Management of Biological and Chemical Constituents for the Advancement of Intensive, Minimal-Exchange, Biofloc-based Shrimp (Litopenaeus vannamei) Aquaculture

Andrew James Ray
University of Southern Mississippi

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MANAGEMENT OF BIOLOGICAL AND CHEMICAL CONSTITUENTS FOR THE
ADVANCEMENT OF INTENSIVE, MINIMAL-EXCHANGE, BIOFLOC-BASED
SHRIMP (*LITOPENAEUS VANNAMEI*) AQUACULTURE

by

Andrew James Ray

Abstract of a Dissertation
Submitted to the Graduate School
of The University of Southern Mississippi
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

December 2012
ABSTRACT

MANAGEMENT OF BIOLOGICAL AND CHEMICAL CONSTITUENTS FOR THE ADVANCEMENT OF INTENSIVE, MINIMAL-EXCHANGE, BIOFLOC-BASED SHRIMP (*LITOPENAEUS VANNAMEI*) AQUACULTURE

by Andrew James Ray

December 2012

Intensive, minimal-exchange, biofloc-based shrimp aquaculture systems may provide a sustainable alternative to traditional shrimp culture. Through a series of experiments, this document explores the effects of several key management strategies on water quality, isotopic distribution, and shrimp production.

An experiment evaluated the effects of managing suspended solids (biofloc) concentration at two levels. It was found that using a higher flow rate to larger settling chambers resulted in significantly lower biofloc and nitrate concentrations, and significantly improved shrimp growth rate. A second experiment compared systems with clear water and systems with biofloc. The filters in the clear water systems prevented biofloc accumulation and cycled nutrients, whereas biofloc systems occasionally contained dangerous concentrations of ammonia and nitrite. Using stable isotope analysis it was estimated that biofloc contributed 72% of the carbon and 42% of the nitrogen found in shrimp from those tanks. A third study was conducted exploring carbohydrate addition as a means of stimulating bacterial nitrogen assimilation. Without carbohydrate addition nitrification proceeded, exemplified by a nitrite spike and an accumulation of nitrate, with carbohydrate addition those compounds were in low concentration. Shrimp production was poor in the treatment receiving molasses, but
similar among the treatment without carbohydrate, and treatments with glycerol and sucrose additions. In a fourth experiment three salinities were evaluated: 10, 20, and 30‰. The pH was lower as salinity increased and nitrite was significantly higher in the 30 versus the 10‰ salinity treatments. Mean shrimp growth rate was 1.9 g wk\(^{-1}\) and the mean feed conversion ratio was 1.3:1; these parameters did not differ significantly between treatments. Lastly, an experiment was conducted to evaluate the utilization of biofloc by juvenile shrimp in a nursery phase. Data suggested that both feed and biofloc contributed carbon to shrimp. A two-source isotope mixing model indicated that between 34 to 50% of the nitrogen in shrimp came from the biofloc.

The results of these studies can help biofloc shrimp culture managers decide how to operate systems. The improved success and continued development of such systems may provide the shrimp aquaculture industry a viable option for ecologically responsible development and intensification.
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Approved:

Jeffrey Lotz
Director

Reginald Blaylock

Kevin Dillon

Eric Saillant

Susan A. Siltanen
Dean of the Graduate School

December 2012
DEDICATION

I dedicate this dissertation to my grandmother Agnes (Ruby) Taylor Ray
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Thank you to my research committee: Jeffrey Lotz, Reginald Blaylock, Kevin Dillon, and Eric Saillant. These gentlemen helped in guiding my research and providing valuable feedback concerning my work.

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LIST OF ABBREVIATIONS

Analysis of variance...........................................................ANOVA
Dissolved oxygen..............................................................DO
Dissimilatory nitrate reduction to ammonia..............................DNRA
Ethylene propylene diene monomer........................................EPDM
Feed conversion ratio........................................................FCR
Five day biochemical oxygen demand....................................BOD₅
Fraction of total contribution................................................f
Fractionation factor..........................................................∆
High density polyethylene..................................................HDPE
Isotope del notation...........................................................δ
Liters per minute..............................................................LPM
Moving bed bioreactor.......................................................MBBR
Nephelometric turbidity unit................................................NTU
Polyvinyl chloride............................................................PVC
Post-larvae.................................................................PL
Raceway...................................................................RW
Recirculating aquaculture system..........................................RAS
Repeated measures ANOVA...............................................RM ANOVA
Standard error of the mean................................................SEM
Thad Cochran Marine Aquaculture Center.............................CMAC
Total ammonia nitrogen....................................................TAN
Total suspended solids.....................................................TSS
United States dollars...............................................................USD

Volatile suspended solids......................................................VSS
CHAPTER I

INTRODUCTION

Shrimp Aquaculture

The aquaculture industry is expanding rapidly to meet the demands of developing nations and a growing human population. Global aquaculture production has grown from below 1 million tons in 1950 to 59.4 million tons in 2004 with a total estimated value of $70.3 billion U.S. dollars (USD) (FAO 2007). In 2005, aquaculture supplied 45.5% of the world’s food fish for human consumption, and by 2015 the industry will need to supply at least 50% according to projected demand and wild fishery contributions (Lowther 2007). At 18.2 billion USD, carps had the highest reported value of all cultured aquatic animals in 2005, followed by shrimp which were valued at 10.6 billion USD. The highest valued single species was the Pacific White Shrimp (*Litopenaeus vannamei*) at 5.9 billion USD (Lowther 2007). The Pacific White Shrimp is the most commonly cultured shrimp and production more than doubled from 982,663 tons in 2003 to 2,296,630 tons in 2007 (FAO 2010).

Shrimp is a popular seafood item in the US and most of the shrimp consumed in the US is farm raised and imported from Thailand, China, Ecuador, or other Asian and Latin American countries. In 2009 the value of shrimp imports totaled 3.778 billion USD, representing a large trade deficit of 3.770 billion USD (USDA 2010). Most of the shrimp aquaculture facilities around the world are composed of extensive ponds in which regular water exchange with the natural environment is conducted to maintain acceptable water quality. The consequences of this practice have included eutrophication of aquatic systems, transmission of disease between both wild and cultured animals, entrainment of
natural biota, and the introduction of chemicals to nearby waterways (Hopkins et al. 1995).

**Intensive, Minimal-exchange Biofloc-based Shrimp Culture**

Intensive, minimal-exchange, biofloc-based shrimp culture systems serve as a potentially more environmentally-friendly alternative to traditional shrimp culture. These systems typically include plastic-lined ponds, raceways, or tanks, controlled feed-nutrient inputs, high animal stocking densities, intense aeration or oxygenation, the accumulation of flocculated (biofloc) particles, and little if any water exchange (Burford et al. 2004; Ray et al. 2009; Wasielesky et al. 2006). Because of dramatically reduced water exchange, the risks of pollution discharge, animal escapement, and disease transmission between cultured and wild animals are lessened. Also, there are opportunities for the culture of marine shrimp at inland locations due, in part, to the tolerance of *L. vannamei* to low salinity water (Roy et al. 2010). With high animal stocking densities comes a relatively small footprint which reduces the need for habitat alteration and lends the ability to cover the systems with green houses, or more rigid structures, which can permit a year round growing season. The potential to provide a continuous supply of fresh shrimp to inland metropolitan areas and locations with cool climates may offer unique marketing opportunities for biofloc systems (Browdy and Moss 2005).

**Microbial Components**

By exchanging little or no water, expensive nutrients from shrimp feeds are retained within biofloc systems. These nutrients create a eutrophic environment in the culture system leading to the proliferation of a dense microbial community. Common microorganisms include bacteria, algae, and zooplankton; fungi, while common, are less
well documented in these systems. A substantial portion of the microbial community is contained on and within biofloc particles. Biofloc is composed of microbes, uneaten feed particles, feces, molts, and other particulate matter, all held together by physiochemical forces of attraction and polymer matrices (Avnimelech 2012; Browdy et al. 2012; Ray et al. 2009). These particles offer ecological advantages to microorganisms such as refuge from predators, direct access to organic and inorganic nutrients, and substrate for bacteria (De Schryver et al. 2008).

Under the appropriate conditions, biofloc particles and the associated microbial community can provide nutritional benefits to shrimp, thereby recycling some nutrients and potentially helping to lower shrimp feeding costs. Using stable isotope techniques, Burford et al. (2004) demonstrated that shrimp can consume the microorganisms in biofloc systems. A review of some specific nutritional components, improved feed conversion efficiencies, and growth enhancement effects of biofloc was provided by Browdy et al. (2012). By increasing the feeding efficiency and supplying supplemental nutrients to shrimp, biofloc helps to augment system sustainability and opens opportunities for non-traditional feeds. For example, Ray et al. (2010a) demonstrated that no significant differences in shrimp production were found when shrimp were fed a fish-free, soybean-based diet versus a traditional fish meal-based diet in biofloc systems.

As with clear water recirculating aquaculture systems (RAS), bacteria are a vital microbial component of biofloc systems. However, a major difference between these and clear water RAS is that biofloc bacteria are primarily contained within the water column rather than in external filtration units. Two categories of bacteria are particularly beneficial for water quality maintenance in biofloc systems: nitrifying bacteria and
heterotrophic, nitrogen-assimilating bacteria. Nitrifying bacteria are chemoautotrophic organisms that obtain energy through the oxidative transformations of ammonia (NH$_3$) to nitrite (NO$_2$) and nitrite to nitrate (NO$_3$). Bacterial genera such as _Nitrosomonas, Nitrosococcus, Nitrosospira, Nitrosolobus, and Nitrosovibrio_ are capable of oxidizing NH$_3$ to form NO$_2$. The genera _Nitrobacter, Nitrococcus, Nitrospira, and Nitrospina_ can convert NO$_2$ to NO$_3$. When nitrification functions reliably both of the toxic compounds NH$_3$ and NO$_2$ are converted to the relatively non-toxic NO$_3$ molecule. However, nitrification can be slow to establish and each of the two essential reactions of the process must function properly to avoid lethal conditions (Browdy et al. 2012).

