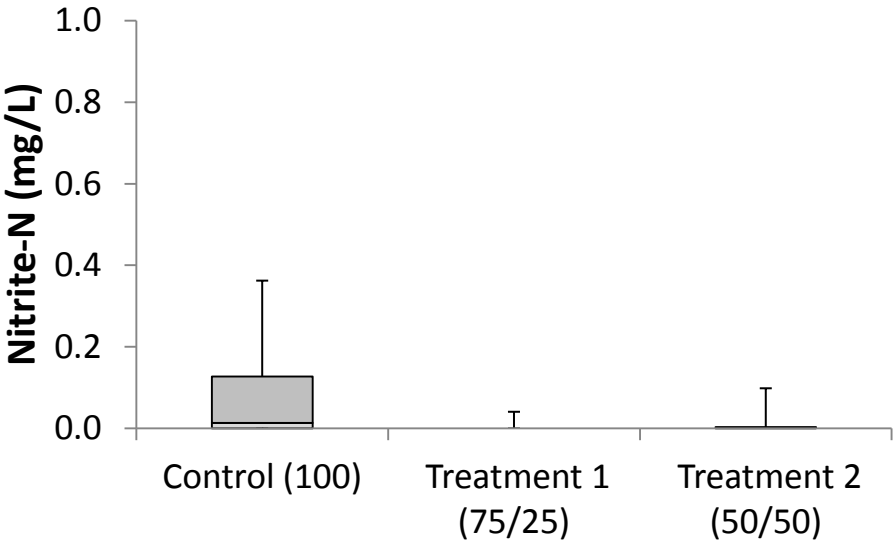


Figure A4.2: Box plot of nitrite-N concentrations in daily, or 24-hour, grab samples from RAS Feeding Experiment Trial #1. Data shown is from the tanks

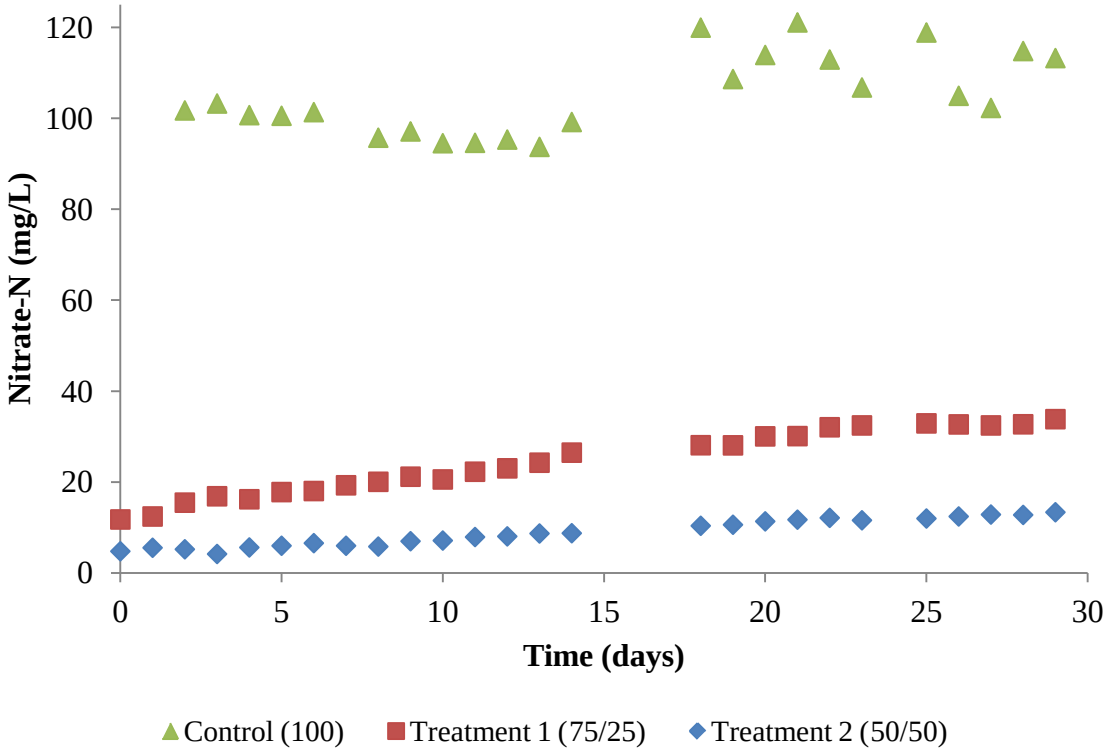
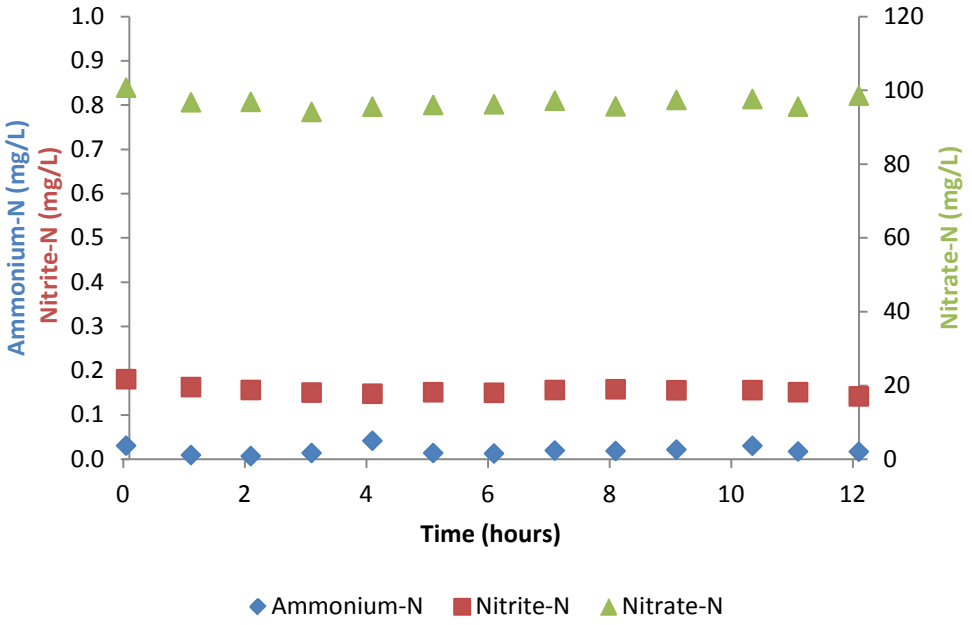
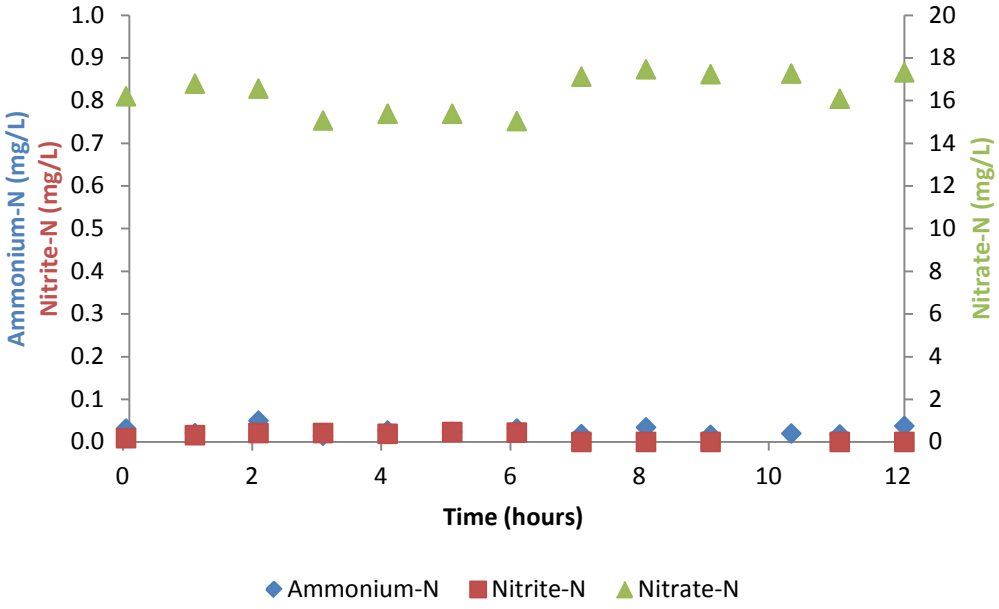


Figure A4.3: Nitrate-N concentrations in daily, or 24-hour, grab samples from RAS Feeding Experiment Trial #1. Data shown is from the tanks

A. Control (100)



B. Treatment-1 (75/25)



C. Treatment-2 (50/50)

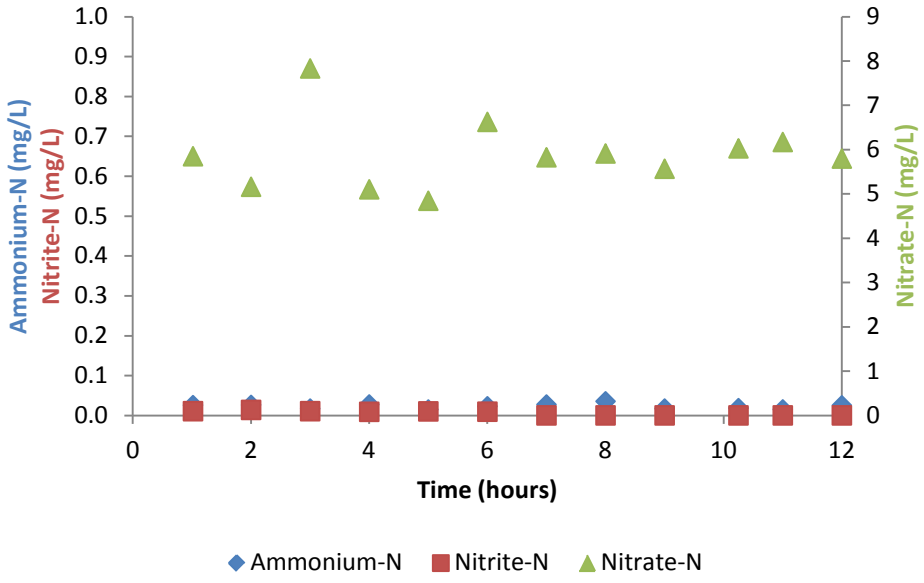
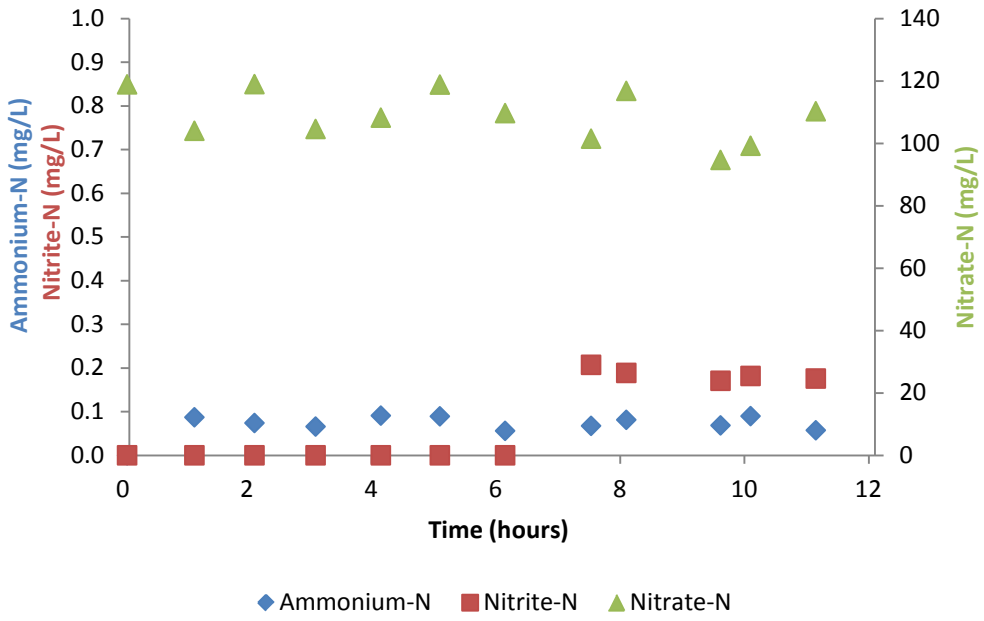
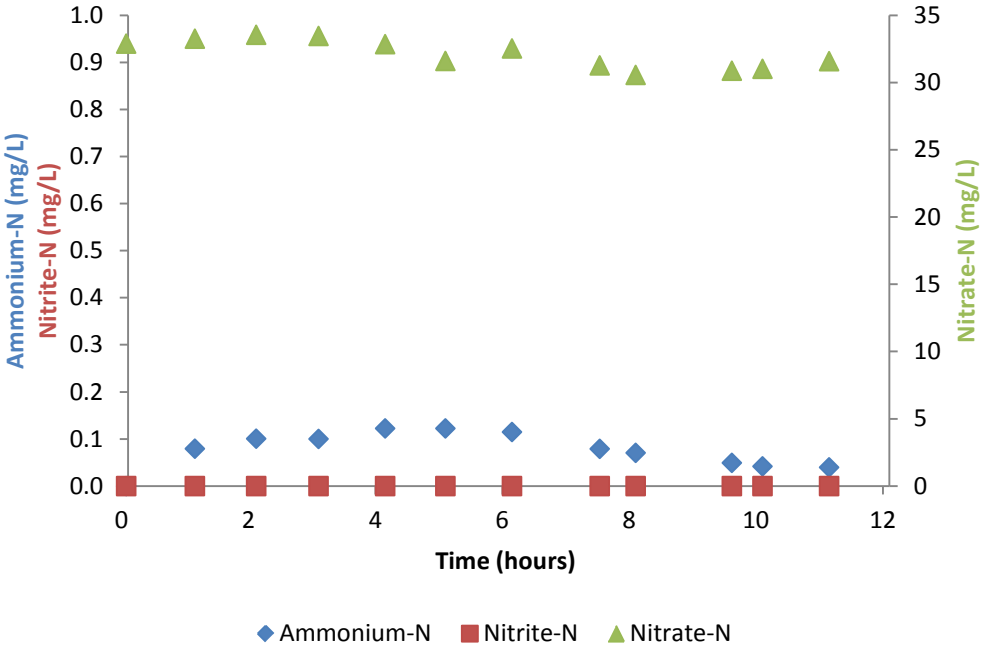


Figure A4.4: Inorganic nitrogen time series from RAS Feeding Experiment Trial #1; (A) Control, (B) Treatment-1, and (C) Treatment-2. Samples were collected on Day 4. Data shown is from the tanks.

A. Control (100)



B. Treatment-1 (75/25)



C. Treatment-2 (50/50)

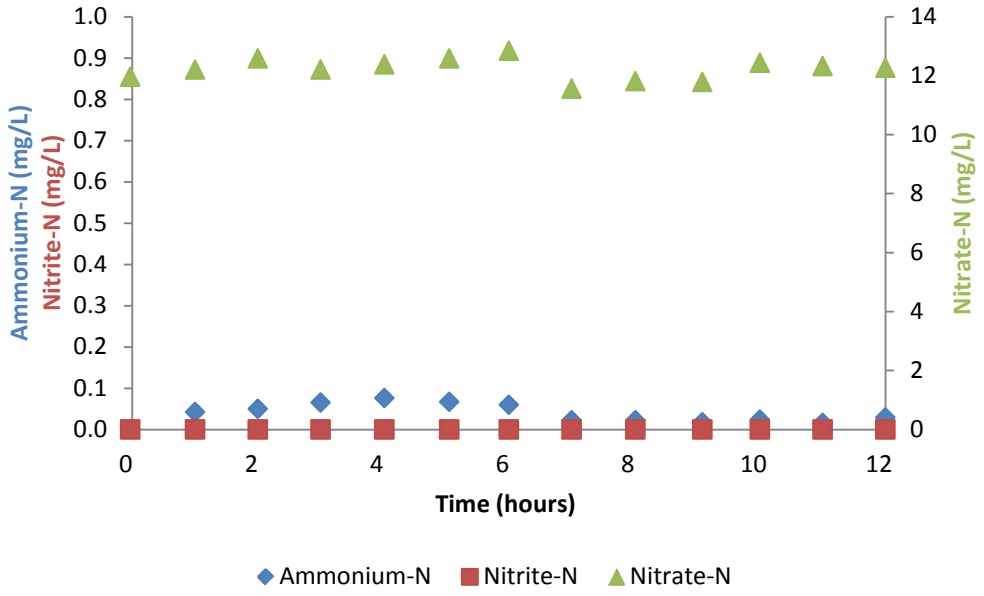


Figure A4.5: Inorganic nitrogen time series from RAS Feeding Experiment Trial #1; (A) Control, (B) Treatment-1, and (C) Treatment-2. Samples were collected on Day 25. Data shown is from the tanks