An alternative to relying on nitrification is managing biofloc systems to favor heterotrophic bacterial assimilation of NH$_3$. This can be performed during the initial establishment of nitrification when NH$_3$ and nitrite can reach elevated concentrations (Ray et al. 2009), or assimilation can be encouraged throughout the culture cycle (Avnimelech 2012). A wide range of bacterial taxa are capable of assimilating NH$_3$ as a nitrogen source to build cellular proteins. This process can be encouraged by elevating the carbon: nitrogen ratio (C:N) in the system (Avnimelech 1999; Ebeling et al. 2006). When labile organic carbon is added to the water column, heterotrophic bacteria consume it and grow rapidly. To grow and reproduce, the bacteria also need nitrogen to build proteins and this is readily acquired from dissolved inorganic nitrogen compounds. The assimilation process is fast and efficient, but consumes a large amount of oxygen and leads to a substantial accumulation of bacterial biomass compared to nitrification (Browdy et al. 2012). If the bacterial biomass is not removed from the system, bacteria will eventually die and return NH$_3$ to the water column. However, there are reports that
increased bacterial abundance corresponds to greater availability of microbial proteins for cultured animals (Avnimelech 2012; Burford et al. 2004; De Schryver et al. 2008).

Algae are another potentially important group of organisms in biofloc systems. Both micro and macro-algae occur, although any macro-algae within reach of shrimp are typically grazed quickly (Browdy et al. 2012). Commonly documented micro-algae taxa include chlorophytes, diatoms, dinoflagellates, and cyanobacteria (blue-green algae) (Ju et al. 2009; Ray et al. 2010b). Similar to heterotrophic bacteria, algae assimilate inorganic nitrogen to build cellular proteins. Algae are photoautotrophic, obtaining energy through the process of photosynthesis. This process consumes carbon dioxide (CO\textsubscript{2}) and generates oxygen (O\textsubscript{2}) in the presence of light. Photosynthesis helps to augment dissolved oxygen (DO) concentrations in aquatic systems during the daylight hours; however, DO is consumed during times of darkness. In algal-dominated systems, the reduction of CO\textsubscript{2} can increase pH and may help reduce the need for buffering agents that are commonly used in biofloc systems (Ray et al. 2009).

Some algal taxa have been shown to provide potential benefits for shrimp culture. Diatoms are an important food for shrimp because they are generally rich in essential nutrients such as fatty acids and, if they are accessible to shrimp, can improve growth rates (Ju et al. 2009; Officer and Ryther 1980; Volkman et al. 1989). Chlorophytes can become highly abundant in biofloc systems, possibly contributing to substantial oxygen production (Ray et al. 2010b). Other taxa such as cyanobacteria and certain dinoflagellates, can pose potential hindrances to shrimp culture. Cyanobacteria have been especially problematic; they can cause off-flavors and produce toxins that may reduce shrimp growth or result in mortality (Alonso-Rodriquez and Paez-Osuna 2003;
Potentially toxic dinoflagellates can also bloom in eutrophic aquatic systems including aquaculture facilities (Alonso-Rodriquez and Paez-Osuna 2003).

Various forms of zooplankton are common in biofloc systems. Using light microscopy these organisms can be seen grazing on and within the biofloc particles; some common zooplankton include rotifers, ciliates, and nematodes (Ray et al. 2010b). Zooplankton may play an important role in energy transfer because they consume bacteria and algae and are often eaten by shrimp (Focken et al. 1998). Along with bacteria and shrimp, zooplankton consume oxygen through the process of respiration and dense communities may add substantially to the overall oxygen demand of biofloc systems.

Microorganism and System Management

As research in biofloc systems has progressed, shrimp culture has become more intensive due to economic and ecological necessity (Browdy et al. 2012). At higher shrimp density, a small areal footprint will be required relative to that of extensive culture systems. Shrimp food is the only source of nutrients in these biofloc systems and the amount of food applied corresponds directly to shrimp density. Therefore, as shrimp density and nutrient inputs increase, the reliance on microbial organisms for nutrient cycling also increases.

Current research has focused on applying management strategies to biofloc systems as a means of controlling the abundance or composition of microorganisms and enhancing the performance of cultured animals. One such management tactic is to control the concentration of biofloc particles in the water column. The abundance of
particles increases as the culture cycle progresses due to continued nutrient input. As mentioned, these biofloc particles may offer distinct advantages for system function and animal nutrition; however, some control over their concentration seems necessary.

Excessive suspended solids may increase biochemical oxygen demand (BOD) and leave inadequate dissolved oxygen available for shrimp (Beveridge et al. 1991). Too many particles can lead to gill clogging (Chapman et al. 1987) and suppress beneficial algal growth while increasing the abundance of potentially harmful microbes (Hargreaves 2006; Brune et al. 2003; Alonso-Rodriquez and Paez-Osuna 2003). A variety of methods are commonly used to control suspended solids concentrations in RAS. These include bead filters, sand filters, swirl separators, screens, and foam fractionators; the application of each depends on filtration needs and particle characteristics. One of the most economical techniques for removing suspended solids in biofloc systems is sedimentation, in which particles are simply allowed to settle out of the water column using gravity.

In an experiment conducted using 6200-L, outdoor tanks stocked at 460 shrimp m$^{-3}$ Ray et al. (2010a) demonstrated that using simple side stream settling chambers decreased suspended solids concentrations by 59% and increased final shrimp biomass (kg m$^{-3}$) by 41% compared to tanks without settling chambers. Exactly which factors contributed to increased shrimp production is unclear. However, in a similar study, Ray et al. (2010b) showed that using the same side stream settling chambers significantly reduced the abundance of rotifers, nematodes, cyanobacteria, and bacteria from biofloc culture tanks.
As discussed previously, another method of controlling the composition and function of microorganisms in biofloc systems is raising the C:N ratio to favor heterotrophic nitrogen assimilation. Some system managers stop adding carbohydrates after overcoming the initial spikes in NH$_3$ and NO$_2$ (Ray et al. 2009) while others contend that the continual addition of carbohydrate to the water column creates a nutritious bacterial biomass that can contribute to enhanced animal growth (Hari et al. 2004).

Nitrifying and heterotrophic bacteria may offer different nutritional qualities to shrimp. The type of carbohydrate added to culture water may also contribute to differences in the nutritional quality of heterotrophic bacteria (Avnimelech 2012; Crab et al. 2010a). Managers have used a variety of carbohydrate sources during animal culture such as molasses (Samocha et al. 2007), tapioca flour (Hari et al. 2004), and wheat meal (Avnimelech 1999). Crab et al. (2010a) recently demonstrated that growing bioflocs in an experimental reactor using glycerol could produce bioflocs with a higher protein and vitamin C content than those grown with glucose or acetate. Glycerol is a byproduct of the biodiesel manufacturing process, potentially making it an environmentally and economically sustainable product (Thompson and He 2006). Kuhn et al. (2009) successfully used sucrose as a carbon source to generate bioflocs that were a suitable replacement for fishmeal and soybean meal in shrimp diets.

An advantage to operating shrimp culture systems in which heterotrophic bacterial assimilation dominates the nitrogen pathways, is that the toxic compounds NH$_3$ and NO$_2$ can be alleviated. However, the potential drawbacks of increased oxygen demand and increased solids production must be considered.
Nitrification is commonly relied upon in closed aquaculture systems. When functioning properly the cycle requires no inputs other than animal feed, aside from a buffering agent to maintain alkalinity. The process consumes less oxygen than heterotrophic assimilation (Ebeling et al. 2006). However, at high concentrations NO₃, the final product of nitrification, can become toxic to shrimp. At a salinity of 11‰, Kuhn et al. (2010) found that 435 mg NO₃-N L⁻¹ significantly reduced shrimp biomass, and survival and growth were reduced at a concentration of 910 mg NO₃-N L⁻¹. In comparison, Lin and Chen (2001) recommended a safe level of total ammonia nitrogen (TAN) to be 2.4 mg TAN L⁻¹ at 15‰ salinity and 8.1 pH, and Lin and Chen (2003) recommended a safe concentration of nitrite to be 6.1 mg NO₂-N L⁻¹ at 15‰ salinity and a pH of 8.0.

Salinity

A key factor in the development of sustainable inland marine shrimp aquaculture is the ability to culture shrimp at the lowest possible salinities. Culture of shrimp on the coast with direct access to saltwater is more expensive and environmentally destructive than culture even several kilometers inland (Hopkins et al. 1995). The price of marine salts and pumping or hauling seawater substantial distances can reduce financial returns. In addition, lower salinity may provide options for reuse of removed biofloc which is currently considered waste. Waste reuse options include terrestrial plant fertilization (Dufault et al. 2001) or feed ingredients for aquatic animals (Kuhn et al. 2009), both of which benefit from a lower salinity.

Most of the work conducted concerning low salinity marine shrimp aquaculture has been in extensive systems. Operating low salinity minimal-exchange, intensive
biofloc systems will likely pose unique challenges. Jiang et al. (2000) found that ammonia-nitrogen excretion by *L. vannamei* was significantly lower when shrimp were held near their isosmotic point at 25‰ salinity than at 10‰ salinity. Such a finding may have substantial implications for minimal-exchange systems in which inorganic nitrogen compounds can easily reach toxic concentrations. The toxicity of inorganic nitrogen compounds to shrimp is lower at higher salinities (Schuler et al. 2010; Kuhn et al. 2010). It is possible that metals such as boron also could be more toxic to shrimp at lower salinities (Li et al. 2008). Metals can be introduced through the fish meal commonly found in shrimp feed.

**Objectives**

The overall objectives of this dissertation are to provide an examination of the effects of management techniques and culture conditions on system and shrimp performance. In the first two chapters the effects of biofloc particle concentration are explored, first in commercial-scale systems with two levels of particle management, and secondly in mesocosm systems half of which contained biofloc and half of which contained no biofloc. The third chapter is an examination of the differences in systems with no carbohydrate addition and systems with three unique carbohydrate sources added to them. Chapter IV describes a study in which shrimp are cultured in seawater diluted to three different salinities. Lastly, the fifth chapter examines the potential contribution of biofloc particles to juvenile shrimp in nurseries. The evaluation of these management techniques and culture conditions is intended to assist biofloc system managers in making informed management decisions. The continued development of intensive, biofloc-based culture systems may assist in the responsible intensification of the shrimp industry.
CHAPTER II

WATER QUALITY DYNAMICS AND SHRIMP (*LITOPENAEUS VANNAMEI*)

PRODUCTION IN INTENSIVE, MESOHALINE CULTURE SYSTEMS

WITH TWO LEVELS OF BIOFLOC MANAGEMENT

Introduction

Intensive, minimal-exchange shrimp culture systems have little, if any, water exchange and high animal stocking densities. Decreased water exchange reduces pollutant discharge, disease exchange between wild and captive stocks, and introductions of exotic species to the wild. With little water exchange and the tolerance of *Litopenaeus vannamei* to low and moderate salinities, these systems can be sited at inland locations, preserving coastal ecosystems and offering fresh marine shrimp to areas that otherwise could not access such a commodity (Browdy and Moss 2005).