TOC 24-hour grab samples

In the control, TOC ranged from 3.6 to 27.6 mg/L with an average of 8.1 mg/L, while TOC in treatment-1 ranged from 5.0 to 36.3 mg/L with an average of 11.5 mg/L and TOC in treatment-2 ranged from 4.8 to 38.7 with an average of 9.5 mg/L (Figure A4.6). The maximum value occurred on day 20 for all treatments. For comparison to COD, the TOC values were converted to COD using a conversion ratio of 2.7 g COD/g TOC (Table 5). This data indicates that there was not a lot of dissolved organic carbon matter in the system. TOC and suspend solids concentrations (Table A4.2) indicate that organic carbon was relatively low through the experimental period.

Table A4.2: Measured TOC values converted to COD for grab samples

	Control		Treatment 1		Treatment 2	
	TOC	COD	TOC	COD	TOC	COD
Average (mg/L)	8.1	21.9	11.5	31.1	9.5	25.7
Minimum(mg/L)	3.6	9.7	5.0	13.5	4.8	13.0
Maximum (mg/L)	27.6	74.5	36.3	98.0	38.7	104.5

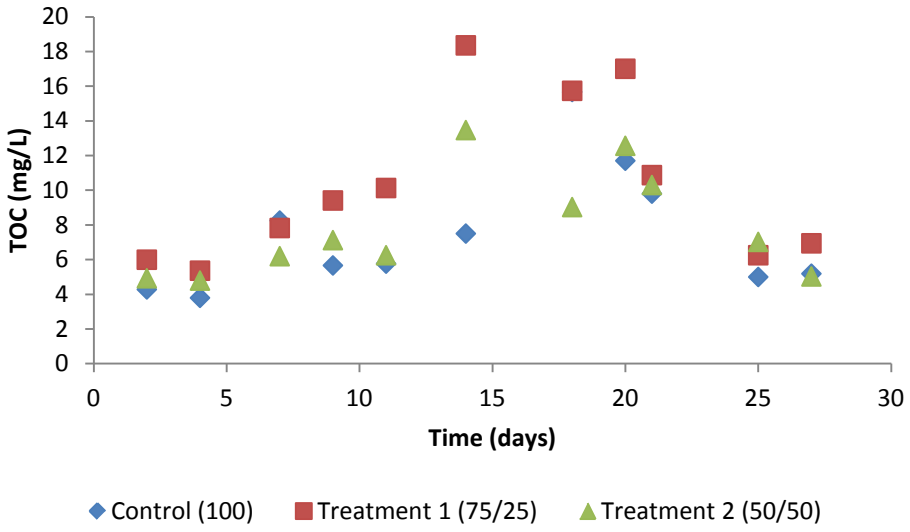
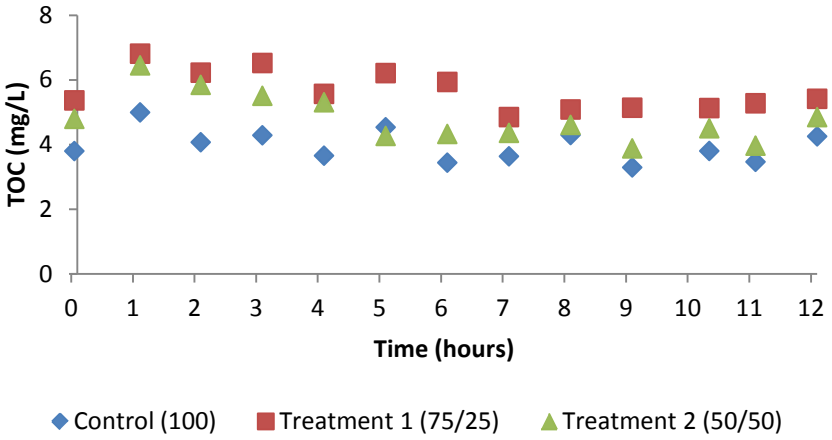


Figure A4.6: TOC 24-hour grab samples from RAS Feeding Experiment Trial #1. Data shown is from the tanks

TOC 12-hour time series concentration between feedings

The purpose of the TOC time series analysis was to observe concentrations during a feeding cycle that were not captured by the grab samples (Figure A4.6). Time series samples were only collected from the tank at the same time as the inorganic nitrogen time series samples (Figure A4.7). There is a general decreasing trend for all treatments over the 12-hour period. Values ranged from 3 to 15 mg/L TOC (8 to 40 mg/L COD).

A. Day 4



B. Day 25

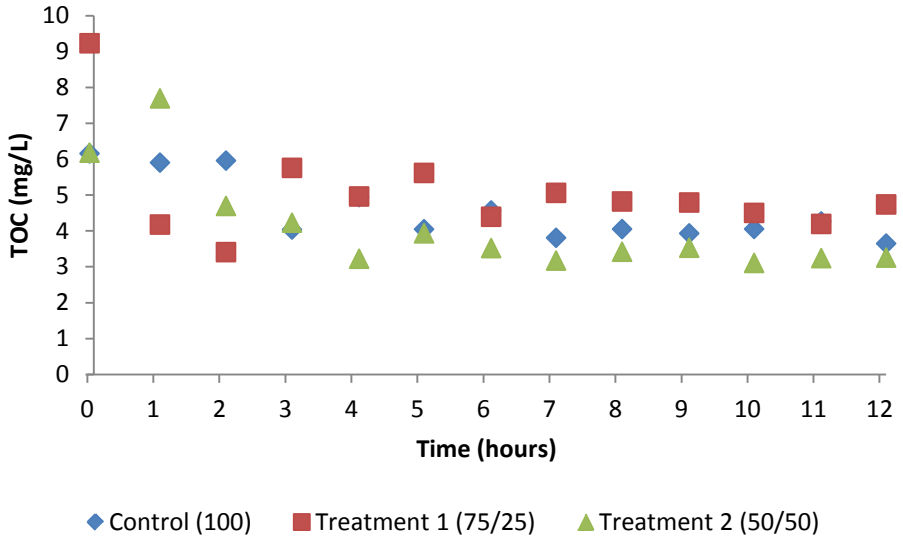


Figure A4.7: TOC 12-hour time series (A) Day 4 and (B) Day 25 from RAS Feeding Experiment Trial #1. Samples collected in one hour intervals after feeding. Data shown is from the tanks

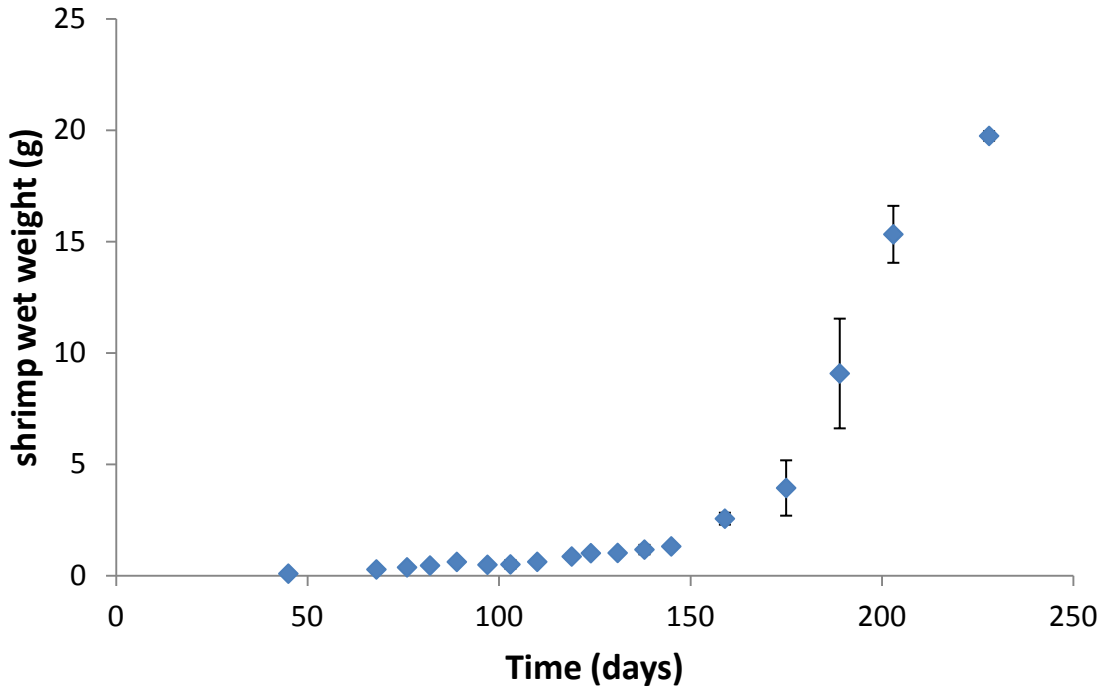


Figure A4.8: Growth curve from preliminary shrimp growth trial. Shrimp were feed 100% commercial feed (46% protein, Melick Aquafeed, Catawissa, PA) at 8% body weight. Wet weight was measured weekly. Amount of feed provided was adjusted weekly based on wet weight measurements and assuming 100% survival. Error bars show standard deviation

Table A4.3: Cost comparison of producing one kilogram of shrimp

	100% Commercial feed		75% Commercial feed		50% Commercial feed	
FCR*	feed consumed (g)	Price to produce kg shrimp	feed consumed** (g)	Price to produce kg shrimp	feed consumed*** (g)	Price to produce kg shrimp
1.0	1000	$0.62	953	$0.59	730	$0.45
1.5	1500	$0.93	1429	$0.88	1095	$0.68
2.0	2000	$1.24	1905	$1.18	1460	$0.90
2.5	2500	$1.55	2381	$1.47	1825	$1.13
3.0	3000	$1.85	2858	$1.77	2190	$1.35
3.5	3500	$2.16	3334	$2.06	2555	$1.58
4.0	4000	$2.47	3810	$2.36	2920	$1.81

*FCR = feed conversion ratio (amount of feed consumed/ amount of shrimp weight gained)

**$(\text{feed consumed})_{100}$*0.75*1.27 where 1.27 = 1+ (88-64)/88 to account for the increased time to grow to 20 g

***$(\text{feed consumed})_{100}$*0.50*1.46 where 1.46 = 1+ (119-64)/119 to account for the increased time to grow to 20 g

Table A4.4: Energy Consumption in Control (100)