High animal stocking densities reduce the footprint of culture systems, but also necessitate large nutrient inputs. These nutrients lead to eutrophication within the systems and, in response, a dense microbial community develops, much of which is contained on and within biofloc particles (Avnimelech 2012; Ray et al. 2010b). The microbial community in intensive, minimal-exchange culture systems is responsible for cycling nutrients, most importantly nitrogen compounds. Feed decomposition and animal excretions contribute to ammonia, which is toxic to shrimp. Algae and heterotrophic bacteria can directly assimilate ammonia to build cellular proteins, and nitrifying bacteria can oxidize ammonia to form nitrite and nitrate (Ebeling et al. 2006). Each of these three groups contribute to detoxifying nitrogenous waste, but each has drawbacks: algae are limited in the amount of nitrogen they can remediate (Brune et al. 2003), heterotrophic
bacteria require substantial amounts of oxygen to assimilate ammonia (Browdy et al. 2012), and nitrifying bacteria can be slow to establish, resulting in spikes of toxic ammonia and nitrite (Ray et al. 2009).

To stimulate the rapid uptake of ammonia by heterotrophic bacteria, labile organic carbon sources such as sucrose can be added to the culture water (Avnimelech 2012; Crab et al. 2007; De Schryver et al. 2008). A carbon: nitrogen ratio (C:N) of system inputs (feed and carbohydrates) above approximately 10 should result in efficient ammonia assimilation (Avnimelech 1999; Ebeling et al. 2006). To effectively assimilate ammonia, these bacteria must expand in abundance, and the nitrogen they assimilate is not taken out of the system unless the bacteria are removed.

The microbial community not only detoxifies nutrients, but also can recycle those nutrients and provide benefits for animal growth and feed conversion ratios (FCR) (Ju et al. 2009; Moss 1995; Wasielesky et al. 2006). Although there are clear benefits to having an in situ microbial community, some control over these organisms and the biofloc particles they are associated with may be necessary. Using 6200-L outdoor tanks, half with simple settling chambers and half without, Ray et al. (2010a) demonstrated that managing biofloc concentration could significantly improve shrimp growth rate, FCR, and biomass production. Also, the authors showed that settling chambers contributed to significantly decreased nitrate and phosphate concentrations and significantly increased alkalinity concentration in the shrimp culture systems.

The purpose of the current project was to help refine optimal biofloc concentration and evaluate simple management and engineering considerations for regulating that concentration to achieve advantageous water quality dynamics and shrimp
production in commercial-scale systems. A detailed analysis of the effects that settling chambers can have on important water quality parameters is provided. Mesohaline conditions were used to enhance the sustainability and potential inland development of intensive, minimal-exchange systems.

Methods

Experimental Setting

This project was conducted at the University of Southern Mississippi’s Thad Cochran Marine Aquaculture Center (CMAC), a part of the Gulf Coast Research Laboratory, located in Ocean Springs, Mississippi, USA. At the CMAC is a commercially-scaled minimal-exchange, intensive shrimp culture facility which was described by Ogle et al. (2006). Briefly, it consists of twelve, 3.2 m x 30.1 m, rectangular, cement block, high density polyethylene (HDPE)-lined raceways, eleven of which were used for this project, including those used during the nursery phase. The raceways are covered by six dome-shaped greenhouse structures covered in clear plastic sheeting (two raceways per greenhouse structure), each connected to a central, wood-frame structure that houses a harvest basin. Each raceway has a dirt floor beneath the liner, which is gently sloped toward the harvest basin.

Shrimp Source, Nursery, and Feeds

*Litopenaeus vannamei* post-larvae (PL 12) were obtained from Shrimp Improvement Systems, LLC (Islamorada, Florida, USA). These shrimp were stocked into three of the above mentioned raceways at a density of 2986 shrimp m$^{-3}$ to begin a nursery phase. The nursery raceways were maintained at a volume of 60 m$^3$ and a salinity of between 19 and 24‰ with no water exchange. The water used for the nursery
phase had been used the previous year for culturing shrimp, but solids were settled from the water and it was passed continuously through a foam fractionator at a flow rate of approximately 150 L min$^{-1}$, for one month prior to use.

Each nursery received blown air from a 746 W regenerative blower (Sweetwater®, Aquatic Ecosystems Inc., Apopka, Florida, USA) delivered through thirty six, 15.2 cm long ceramic air diffusers. Shrimp were fed PL Raceway Plus #1 between stages PL 12 and PL 18, and PL Raceway Plus #2 between stages PL 19 and PL 30 (Zeigler™ Brothers Inc., Gardners, Pennsylvania, USA). Both of these feeds were guaranteed by the manufacturer to provide a minimum of 50% protein and 15% fat, and a maximum of 1% fiber, 12% moisture, and 7.5% ash. Shrimp were then fed Zeigler™ Hyperintensive-35 for the remainder of the nursery phase and throughout the duration of this project. The Hyperintensive feed was analyzed by Clemson University’s Agricultural Services Laboratory (Clemson, South Carolina, USA) and found to contain 33.4% crude protein, 10.4% fat, 8.6% moisture, and 6.6% ash.

During the nursery phase, dissolved total ammonia nitrogen (TAN) and nitrite-nitrogen (NO$_2$-N) concentrations were monitored. TAN was assessed using Hach method 8155 (Hach Company 2003) and NO$_2$-N was measured using the spectrophotometric procedure outlined by Strickland and Parsons (1972). Absorbance was measured at 655 nm for TAN and 543 nm for NO$_2$-N using a Hach DR 3800 spectrophotometer (Hach Company, Loveland, Colorado, USA). Shrimp were cultured in the nursery raceways for 39 days and then stocked into the experimental raceways. In response to NO$_2$-N concentrations above 2 mg L$^{-1}$ during the nursery, sucrose was added to stimulate nitrogen assimilation by heterotrophic bacteria.
Experimental Systems

Eight of the raceways described above were used for this experiment with the following modifications. Each experimental system had a central wall of plastic sheeting suspended between two pieces of PVC pipe. The top pipe was suspended with ropes from the greenhouse structure and the bottom pipe was weighted internally with rubber-coated iron bars and water. Water was propelled around the central wall of each raceway using a combination of four airlift mechanisms and a 560 W water pump delivering water to eighteen, 1.3 cm diameter Venturi nozzles (Turbo-Venturi®, Kent Marine, Franklin, Wisconsin, USA) throughout the raceway (Figure 1) and to the settling chambers. Each airlift mechanism consisted of three, 15.2 cm long ceramic air diffusers oriented parallel to the water flow and receiving air from a regenerative blower described above. The airlifts were constructed of a 2.5 cm diameter PVC frame which held the diffusers approximately 6 cm above the raceway floor. Above the diffusers was a sheet of EPDM rubber held by the PVC frame and oriented at an approximately 35° angle relative to the water movement. Air from the diffusers traveled vertically and contacted the EPDM, which served as a deflector to project the air, and the water traveling with it, horizontally forward.
Figure 1. The configuration of each of the eight experimental raceways. Four air lifts and eighteen Venturi nozzles propelled water around the central wall.

The Venturi nozzles were located near the bottom of each raceway, each connected to a 1.3 cm diameter vertical pipe that was connected to a 5 cm diameter pipe that circumvented the raceway. Each Venturi had tubing attached to the gas injection point which then attached to another pipe, 2.5 cm in diameter that circumvented the raceway. This pipe had two valves to allow ambient air to be drawn in and a point where pure oxygen gas could be injected; this allowed air, pure oxygen, or a combination of the two to be injected into the raceway water through the Venturi nozzles.

Experimental Design

The eight raceways used for this experiment were each randomly assigned to one of two treatments, each treatment containing four replicate raceways. The low solids
treatment (T-LS) was designed to have a lower suspended solids concentration in the raceway water column than the high solids treatment (T-HS). Two factors were different between the treatments: settling chamber volume and the flow rate to those settling chambers. Raceways belonging to the T-LS treatment had settling chambers 1700 L in volume which received water at a rate of 20 L min\(^{-1}\). Raceways belonging to the T-HS treatment had settling chambers with a volume of 760 L, 45% that of the T-LS settling chambers, and received a flow rate of 10 L min\(^{-1}\), half that of the T-LS settling chambers (Table 1). Both types of settling chambers had conical bottoms and the depth of each was similar.

Table 1

*The settling chamber volumes and flow rates. These are the factors that differed between the two treatments.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>T-LS (Low Solids)</th>
<th>T-HS (High Solids)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Settling Chamber Volume (L)</td>
<td>1700</td>
<td>760</td>
</tr>
<tr>
<td>Flow Rate to Settling Chambers (L min(^{-1}))*</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>

The retention time of the T-LS settling chambers was 85 minutes and the volume of a raceway flowed through those chambers every 41.7 hours. The retention time of the T-HS settling chambers was 76 minutes and the 50 m\(^3\) volume of a raceway flowed through them every 83.3 hours. The 1700 L, T-LS settling chambers were 3.4% of the volume of a raceway, proportionately similar to those used by Ray et al. (2010a) which were 3.2% the volume of the culture tanks. The T-HS settling chambers were 1.5% the volume of the raceways. Flow to all settling chambers was continuous, except during approximately four hours when the settled material was being drained once per week. Flow rates were monitored twice per week by using a stop watch to measure how fast a one liter beaker
filled. Flow rate was adjusted with a valve in the supply line to each settling chamber (Figure 2). Flow rates were reset for approximately two weeks due to pipe clogging. During this period of time the flow rates for T-LS and T-HS settling chambers were 10 and 5 L min$^{-1}$, respectively.

\textit{Figure 2}. The design of the settling chambers used to manage biofloc concentration. The structures that support the chambers are not shown in this diagram. The T-LS settling chambers received a continuous flow rate of 20 LPM while T-HS settling chambers received a flow rate of 10 LPM.

Settled material was removed from each chamber once per week by opening a valve at the bottom of the chamber (Figure 2). The material was allowed to flow until the color and consistency was approximately that of the corresponding raceway, implying that the settled solids had been removed and clarified water was starting to flow. The volume of material removed from each settling chamber was measured, as was the total suspended solids (TSS) and volatile suspended solids (VSS) of the material. Both TSS
and VSS were measured by diluting as needed with deionized water and following ESS Method 340.2 (ESS 1993).

**Water Quality**

Twice per day, at approximately 0730 and 1600 h, temperature, dissolved oxygen (DO), pH, and salinity in the raceways were measured using a YSI Model 556 Handheld Instrument (YSI Incorporated, Yellow Springs, Ohio, USA). Once per week TSS, VSS, turbidity, TAN, NO$_2$-N, orthophosphate (PO$_4$), and alkalinity (as CaCO$_3$) were measured in each experimental raceway. Once every week except weeks one, two, and twelve the concentration of nitrate-nitrogen (NO$_3$-N) was measured. Raceway water samples were collected approximately 4 cm below the water surface, near the intake of the pumps that distributed water around the raceways and to the settling chambers. At the same time samples for these analyses were taken, samples of the settling chamber effluent (the water returning to each raceway from its respective settling chamber) were collected for analyses. The settling chamber return lines were located at the end of the raceways opposite that of the pump intake. The purpose of analyzing the settling chamber effluent was to compare the chemistry of this water to that of the raceways and assess what effect each settling chamber was having on water quality. However, not all chemistry analyses were performed for settling chamber effluent every week due to resource limitations. In the settling chamber effluent, TSS and VSS were both measured during weeks two through eight, turbidity was measured during weeks three through eight and ten through twelve, TAN was measured during weeks two through eight and week thirteen, NO$_2$-N was measured every week, NO$_3$-N was measured during weeks six through eleven and
week thirteen, $\text{PO}_4$ was measured during weeks one through eight, and alkalinity was measured during weeks one through nine and weeks eleven and twelve.

The methods used to assess TSS and VSS are referenced in section above, and turbidity was measured in Nephelometric Turbidity Units (NTU) using a Micro 100 Turbidimeter (HF Scientific, Fort Myers, Florida, USA). The methods used to measure TAN and NO$_2$-N concentrations are described above. The concentration of NO$_2$-N plus NO$_3$-N was determined using the chemiluminescence detection described by Braman and Hendrix (1989), then NO$_3$-N was calculated by subtracting NO$_2$-N concentration. The concentration of $\text{PO}_4$ was measured using the PhosVer 3 (ascorbic acid) method outlined in Hach Method 8048 (Hach Company 2003) and absorbance was measured at 890 nm using the Hach DR 3800 spectrophotometer. Alkalinity was measured following the Potentiometric Titration to Preselected pH procedure outlined in section 2320 B by the APHA (2005).

*Shrimp Culture*

The eight experimental raceways were each filled with 50 m$^3$ of water; 15 m$^3$ of which was inoculant from the nursery raceways, 20 m$^3$ was previously bleached water from Davis Bayou, a tributary of The Mississippi Sound adjacent to the CMAC in Ocean Springs, Mississippi, USA, and 15 m$^3$ was artificial seawater made with municipal water and a mixture of Fritz Super Salt Concentrate (Fritz Pet Products, Mesquite, Texas, USA) and sodium chloride (Morton® Purex® Salt, Morton® Salt, Chicago, Illinois, USA). Once every two weeks, during the experiment, artificial seawater was added to each raceway to replace the volume removed by the settling chambers. Once per week municipal fresh
water was added to replace evaporation. Saltwater or freshwater were added in an effort to maintain a salinity of 16‰ and a volume of 50 m³ throughout the study.

Shrimp were stocked into the experimental raceways at a density of 250 m⁻³ and a mean ± SEM weight of 0.72 ± 0.20 g. Feeding was based on an estimated feed conversion ratio (weight of feed provided/shrimp population weight gain), which was calculated by estimating the shrimp population (assuming 10% stocking mortality then 1% mortality per week, along with routine dip net sampling to check for uneaten feed and dead shrimp) and sampling individual weights weekly. The feed conversion ratio (FCR) was multiplied by the expected weekly growth, which was then multiplied by the estimated shrimp population, to generate a weekly feeding amount. The Zeigler™ Hyperintensive-35 feed used is described in section 2.2 and was dispersed evenly into the raceways five times per day at uniformly spaced times of approximately 0700, 0930, 1200, 1430, and 1700 h. Each feed portion was weighed on a digital balance, and each raceway received the same amount of feed during the study. Shrimp weights were measured once per week by weighing five groups of ten shrimp from each raceway; these shrimp were collected from various locations throughout the raceways using a dip net. Nets were moved through the water very quickly during sampling to ensure representative sampling of shrimp. Shrimp were grown for 13 weeks.

Household, granulated sucrose was added to each raceway at least two times per day. The carbon content of the sucrose was 41%. Sucrose had little or no liquid mass; after drying in an oven at 60⁰ C for five days, there was no appreciable change in mass. The frequency at which sucrose was added depended on the raceway DO concentration, as sucrose addition led to a substantial decrease in DO concentration within 1.5 hours of
addition which could potentially cause stress for shrimp. The reason for adding sucrose was to stimulate the uptake of TAN by heterotrophic bacteria; De Schryver et al. (2008) suggested that sucrose is a fast-acting carbohydrate for this purpose. Every addition of sucrose was weighed on a digital balance, and each raceway received the same amount of sucrose. In response to unstable DO concentrations due, at least in part, to the sucrose inputs, pure oxygen gas was injected through the Venturi nozzles intermittently beginning week two and continuously beginning week five.

Data Management and Statistical Analyses

The data reported in this document are presented as mean ± SEM, and in many cases the range is given in parentheses. The statistical software used for this study was Systat Version 13 (Systat Software, Inc., Chicago, Illinois, USA). To test the sphericity assumption of repeated measures (RM) ANOVA tests SAS/STAT® software was used (SAS Institute Inc., Cary, NC, USA). The amount of both saltwater and freshwater added to raceways, the volume of material removed with settling chambers, and the dry weight of material removed with settling chambers were each compared between treatments using a two sample t-test.

The TSS, VSS, turbidity, TAN, NO$_2$-N, NO$_3$-N, PO$_4$, and alkalinity data for the raceways were compared using a RM ANOVA for each parameter. The one-way RM ANOVAs used to analyze data from this study were fixed model with two levels based on the two treatments. The NO$_3$-N data had to be transformed to meet the normality assumption of the ANOVA, this was accomplished by calculating the log$_{10}$ values of the data followed by the exponential function.
To assess the effects that the settling chambers had on water quality, the percent change in water quality values of water entering the settling chambers (influent: water in the raceways) and water exiting the settling chambers (effluent) was calculated. These calculations were made for each date that both raceway water and settling chamber effluent were analyzed for a given water quality parameter. The mean percent change for each treatment was calculated for each date and organized over time; a RM ANOVA was then used to test for differences between the two treatments. If no significant differences were found in percent change between treatments the overall influent mean and effluent mean (combining treatments) for each date was determined. These data were organized over time and a RM ANOVA was used to determine whether differences existed between overall settling chamber influent and effluent when the treatments were pooled. The NO$_3$-N concentration of influent versus effluent was also compared within each treatment.

Results

_Nursery_

On the last day of the nursery, nitrite concentration in the three nursery raceways was 14.5 ± 4.2 mg NO$_2$-N L$^{-1}$. It is unclear what events may have caused this spike in nitrite; other water quality parameters were within an acceptable range for _L. vannamei_ culture. Sucrose had been added regularly in an effort to encourage nitrogen assimilation by heterotrophic bacteria. Ten days prior to the final day of the nursery, nitrite concentration had been 2.0 ± 0.5 mg NO$_2$-N L$^{-1}$. Excessive mortality was observed during the last two days of the nursery phase and overall nursery survival was 25%; many of the surviving shrimp appeared lethargic.
C:N Management, Water Use, and Settling Chambers

A raceway belonging to the T-LS treatment developed a substantial leak during the experiment. The volume of saltwater added to this raceway to maintain a consistent total raceway volume was 29% greater than the mean volume added to the other raceways in the treatment. Including the original 50 m^3 of water placed in all raceways, the leaking tank required 76.86 m^3 and the other tanks in the T-LS treatment required 59.58 ± 0.79 m^3 of 16‰ salinity water. Because of the leak and the resultant water exchange to maintain volume, data generated from this raceway and the corresponding settling chamber were excluded from all data analyses and conclusions of this study.

A total of 353.5 kg of feed (wet weight) and 184.3 kg of sucrose were added to each raceway throughout the study. The sucrose used contained 41% C and the feed contained 44.4 % C, 5.3% N, and 8.6% moisture (as determined by the Clemson Agricultural Services Laboratory, Clemson, South Carolina, USA). Therefore, on a dry weight basis, considering only sucrose and feed inputs (excluding shrimp, original water, and the small amount of replacement water for evaporation and particle removal) the C:N of inputs was 12.4.

The total amount of 16‰ salinity water used for the T-LS raceways was 59.58 ± 0.79 m^3 and that used for the T-HS raceways was 56.35 ± 0.64 m^3; these numbers include the 50 m^3 initially placed in the raceways. A significantly (P = 0.030) greater volume of this mesohaline water was used for the T-LS raceways due to the larger volume of material removed with settling chambers. The total amount of freshwater used to replace evaporation was 14.72 ± 1.38 m^3 for the T-LS raceways and 15.70 ± 1.04 m^3 for the T-HS
raceways. There was no significant difference in freshwater input between the two treatments ($P = 0.602$).

At the end of week four, the settling chamber flow rate was reset because the pipes that delivered water to the chambers had become clogged. At this point, the flow rates were reset to $10 \text{ L min}^{-1}$ for the T-LS settling chambers and $5 \text{ L min}^{-1}$ for the T-HS settling chambers. At the beginning of week seven the flow rates were set back to the original values of $20$ and $10 \text{ L min}^{-1}$ after a method of regularly cleaning the supply pipes was developed.

A total of $10.26 \pm 0.34 \text{ m}^3$ of settled material was removed from the T-LS raceways using the settling chambers and $7.97 \pm 0.18 \text{ m}^3$ of settled material was removed from the T-HS raceways, constituting a significant difference ($P = 0.009$) between treatments. The mean TSS of material drained from the bottom of the T-LS settling chambers was $27.44 \pm 9.21 \text{ g L}^{-1}$ and it was $22.26 \pm 9.33 \text{ g L}^{-1}$ from T-HS settling chambers. There was no significant difference ($P = 0.244$) between the two treatments in terms of the mean TSS concentration of this removed material.

The total dry weight of solids removed from each T-LS raceway was $268.70 \pm 31.10 \text{ kg}$ which was $71\%$ greater than that removed from each T-HS raceway ($156.71 \pm 7.45 \text{ kg}$). However, the results of a two sample t-test indicate that these amounts were not significantly different ($P = 0.062$). Most of this material was volatile solids; the weight of volatile solids removed from each T-LS raceway was $222.72 \pm 24.35 \text{ kg}$, and volatile solids removed from the T-HS raceways was $133.05 \pm 1.95 \text{ kg}$.