Equipment	kW	90% Efficient Equipment (kW)	# of Units per RAS	# Days Used/cycle	# Days Used x # of Units	# Hours Used	Total Electrical Use/cycle (kWh/cycle)
Air pump for tank	0.1	0.11	1	63	63	1512	168
Air pump for biofilters	0.018	0.02	4	63	252	6048	121
Heater	0.05	0.06	1	63	63	1512	84
Water pump	0.075	0.08	1	63	63	1512	126
						Total Electrical Use (kWh/cycle):	499
						Electricity cost ($0.1/kWh, Capdet Works):	$49.90

Table A4.5: Energy Consumption in Treatment-1 (75/25)

Equipment	kW	90% Efficient Equipment (kW)	# of Units per RAS	# Days Used/cycle	# Days Used x # of Units	# Hours Used	Total Electrical Use/cycle (kWh/cycle)
Air pump for tank	0.1	0.11	1	88	88	2112	235
Air pump for biofilters	0.018	0.02	4	88	352	8448	169
Heater	0.05	0.06	1	88	88	2112	117
Water pump	0.075	0.08	1	88	88	2112	176
						Total Electrical Use (kWh/cycle):	697
						Electricity cost: ($0.1/kWh, Capdet Works):	$69.70

Table A4.6: Energy Consumption in Treatment-2 (50/50)

Equipment	kW	90% Efficient Equipment (kW)	# of Units per RAS	# Days Used/cycle	# Days Used x # of Units	# Hours Used	Total Electrical Use/cycle (kWh/cycle)
Air pump for tank	0.1	0.11	1	119	119	2856	317
Air pump for biofilters	0.018	0.02	4	119	476	11424	228
Heater	0.05	0.06	1	119	119	2856	159
Water pump	0.075	0.08	1	119	119	2856	238
						Total Electrical Use (kWh/cycle):	942
						Electricity cost ($0.1/kWh, Capdet Works):	$94.25

RAS Feeding Experiment Trial #2

Table A4.7: Water quality in RAS fedding experiment trial #2

	Control (100)		Treatment 1 (75/25)		Treatment 2 (50/50)	
	average	std dev*	average	std dev	average	std dev
Temperature (°C)	24.9	0.3	25.2	0.2	24.8	0.4
Salinity (psu)	26.7	3.9	26.5	1.3	25.7	1.3
pH	8.11	0.08	8.08	0.09	8.11	0.09
Alkalinity (mg/L CaCO3)	264	39	234	40	188	46
TSS (mg/L)	78.2	10.0	82.3	11.2	104.4	32.5
VSS (mg/L)	19.7	3.0	19.0	3.2	23.0	5.7
TOC (mg/L)	13.50	2.80	12.30	3.70	8.85	1.84

*std dev = standard deviation

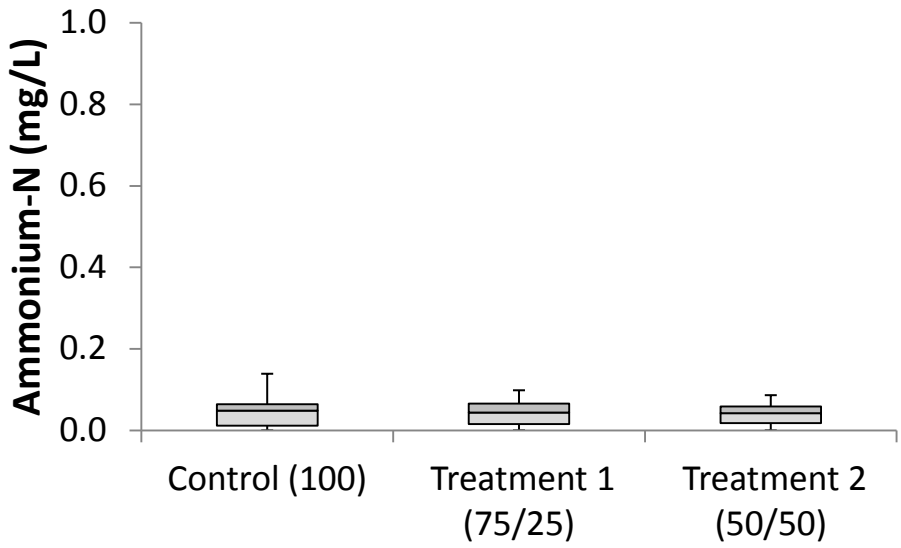


Figure A4.9: Box plot of ammonia-N concentrations in daily, or 24-hour, grab samples from RAS Feeding Experiment Trial #2. Data shown is from the tanks