**Raceway Water Quality**
The values of the four water quality parameters measured twice per day (temperature, DO, pH, and salinity) are presented in Table 2. These parameters remained within an acceptable range for the growth of *L. vannamei*. The DO concentration was maintained through pure oxygen gas injection during most of the experiment.

Table 2

*Temperature, DO, pH, and salinity recorded in the two treatments. Results are presented as mean ± SEM (range).*

<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
<th>T-LS</th>
<th>T-HS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature (°C)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>29.2 ± 0.1 (25.9-32.2)</td>
<td>28.9 ± 0.1 (26.1-31.5)</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>30.7 ± 0.1 (27.0-33.8)</td>
<td>30.3 ± 0.1 (27.0-33.0)</td>
<td></td>
</tr>
<tr>
<td><strong>Dissolved Oxygen (mg L⁻¹)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>7.9 ± 0.1 (4.2-13.4)</td>
<td>7.2 ± 0.1 (4.2-11.7)</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>6.2 ± 0.1 (2.9-10.7)</td>
<td>6.1 ± 0.1 (2.7-10.7)</td>
<td></td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>7.6 ± 0.0 (6.7-8.3)</td>
<td>7.6 ± 0.0 (7.1-8.3)</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>7.4 ± 0.0 (7.1-8.5)</td>
<td>7.5 ± 0.0 (7.1-8.5)</td>
<td></td>
</tr>
<tr>
<td><strong>Salinity (‰)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>16.3 ± 0.0 (15.6-18.3)</td>
<td>16.3 ± 0.0 (15.0-18.4)</td>
<td></td>
</tr>
<tr>
<td>PM</td>
<td>16.2 ± 0.0 (15.5-18.4)</td>
<td>16.2 ± 0.0 (15.0-18.4)</td>
<td></td>
</tr>
</tbody>
</table>

The TSS and VSS concentrations, and the turbidity of water in the T-LS raceways were each significantly lower (P = 0.003, 0.000, and 0.001, respectively) than those of the T-HS raceways during the experiment (Figure 3).
During the second week of the experiment TSS, VSS, and turbidity were each higher in the T-LS raceways. This coincided with a large amount of thick, green material developing on the water surface of the T-HS raceways. Four spray bars that injected raceway water onto the water surface were then installed in every raceway and appeared to effectively homogenize the T-HS systems.
During weeks seven through nine of the experiment, two of the T-HS raceways changed substantially in color. Based on visual observation, these two raceways had a milky brown appearance, while the other two T-HS raceways were green in color. This coincided with a substantial increase in turbidity values for the two milky brown raceways. During this three week period turbidity was 262.1 ± 22.7 NTU in the milky brown raceways, and 120.1 ± 12.3 NTU in the green raceways. By week ten, each of the T-HS raceways appeared green in color again and turbidity was 108.4 ± 6.8 NTU in the four raceways. This event had a substantial impact on the turbidity data for the T-HS treatment, but did not seem to impact TSS or VSS values (Figure 3).

The concentration of TAN was significantly greater in the T-LS treatment ($P = 0.021$). The TAN concentration in T-LS raceways was highest on week 12 (Figure 4a). At this time, TAN concentration in the three T-LS raceways was 15.0, 4.8, and 1.8 mg TAN L$^{-1}$. Between the week 11 and week 12 sampling, 580 dead shrimp were removed from the raceway with a week 12 concentration of 15.0 mg TAN L$^{-1}$. Dead shrimp were first found in this raceway the day of week 11 sampling; that day TAN concentration was 0.8 mg L$^{-1}$. This appears to indicate that the rise in TAN concentration was a result of shrimp mortality and not the cause of that mortality; it is unclear what may have caused the mortality. Shrimp were monitored daily and no dead shrimp were found in the other two T-LS raceways during this time.
Figure 4. The concentrations of total ammonia nitrogen (TAN) (a), nitrite–nitrogen (NO\textsubscript{2}–N) (b), and nitrate–nitrogen (NO\textsubscript{3}–N) (c) over time in the raceways. Data points represent treatment means; error bars are one standard error around the mean.

The concentrations of both NO\textsubscript{2}–N and NO\textsubscript{3}–N were significantly (P = 0.000 and 0.007, respectively) greater in the T-HS treatment (Figure 4). All raceways started with a relatively high NO\textsubscript{2}–N concentration because of inoculant water reuse from the nurseries.

NO\textsubscript{2}–N was especially higher in the T-HS raceways during the first half of the experiment (Figure 4b); however, it eventually subsided. Beginning week six, NO\textsubscript{3}–N
concentration began to increase in the T-HS raceways and continued to increase during the following weeks (Figure 4c).

PO₄ concentration gradually increased in both treatments (Figure 5a), but was significantly greater (P = 0.003) in the T-LS treatment versus the T-HS treatment (Table 3).

There were no significant differences between alkalinity concentrations (Figure 5b) in the two treatments (Table 3), although a low probability value suggests that there may have been a subtle difference (P = 0.055), with T-LS systems containing a slightly higher concentration.

Figure 5. The concentrations of orthophosphate (PO₄) (a), and alkalinity (as CaCO₃) (b) over time in the raceways. Data points represent treatment means and error bars are one standard error around the mean.
Table 3

The overall mean ± SEM (range) of water quality parameters. These parameters were measured in the water of each raceway and, at the same time, in the effluent returning from the settling chambers to the raceways. Different superscript letters in a row indicate significant differences (P ≤ 0.02) in raceway water between treatments. Different superscript numbers in a row indicate significant differences (P ≤ 0.01) between settling chamber effluent and raceway water. A comparison is not being made between settling chambers belonging to different treatments.

<table>
<thead>
<tr>
<th>Location</th>
<th>T-LS Raceways</th>
<th>T-LS Settling Chambers</th>
<th>T-HS Raceways</th>
<th>T-HS Settling Chambers</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSS (mg L⁻¹)</td>
<td>196.8 ± 15.3 (70.0-630.0)ᵃ</td>
<td>98.1 ± 11.0 (15.0-225.0)ᵇ</td>
<td>313.0 ± 17.0 (85.0-755.0)ᵇ</td>
<td>122.5 ± 10.4 (45.0-250.0)ᵇ</td>
</tr>
<tr>
<td>VSS (mg L⁻¹)</td>
<td>131.9 ± 7.9 (10.0-225.0)ᵃ</td>
<td>70.2 ± 12.0 (5.0-175.0)ᵇ</td>
<td>248.1 ± 15.1 (15.0-650.0)ᵇ</td>
<td>82.5 ± 11.6 (5.0-235.0)ᵇ</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>43.8 ± 2.8 (12.1-88.5)ᵃ</td>
<td>19.1 ± 2.0 (4.6-55.6)ᵇ</td>
<td>105.6 ± 9.9 (13.9-356.7)ᵇ</td>
<td>68.6 ± 12.5 (5.7-332)ᵇ</td>
</tr>
<tr>
<td>Ammonia (mg TAN L⁻¹)</td>
<td>1.5 ± 0.4 (0.0-15.0)ᵃ</td>
<td>2.1 ± 0.3 (0.2-7.3)ᵇ</td>
<td>0.4 ± 0.4 (0.0-2.1)ᵇ</td>
<td>1.5 ± 0.3 (0.0-9.7)ᵇ</td>
</tr>
<tr>
<td>Nitrite (mg NO₂-N L⁻¹)</td>
<td>2.5 ± 0.4 (0.0-10.1)ᵃ</td>
<td>2.1 ± 0.5 (0.0-10.1)ᵇ</td>
<td>3.2 ± 0.4 (0.0-10.0)ᵇ</td>
<td>3.0 ± 0.4 (0.0-10.0)ᵇ</td>
</tr>
<tr>
<td>Nitrate (mg NO₃-N L⁻¹)</td>
<td>0.4 ± 0.3 (0.0-8.7)ᵃ</td>
<td>0.4 ± 0.2 (0.0-4.3)ᵇ</td>
<td>7.1 ± 1.3 (0.0-26.0)ᵇ</td>
<td>6.2 ± 1.2 (0.0-23.6)ᵇ</td>
</tr>
<tr>
<td>Orthophosphate (mg PO₄ L⁻¹)</td>
<td>39.9 ± 3.1 (7.9-79.4)ᵃ</td>
<td>31.3 ± 2.2 (10.7-48.3)ᵇ</td>
<td>33.6 ± 2.3 (8.4-71.1)ᵇ</td>
<td>29.4 ± 1.9 (6.5-59.1)ᵇ</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO₃ L⁻¹)</td>
<td>294.3 ± 3.9 (252.5-374.0)ᵃ</td>
<td>302.5 ± 4.3 (266.0-383.0)ᵇ</td>
<td>278.2 ± 4.4 (207.0-346.0)ᵇ</td>
<td>287.7 ± 4.9 (211.5-349.0)ᵇ</td>
</tr>
</tbody>
</table>

Effects of Settling Chambers

Table 3 contains the parameters that were measured in raceway water and simultaneously in settling chamber effluent returning to the raceways. There were no significant differences between the two treatments in the percent change of TSS, VSS, or turbidity levels entering and exiting settling chambers (P = 0.326, 0.068, and 0.503, respectively). Each of these parameters was significantly reduced by settling chambers in both treatments (P = 0.000, 0.001, and 0.001, respectively).

In terms of the percent change in TAN concentration between influent water from each raceway and effluent water from the settling chambers over time, there was no significant difference between the two treatments (P = 0.077). Combining data from the two treatments, and analyzing the overall influent TAN concentration into the settling chambers, and the effluent TAN concentration leaving the settling chambers (Figure 6a).
revealed that the effluent contained a significantly greater TAN concentration ($P = 0.004$).

![Graphs a, b, and c showing the mean ± SEM concentrations of TAN (a), NO$_2$-N (b), and NO$_3$-N (c) in the settling chamber influent (raceway water) versus effluent (returning from the settling chambers) over time. Graphs a and b represent mean concentrations of influent and effluent combined from all raceways and settling chambers. Due to relatively low NO$_3$-N concentration in the T-LS raceways, graph c represents data only from systems in the T-HS treatment.](image)

**Figure 6.** The mean ± SEM concentrations of TAN (a), NO$_2$-N (b), and NO$_3$-N (c) in the settling chamber influent (raceway water) versus effluent (returning from the settling chambers) over time. Graphs a and b represent mean concentrations of influent and effluent combined from all raceways and settling chambers. Due to relatively low NO$_3$-N concentration in the T-LS raceways, graph c represents data only from systems in the T-HS treatment.

There were no significant differences between the two treatments with regard to the percent change in NO$_2$-N concentration between the settling chamber influent and effluent ($P = 0.826$). Pooling data from the two treatments, the concentration of NO$_2$-N
in settling chamber effluent was significantly ($P = 0.001$) reduced compared to that of the influent (Figure 6b).

There was no significant difference between the two treatments in terms of the percent change of settling chamber influent versus effluent NO$_3$-N concentration ($P = 0.584$); settling chamber effluent contained a lower NO$_3$-N concentration than influent in both treatments. However, throughout the experiment the concentration of NO$_3$-N in T-LS raceways was low (Figure 4c); there were only small differences in settling chamber effluent and influent concentrations. Nonetheless, the percent change was similar to that of the T-HS raceways. When data from each treatment were analyzed individually no significant difference ($P = 0.080$) between the influent and effluent NO$_3$-N concentrations of the T-LS settling chambers was found (Table 3). However, in the T-HS systems there was significantly ($P = 0.005$) lower NO$_3$-N concentration in the effluent of the settling chambers versus the influent (Figure 6c).

There were no significant differences in the percent change of PO$_4$ concentration in settling chamber effluent and influent between the two treatments ($P = 0.138$). Regardless of treatment, settling chambers were returning a significantly higher ($P = 0.010$) concentration of PO$_4$ in the effluent than what was entering the chambers (Figure 7a).
Figure 7. The mean ± SEM concentrations of orthophosphate (PO$_4$) (a) and alkalinity (b) in the settling chamber influent (raceway water) and effluent (return water from settling chambers). Data are combined from all raceways and settling chambers regardless of treatment.

There was no significant difference between the two treatments in terms of the percent change in alkalinity entering and exiting the settling chambers (P = 0.118). In both treatments the settling chamber effluent had a significantly higher (P = 0.003) alkalinity concentration than the influent (Figure 7b).

Shrimp Production

Table 4 presents shrimp production parameters for the two treatments. Shrimp in the T-LS treatment weighed significantly (P = 0.019) more than those in the T-HS treatment at the end of the thirteen week experiment (22.1 ± 0.3 g versus 17.8 ± 0.2 g,
respectively). This was the result of a significantly greater ($P = 0.019$) growth rate in the T-LS treatment ($1.7 \pm 0.0 \text{ g wk}^{-1}$) versus the T-HS treatment ($1.3 \pm 0.1 \text{ g wk}^{-1}$) (Figure 8).

Table 4.

*Shrimp production in the two treatments. Data are presented as mean ± SEM (range); different letters between columns indicate significant differences between treatments ($P \leq 0.020$).*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Low Solids (T-LS)</th>
<th>High Solids (T-HS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean Final Weight (g)</td>
<td>$22.1 \pm 0.3 (21.7-22.7)^a$</td>
<td>$17.8 \pm 0.2 (15.3-19.7)^b$</td>
</tr>
<tr>
<td>Growth Rate (g week$^{-1}$)</td>
<td>$1.7 \pm 0.0 (1.6-1.7)^a$</td>
<td>$1.3 \pm 0.1 (1.1-1.5)^b$</td>
</tr>
<tr>
<td>Percent Survival</td>
<td>$49.7 \pm 3.1 (43.9-54.5)$</td>
<td>$49.4 \pm 5.9 (41.7-66.5)$</td>
</tr>
<tr>
<td>Biomass (kg m$^{-3}$)</td>
<td>$2.8 \pm 0.1 (2.5-3.0)$</td>
<td>$2.2 \pm 0.4 (1.8-3.3)$</td>
</tr>
<tr>
<td>Feed Conversion Ratio</td>
<td>$2.5 \pm 0.1 (2.3-2.7)$</td>
<td>$3.3 \pm 0.4 (2.0-4.0)$</td>
</tr>
</tbody>
</table>

*Figure 8. Mean ± SEM weekly individual shrimp weights.*

There were no significant differences in survival, feed conversion ratio (FCR), or final shrimp biomass between treatments. Survival was $49.7 \pm 3.1 \%$ in the T-LS treatment and $49.4 \pm 5.9 \%$ in the T-HS treatment. Due to low survival, populations of
shrimp in the raceways were overestimated, resulting in higher than expected feed conversion ratios (FCRs). The FCR in the T-LS treatment (2.5 ± 0.1) was lower than that of the T-HS treatment (3.3 ± 0.4), although that difference was not significant (P = 0.180). Similarly, biomass production was higher in the T-LS treatment (2.8 ± 0.1 kg m\(^{-3}\)) than the T-HS treatment (2.2 ± 0.4 kg m\(^{-3}\)), although not significantly different (P = 0.284).

Discussion

**General Effects of Settling Chambers**

Because TSS, VSS, and turbidity were significantly lower in the T-LS raceways, this is interpreted to mean that there was significantly reduced biofloc concentration in the T-LS raceways compared to that of the T-HS raceways.

The two sizes of settling chambers and two flow rates caused a similar percent change in TSS, VSS, and turbidity as water passed through them. Overall TSS, VSS, and turbidity values were significantly decreased using settling chambers (P = 0.000, 0.001, and 0.001, respectively). These data (Table 3) indicate that although the two sizes and flow rates of settling chambers affected TSS, VSS, and turbidity similarly in the return water, the two times greater flow rate of T-LS settling chambers led to lower values for these parameters.

Both the T-LS and T-HS settling chambers were causing a similar percent increase in TAN concentration of return water (Table 3). However, twice the water volume flowed through the T-LS settling chambers, possibly resulting in the significantly greater TAN concentration in T-LS raceways (Figure 4a).
Settling chambers from both treatments were reducing NO$_2$-N concentration by a similar percentage. However, possibly due to the faster flow rate through T-LS settling chambers, there was a significantly lower NO$_2$-N concentration in the T-LS raceways (Figure 4b).

With the exception of NO$_3$-N, the changes in concentration of water quality parameters were similar with both the large (T-LS) and the small (T-HS) settling chambers. The differences in raceway water quality between the two treatments may have resulted from the different settling chamber flow rates, the different retention times between the two treatments, or a combination of the two. A greater volume of water was treated by the T-LS settling chambers due to the faster flow rate, therefore intensifying the effects those settling chambers had on the raceways overall. Settling chamber retention times were similar between the two treatments (85 minutes in the T-LS settling chambers and 76 minutes in the T-HS settling chambers). However, this nine minute difference in retention time may have affected denitrification in the two systems. Menasveta et al. (2001) described an effective denitrification system with a hydraulic retention time of 86 minutes. These authors recommended a retention time between 60 and 120 minutes, and pointed out that retention times in the higher end of that range are generally more effective. Further research should be conducted to help determine the effects that settling chamber retention time can have on denitrification in biofloc-based aquaculture systems. Also worthy of consideration is that a smaller settling chamber will fill with settled material faster and therefore may require more frequent draining.

*Potential Processes Within Settling Chambers*
In closed aquaculture systems NO\textsubscript{3}-N may accumulate to concentrations that can inhibit shrimp survival and growth (Kuhn et al. 2010). Given the proper conditions, anaerobic denitrifying bacteria can convert NO\textsubscript{3}-N or NO\textsubscript{2}-N to nontoxic N\textsubscript{2} gas which is then released into the atmosphere (Hamlin et al. 2008). These bacteria are also capable of assimilating PO\textsubscript{4} and they typically generate alkalinity. However, in suboptimal anaerobic conditions some bacteria can reduce NO\textsubscript{3}-N back to the toxic TAN compound through the dissimilatory nitrate reduction to ammonia (DNRA) process (van Rijn et al. 2006).

The chemical evidence in this study suggests that DNRA may have been occurring in settling chambers of both treatments because TAN was being produced in these devices. However, in the T-LS treatment only low concentrations of NO\textsubscript{3}-N entered the settling chambers. The observed TAN concentrations exiting the chambers (Table 3) were likely not produced solely by DNRA. In the T-HS treatment the mean reduction of NO\textsubscript{3}-N concentration by settling chambers was 0.9 mg L\textsuperscript{-1}, but the mean increase in TAN concentration was 1.1 mg L\textsuperscript{-1}. Furthermore, NO\textsubscript{2}-N concentration was being reduced by the settling chambers (Table 3). This may indicate that rather than DNRA occurring, the settled biofloc in these chambers may have been decomposing, thereby generating TAN while denitrification was occurring simultaneously and causing a decrease in NO\textsubscript{3}-N and NO\textsubscript{2}-N concentrations. Another possibility is that these inorganic nitrogen compounds were being converted to organic biomass in the settling chambers, a portion of which was decomposing.

Any combination of the above mentioned processes may have occurred in settling chambers; however, it is probable that at least some level of denitrification was occurring
in the T-HS settling chambers due to the significant increase of alkalinity and the significant decrease of NO$_3$-N concentration within the chambers. Ray et al. (2010a) documented a significant reduction in NO$_3$-N and significant increase of alkalinity in systems with settling chambers compared to those without. The results of their study and the current one indicate that these simple filtration systems can not only remove particles, but may also serve as denitrification chambers. This dual function may make settling chambers an attractive filtration option for intensive aquaculture.

*Nitrogen Cycling*

NO$_3$-N accumulated in the T-HS treatment raceways; however, little NO$_3$-N accumulated in the T-LS raceways. It is postulated that nitrification was occurring in the T-HS treatment and that in the T-LS systems, either denitrification was occurring in the settling chambers, nitrification was reduced in the raceways, algae were assimilating a relatively greater proportion of NO$_3$-N in the raceways, or a combination of these events was occurring. In support of the hypothesis that denitrification was taking place in the T-LS settling chambers, a significant amount of alkalinity was generated in those chambers, a sign of denitrification. However, there was no significant decrease in NO$_3$-N concentration made by the T-LS settling chambers, indicating that denitrification may not have been occurring in the chambers. It is possible that denitrification was occurring at a rate so rapid that substantial concentrations of NO$_3$-N were not allowed to accumulate in T-LS systems, and therefore changes in concentration were not detected.

In support of the hypothesis that nitrification was reduced in T-LS raceways it is noted that in addition to the lack of NO$_3$-N, the concentrations of TAN and NO$_2$-N in the T-LS raceways appeared less stable than in the T-HS raceways (Figure 4). This could be
a sign that neither ammonia-oxidizing bacteria nor nitrite-oxidizing bacteria, both critical for nitrification, were functioning properly or perhaps were not present in adequate abundance. One explanation for such reduced function in the T-LS raceways is that the relatively low concentration of biofloc may not have provided sufficient surface area for nitrifying bacteria.