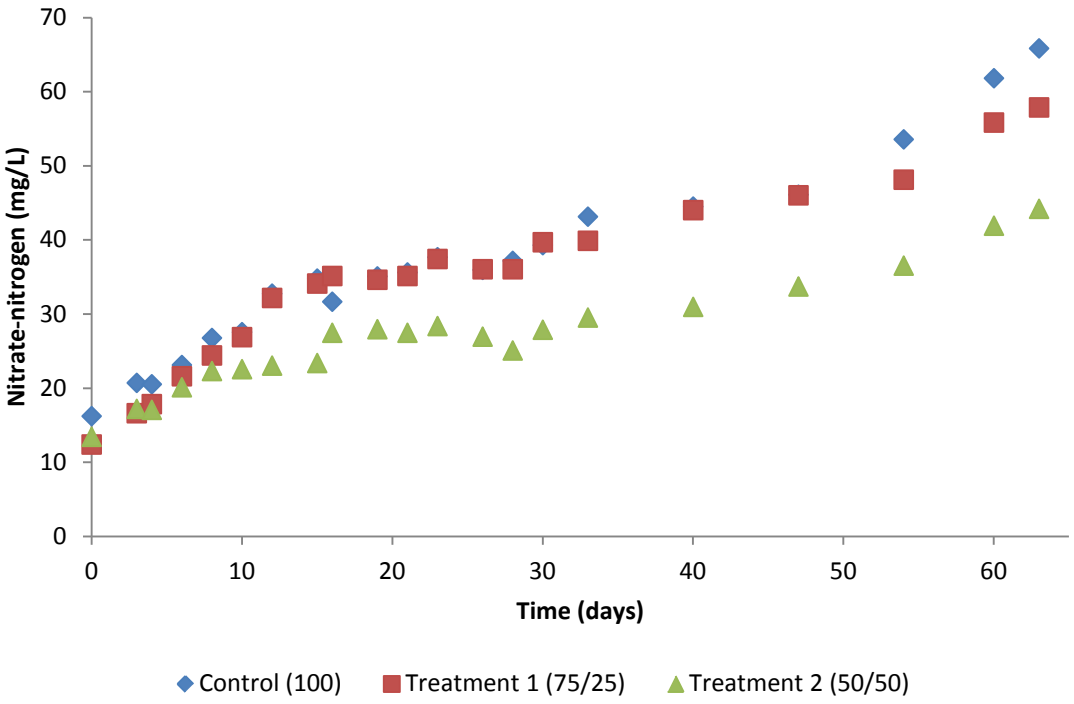


Figure A4.10: Nitrate-N concentrations in daily, or 24-hour, grab samples from RAS Feeding Experiment Trial #2. Data shown is from the tanks

Table A4.8: Growth rate for Trial #2

Time	Control (100)	Treatment 1 (75/25)	Treatment 2 (50/50)
Day 0 - 33	0.0024	0.0015	0.0010
Day 33-68	0.0241	0.0128	0.0083
Day 0 - 68	0.0136	0.0073	0.0047

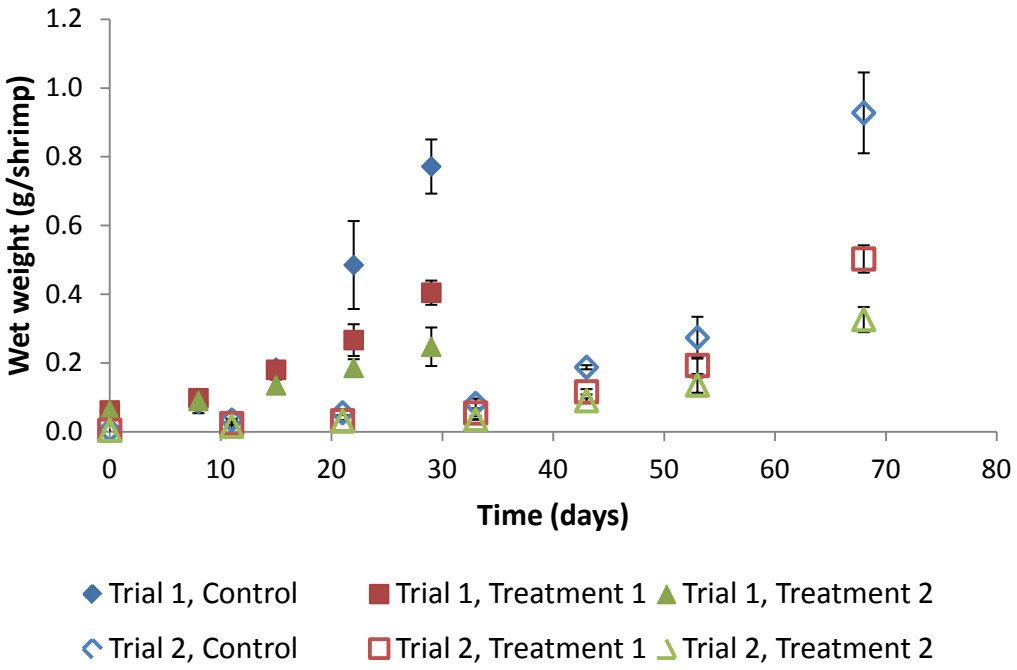


Figure A4.11: Growth curve for shrimp in feeding Trials 1 and 2.