Another possibility is that the decreased concentration of particles in the T-LS raceways allowed greater light penetration and led to greater algal productivity than in T-HS raceways. As a result, algae in T-LS systems may have assimilated a relatively greater amount of NO$_3$-N, thereby leading to the decreased concentration of this compound. Because of the high rate of solids removal, algae were likely removed continually, thereby removing the nitrogen they had assimilated.

*Shrimp Production*

The relatively low survival during this project was attributable, in part, to the high NO$_2$-N concentrations during the nursery phase, during which a large proportion of shrimp died. Shrimp that were stocked into the experimental raceways appeared lethargic and it is probable that many of them succumbed to a combination of NO$_2$-N toxicity and handling stress early in the experiment. Survival was also likely impacted by the fluctuating concentrations of ammonia and nitrite during this study. The lower than expected survival during the study contributed to an over estimation of the shrimp population and consequently high FCRs.

The FCR of 3.3 obtained in the high solids treatment of the current study is unacceptably high for intensive shrimp culture systems. The FCR of 2.5 obtained in the low solids treatment is at the highest end of the range reported by Venero et al. (2009);
these authors summarized FCR values ranging from 1.2 to 2.5 during four recent, intensive shrimp production trials that were considered successful.

Although growth rate and final individual shrimp weight were both significantly greater in the T-LS raceways versus T-HS, the final shrimp biomass was not significantly greater. This appears to be due to variability in biomass among the T-HS raceways, caused primarily by one raceway. This T-HS raceway had a final shrimp biomass of 3.3 kg m$^{-3}$, whereas the other three raceways were in the range of 1.8-2.0 kg m$^{-3}$. Shrimp from the raceway also had a high growth rate (1.5 g wk$^{-1}$) and final weight (19.7 g), but those parameters were not as far from the range of the other raceways in the treatment (1.1-1.4 g wk$^{-1}$ and 15.3-19.4 g, respectively). The variability in final biomass likely prevented a statistical difference from being detected between treatments.

A mesohaline salinity of 16‰ was used during this study primarily to help facilitate the inland production of marine shrimp. The decision of what salinity to culture *L. vannamei* may have implications for shrimp growth. Bray et al. (1994) found that shrimp cultured in 15‰ salinity grew significantly larger than shrimp cultured at 25 and 35‰. This seems to contradict a study by Yan et al. (2007) who found an increasing trend in shrimp growth rate in systems with increasingly high salinities of 11, 21, and 31‰. Higher salinity can help protect animals from high concentrations of toxic nitrogen compounds, which can be problematic in intensive culture systems.

Between weeks seven and nine there was a decline of shrimp growth in the T-HS treatment (Figure 8). This was the case in all of the T-HS raceways, and was not specific to the raceways that changed color during this time. This was approximately the time that nitrate began to accumulate in the T-HS raceways. Possibly nitrate accumulation
was indicative of a shift in the bacterial community that negatively affected shrimp growth during this time.

It is unclear why shrimp growth rate was significantly greater in the low solids treatment versus the high solids treatment during this study. The T-LS animals may have had greater access to dissolved oxygen, as a higher particle concentration can lead to gill clogging (Chapman et al. 1987). An increased concentration of particles can suppress the growth of some potentially beneficial algae (Hargreaves 2006) or promote the proliferation of potentially harmful taxa such as cyanobacteria (Alonso-Rodriguez and Paez-Osuna 2003; Ray et al. 2010b). It has been suggested that continually cropping out a portion of the microbial community produces a younger, healthier community that may thereby provide enhanced nutritional benefits to culture animals (Turker et al. 2003). The T-LS raceways may have contained a younger microbial community due to higher cropping rates. Further research is needed to fully understand the ways that solids management affects shrimp growth rate.

Work similar to that conducted in this study should be repeated at higher shrimp stocking densities. Ray et al. (2010a) found a similar trend of increased shrimp growth rate with increased solids management. Although the growth rates they reported were considerably lower than those obtained during this study, the culture systems they used were 88% smaller and the stocking density they used was 84% higher than that of the current study. Venero et al. (2009) summarized four minimal-exchange, superintensive shrimp production trials stocked at between approximately 400 and 800 shrimp m$^{-3}$; these trials were conducted at three U.S.-based research institutions. The growth rate in the low solids treatment of the current study was higher than any reported by these authors.
However, such a growth rate may not be possible at very high, superintensive densities, as it has been demonstrated that stocking density can have an inverse relationship with growth rate (Wyban et al. 1987; Sookying et al. 2011).

Production goals, including culture period length, should be considered carefully by system managers. Growing shrimp to a marketable size in thirteen weeks, as was the case in this project, suggests that four crops of shrimp could be produced annually which may be an economically attractive option for shrimp producers.

Conclusion

This study demonstrates some of the effects that simple management and engineering considerations can have on chemical dynamics and shrimp production in intensive minimal-exchange shrimp culture systems. Raceways with a 20 L min\(^{-1}\) flow rate to 1700-L, external settling chambers had significantly lower TSS, VSS, turbidity, nitrite, and nitrate values than raceways with a settling chamber flow rate of 10 L min\(^{-1}\) and chamber volume of 760-L. The raceways with smaller settling chambers and lower flow rate had significantly lower ammonia and phosphate concentrations.

Nitrification progressed in the treatment with a mean TSS concentration of roughly 300 mg L\(^{-1}\) (high solids), as indicated by an accumulation of nitrate. However, no appreciable amount of nitrate accumulated in the treatment with a mean TSS concentration of approximately 200 mg L\(^{-1}\) (low solids). It is possible that the lower solids concentration may not have provided adequate substrate for nitrifying bacteria, denitrification may have occurred at a rapid rate in the settling chambers of this treatment, algae may have assimilated the nitrate, or a combination of these events may have occurred.
Shrimp grew significantly faster in the low suspended solids treatment and reached a significantly greater mean weight of 22 g during this 13 week study. There were no significant differences in shrimp survival, FCR, or biomass production, regardless of the level of biofloc concentration.

This study indicates that the level at which biofloc concentration is managed can affect nitrogen cycling pathways and shrimp production. Also, settling chambers may be able to perform denitrification along with particle removal, making them a potentially valuable, simple filtration mechanism.
CHAPTER III

SHRIMP (*LITOPENAEUS VANNAMEI*) PRODUCTION, WATER QUALITY, AND C AND N ISOTOPE DYNAMICS IN MINIMAL-EXCHANGE CLEAR WATER VERSUS BIOFLOC CULTURE SYSTEMS

Introduction

Extensive shrimp culture operations have come under scrutiny due, in part to the discharge of pollution and disease exchange between captive and wild animals. Closed systems offer advantages such as reduced or eliminated pollution discharge, little water exchange, and heat conservation. These qualities make them ideal for ecologically responsible aquaculture, with the potential to culture marine species at inland locations near metropolitan markets.

Clear water recirculating aquaculture systems (RAS) take advantage of external filtration devices to maintain adequate water quality. Particular filtration components vary in form and scale, but one or more solids filters and a biological filter are always used, and one or more means of water sanitization is common. Various types of solids filters remove different sizes and amounts of solids. Biological filters typically contain plastic media with ample surface area that acts as substrate for nitrifying bacteria. To discourage potentially harmful microbes from interacting with cultured animals, disinfection procedures can be used such as UV sterilization or ozonation of water.

Advantages of clear water RAS include the reliable culture of animals with a high level of control, consistently functioning biological filtration, and filtration systems that can be scaled to accommodate high animal biomass. The primary disadvantage is the upfront cost of filtration devices.
Another type of closed system, especially well-suited for shrimp, is a biofloc system. In biofloc systems solids are not removed entirely from the culture unit, although solids concentrations are typically regulated, and biological filtration occurs in the system rather than in an external filter. A microbial community is allowed to develop in the water column, and a portion of this community is contained on and in organic particles known as biofloc. Through careful system management the microbial community can be structured to cycle nitrogen in various ways, including through nitrification, heterotrophic bacterial assimilation, algal assimilation, or a combination of 2 or more of these processes (Ebeling et al. 2006; Browdy et al. 2012). Heterotrophic bacterial assimilation has proven effective at the Thad Cochran Marine Aquaculture Center (Ray et al. 2011b). This process is encouraged by adding a labile organic carbon source to elevate the C:N ratio of the culture water (Avnimelech 1999). The advantages of operating a biofloc system are that the costs of filtration components are reduced and a portion of the microbial community may be consumed by shrimp, potentially recycling nutrients from feed (Avnimelech 2006; De Schryver et al. 2008; Wasielesky et al. 2006). Disadvantages of biofloc systems include inconsistent nitrogen cycling and high system oxygen demand.

Direct comparisons of clear water RAS and biofloc systems are lacking in the scientific literature. One purpose of this project was to provide a preliminary comparison of shrimp production and water quality in clear water RAS versus biofloc culture systems.

Secondly, this project investigated some C and N stable isotope dynamics that may be important for shrimp aquaculture nutritional studies. Compounds constructed largely of C such as carbohydrates and lipids, and compounds constructed mostly of N
such as proteins, are integral components of the shrimp diet. Tracking the distribution of C and N isotope ratios can provide estimates of where shrimp are obtaining these elements. When an animal consumes a food item, that animal typically retains a greater portion of heavy C and N isotopes compared to the food item; this is a result of isotopic fractionation (Fry 2006). By utilizing clear water RAS an estimate of isotope fractionation was made by providing only one potential food source. Next, this information was applied to biofloc culture systems to estimate the C and N contributions of applied feed versus the biofloc microbial community in shrimp tissue.

Lastly, in examining stable isotope levels during shrimp nutritional studies some authors have sampled whole shrimp (ex. Burford et al. 2004; Epp et al. 2002) and others have used only shrimp abdomens (tails) (ex. Gamboa-Delgado and LeVay 2009; Parker et al. 1989). A third objective of the current project was to examine differences between isotope levels in whole shrimp versus shrimp tails.

Methods

Two independent studies were conducted for this project. In the first study shrimp were grown in clear water RAS and in biofloc culture systems as a side by side comparison of the two culture types. During the second study shrimp from the project described in Chapter II were used to compare isotopic levels in whole shrimp versus shrimp tails.