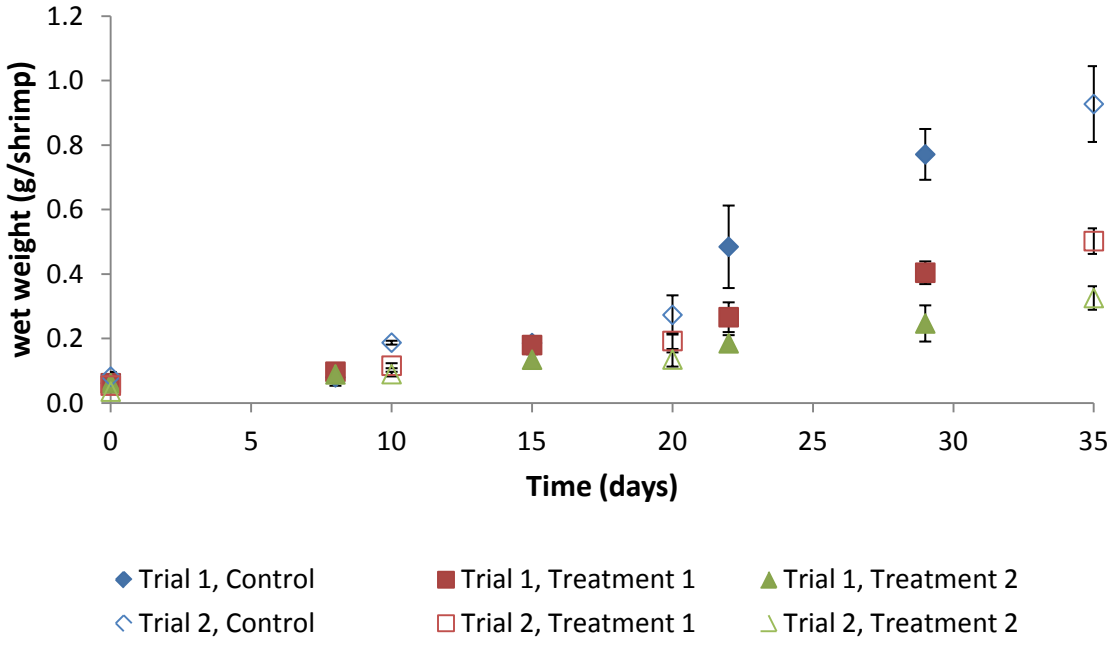


Figure A4.12: Same data as shown in Figure A4.12 with the first 33 days of Trial #2 removed to highlight the reproducibility between the trials of the experiment.

Table A4.9: Cost comparison of producing one kilogram of shrimp

	100% Commercial feed		75% Commercial feed		50% Commercial feed	
FCR*	feed consumed (g)	Price to produce kg shrimp	feed consumed** (g)	Price to produce kg shrimp	feed consumed*** (g)	Price to produce kg shrimp
1.0	1000	$0.62	833	$0.51	590	$0.36
1.5	1500	$0.93	1249	$0.77	885	$0.55
2.0	2000	$1.24	1665	$1.03	1180	$0.73
2.5	2500	$1.55	2081	$1.29	1475	$0.91
3.0	3000	$1.85	2498	$1.54	1770	$1.09
3.5	3500	$2.16	2914	$1.80	2065	$1.28
4.0	4000	$2.47	3330	$2.06	2360	$1.46

*FCR = feed conversion ratio (amount of feed consumed/ amount of shrimp weight gained)

**$(\text{feed consumed})_{100}$*0.75*1.11 where 1.11 = 1+ (126-112)/112 to account for the increased time to grow to 20 g

***$(\text{feed consumed})_{100}$*0.50*1.18 where 1.18 = 1+ (137-112)/137 to account for the increased time to grow to 20 g

Table A4.10: Energy Consumption in Control (100)

Equipment	kW	90% Efficient Equipment (kW)	# of Units per RAS	# Days Used/ cycle	# Days Used x # of Units	# Hours Used	Total Electrical Use/cycle (kWh/cycle)
Air pump for tank	0.1	0.11	1	112	112	2688	299
Air pump for biofilters	0.018	0.02	4	112	448	10752	215
Heater	0.05	0.06	1	112	112	2688	149
Water pump	0.075	0.08	1	112	112	2688	224

Total Electrical Use (kWh/cycle): 887

Electricity cost ($0.1/kWh, Capdet Works): $88.70

Table A4.11: Energy Consumption in Treatment-1 (75/25)

Equipment	kW	90% Efficient Equipment (kW)	# of Units per RAS	# Days Used/ cycle	# Days Used x # of Units	# Hours Used	Total Electrical Use/cycle (kWh/cycle)
Air pump for tank	0.1	0.11	1	126	126	3024	336
Air pump for biofilters	0.018	0.02	4	126	504	12096	242
Heater	0.05	0.06	1	126	126	3024	168
Water pump	0.075	0.08	1	126	126	3024	252

Total Electrical Use (kWh/cycle): 998

Electricity cost ($0.1/kWh, Capdet Works): $99.79

Table A4.12: Energy Consumption in Treatment-2 (50/50)

Equipment	kW	90% Efficient Equipment (kW)	# of Units per RAS	# Days Used/ cycle	# Days Used x # of Units	# Hours Used	Total Electrical Use/cycle (kWh/cycle)
Air pump for tank	0.1	0.11	1	137	137	3288	365
Air pump for biofilters	0.018	0.02	4	137	548	13152	263
Heater	0.05	0.06	1	137	137	3288	183
Water pump	0.075	0.08	1	137	137	3288	274

Total Electrical Use (kWh/cycle): 1085

Electricity cost($0.1/kWh, Capdet Works): $108.50

Batch Experiment

Table A4.13: Water quality data from batch experiment

	B100-1		B100-2		B75-1		B75-2		B50-1		B50-2	
	Average	**Std dev**	**Average**	**Std dev**	**Average**	**Std dev**	**Average**	**Std dev**	**Average**	**Std dev**	**Average**	**Std dev**
Temperature (°C)	27.2	0.4	25.2	0.3	27.3	0.3	26.1	0.4	27.3	0.3	25.1	0.2
Salinity (psu)	30.4	2.9	29.0	3.1	29.4	2.9	29.7	2.9	30.2	3.0	31.4	2.2
pH	8.36	0.05	8.40	0.08	8.39	0.10	8.51	0.09	8.32	0.08	8.48	0.02
Alkalinity (mg/L CaCO3)	390	42	377	53	353	61	370	55	381	53	340	64
TSS (mg/L)	171	75	118	37	126	37	129	28	152	48	154	49
VSS (mg/L)	75.8	50.0	43.2	24.5	46.2	22.7	45.8	22.3	59.3	32.8	63.4	36.3
TOC (mg/L)	15.2	3.7	13.5	2.7	13.6	3.6	12.3	4.2	10.3	4.2	12.2	1.4