Study 1

Shrimp (L. vannamei) postlarvae (PL 12) were obtained from Shrimp Improvement Systems, LLC (Islamorada, Florida, USA). These shrimp were stocked in a nursery raceway at a density of 4000 shrimp m$^{-3}$. The nursery raceway was 3.2 m x 30.1
48

m x 0.52 m (W x L x D), lined with polyethylene, and contained under a greenhouse structure covered by clear plastic. The nursery raceway contained a central wall made of plastic sheeting; water was propelled around this wall using 6 airlift mechanisms described in Chapter II. The nursery was operated as a biofloc system, with sucrose added periodically in response to elevated ammonia concentrations. The nursery was operated at a salinity of approximately 25‰, and shrimp were grown for 83 days.

Each of 4, circular, 1.54 m x 0.8 m (diameter x depth), fiberglass tanks, contained in a greenhouse was randomly assigned to either a clear water (CW) treatment or a biofloc (BF) treatment, such that each treatment contained 2 replicate tanks. At the center of each CW tank was a stand pipe with small openings to allow water and feces through, but prevent food and shrimp from entering. There were openings throughout the area of the standpipe, allowing water to enter primarily from the tank bottom; an external stand pipe controlled tank depth. The internal stand pipe was placed in a drain that fed a 0.16 m³, square, plastic sump. Water was moved from the sump, using a 370 W pump, to a propeller-washed bead filter containing 111 m² of surface area (Model PBF-3, Aquaculture Systems Technologies, LLC, New Orleans, LA, USA). Water then passed through an in-line, 4 kW, titanium heater (Model HTI-4-220, Aqualogic, Inc., San Diego, CA, USA), and then into a moving bed bioreactor (MBBR). The MBBR consisted of a 756-L plastic, covered tank filled with 0.28 m³ of bio-filtration media (Curler X-1 Media, Aquaculture Systems Technologies, LLC). The MBBR was vigorously aerated with blown air from a 746 W regenerative blower (Sweetwater®, Aquatic Ecosystems Inc., Apopka, Florida, USA), delivered through a circular diffuser to ensure that particles did not accumulate in the MBBR. A portion of the water then returned to the sump and the
remainder entered the shrimp culture tank; the flow rate through shrimp culture tanks was approximately 30 LPM. An independent 90 W pump pulled water from the sump to supply a 1.27 m x 0.18 m (height x diameter) foam fractionator; water from the fractionator re-entered the sump. Each of the 2 CW tanks had a completely independent filtration system.

In the BF tanks, stand pipes with no openings were placed in the drains to prevent water from entering. Aeration was provided to the BF tanks through 6 ceramic diffusers receiving air from the blower described above. Pure oxygen gas was continuously supplied to the BF tanks using pressurized tanks of oxygen connected to a fine-pore diffuser located in each tank. Each BF tank contained a 2 kW, submersible, titanium heater. Adjacent to each BF tank was a 15-L container used as a settling chamber. A 15 x 5 x 5 cm ceramic air diffuser was placed in a 5 cm diameter, 20 cm long pipe covered on the bottom by plastic screen with 0.2 cm mesh size, and connected on the top to a 1.9 cm diameter piece of PVC. The pipe and diffuser were placed in an experimental tank, the diffuser received blown air from the regenerative blower described above; this air lift mechanism propelled water into the top of a settling chamber. Inside the settling chamber water traveled down a 5 cm diameter pipe, causing velocity to slow and particles to settle out on the bottom of the chamber; relatively clear water at the top of the chamber flowed out of another 1.9 cm diameter pipe and back into the shrimp culture tank. Settling chambers were operated continuously at approximately 6 LPM, and they were emptied once per week.

The CW systems were filled with artificial seawater made with municipal water and a mixture of Fritz Super Salt Concentrate (Fritz Pet Products, Mesquite, Texas, USA).
and sodium chloride (Morton® Purex® Salt, Morton® Salt, Chicago, Illinois, USA). In the BF systems, 20% of the volume was water from the nursery raceway, intended to “seed” the tanks with biofloc and the associated microbiota, the remainder was artificial seawater made in the same manner as that of the CW systems. In both treatments approximately half of the total system volume was composed of artificial seawater that had been used to culture fish (Cynoscion nebulosus) in clear water RAS previously. Salinity was maintained at approximately 20‰ throughout this experiment.

To ensure that nitrifying bacteria were functioning in the MBBRs of the CW systems, 30 g of NH₄Cl was added to each system every other day, beginning 2 weeks prior to the start of the study. The methods described below were used to measure ammonia and nitrite concentrations before shrimp were stocked, to ensure these compounds were not present at potentially dangerous levels and as an indication that nitrification was occurring in the MBBRs.

At the end of the 83 day nursery shrimp weighed a mean ± SEM of 2.6 ± 0.1 g. At this time, shrimp were hand-counted and placed into the 4 experimental tanks at a stocking density of 250 shrimp m⁻³ after an approximately 1.5 hour acclimation period. The tanks were 1.5 m³ in volume; however, the CW systems contained an additional 1.25 m³ water volume in the filtration systems and the plumbing for those systems, and the BF tanks had an additional 15 L in the settling chambers. The total system volume in each system was taken into consideration when stocking shrimp: the CW tanks each received 688 shrimp and each BF tank received 378 shrimp. Shrimp were grown for 8 weeks in this study.
Shrimp were fed Zeigler Hyperintensive-35 (Zeigler™ Brothers Inc., Gardners, Pennsylvania, USA). Feeding was based on an estimated feed conversion ratio (weight of feed provided/shrimp population weight gain), which was calculated by estimating the shrimp population (assuming 10% stocking mortality then 1% mortality per week, along with routine sampling to check for uneaten feed and dead shrimp) and estimating growth rate. The feed conversion ratio (FCR) was multiplied by the expected weekly growth, which was then multiplied by the estimated shrimp population to arrive at a weekly feeding amount. These calculations were made for the CW tanks, and the quantity given to each CW tank was multiplied by 0.55 to arrive at the amount given to each BF tank. This was because the BF tanks were stocked with 55% the number of shrimp that the CW tanks were stocked with. The intention was to provide the same amount of feed per shrimp in the two systems so that differences in water quality and shrimp production would be due to system differences rather than disparity in the amount of feed provided. Feed was broadcast by hand 3 times per day to each tank. The same amount of feed was provided to each tank within a treatment.

Sucrose (Extra Fine Granulated Cane Sugar, Sysco® Corporation, Houston, TX, USA) was added to the BF tanks to raise the C:N ratio and stimulate heterotrophic bacterial N assimilation. Based on the total feed and sugar inputs, and the C and N concentrations of those items, the overall C:N ratio of inputs to the BF systems was 24.5:1. In response to low alkalinity concentrations in the CW systems, sodium bicarbonate was occasionally added to those tanks.

Twice daily, between 0800 and 0900, and again between 1500 and 1600 hr., temperature, pH, salinity, and dissolved oxygen (DO) were measured in each
experimental tank using a YSI Model 556 Handheld Instrument (YSI Incorporated, Yellow Springs, Ohio, USA). Ammonia concentration (mg TAN L\(^{-1}\)) was measured in each tank during weeks 0 (prior to shrimp stocking), 1, 4, 6, and 7 using Hach method 8155 (Hach Company 2003). Nitrite concentration was measured weeks 0, 1, 2, 3, 4, 5, and 7 using the spectrophotometric procedure outlined by Strickland and Parsons (1972). The concentration of NO\(_2\)-N plus NO\(_3\)-N was determined using the chemiluminescence detection described by Braman and Hendrix (1989), then NO\(_3\)-N was calculated by subtracting NO\(_2\)-N concentration; this was performed weeks 0, 2, 4, and 7. During weeks 1, 4, 5, 6, and 7 concentrations of total suspended solids (TSS) and volatile suspended solids (VSS) were measured in each tank by following ESS Method 340.2 (ESS 1993). During weeks 1, 4, and 7 turbidity was measured in Nephelometric Turbidity Units (NTU) using a Micro 100 Turbidimeter (HF Scientific, Fort Myers, Florida, USA). Also during weeks 1, 4, and 7 alkalinity was measured following the Potentiometric Titration to Preselected pH procedure outlined in section 2320 B by the APHA (2005).

At the time of harvest 1 L of water was collected from each BF tank and centrifuged, 3 shrimp were collected from each of the 4 experimental tanks, and feed and sucrose samples were obtained. Centrifuged biofloc and shrimp were dried, ground, acid washed with 10% HCl to separate organic carbon from carbonate carbon, and thoroughly rinsed. All samples were then completely dried, finely ground, and homogenized prior to analysis. The 3 shrimp from each tank were analyzed as composite samples, representative of each respective tank. Triplicate samples were placed into a Costech Model 4010 Elemental Combustion System which combusted the samples and supplied gaseous C and N (as CO\(_2\) and N\(_2\)) from the samples to a Thermo, Delta V Advantage
Isotope Ratio Mass Spectrometer (Thermo Scientific, Bremen, Germany). The Costech Model 4010 generated %C and %N values, and the mass spectrometer generated $\delta^{13}C$ and $\delta^{15}N$ values, calculated using the following equation:

$$\delta = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$

where $R$ = the ratio of heavy over light isotopes, in this case $^{13}C/^{12}C$ or $^{15}N/^{14}N$. The standard used for C was Pee Dee Belemnite, and that used for N was atmospheric N.

**Study 2**

At the end of the experiment described in Chapter II, also described by Ray et al. (2011b), 2 shrimp were randomly taken from each of the 7 experimental raceways. For each pair of shrimp removed from a raceway, the cephalothorax was removed and discarded from 1 shrimp and the other shrimp remained whole. The whole shrimp and the shrimp abdomens (tails) were then dried and ground, acid washed with 10% HCl, rinsed thoroughly, dried thoroughly, and re-ground and homogenized. The shrimp were then analyzed for %C and %N, as well as C and N stable isotope levels, using the methods described for Study 1.

**Data Management and Analysis**

Water quality and shrimp production data collected from Study 1 are not analyzed statistically. Although it may be possible to detect significant differences in some cases, due to the small number of replicates the chance of committing a type I error is unacceptably high. Instead, data are represented in figures and tables to help convey trends. Using data from replicate tanks and triplicate methodical replicates, the %C, %N, $\delta^{13}C$, and $\delta^{15}N$ values between the two treatments were compared using two-sample t-tests.
In the clear water treatment of Study 1 it was assumed that applied feed was the only source of C and N to shrimp. Based on this assumption, fractionation factors ($\Delta$) were calculated using the following equation (Fry 2006).

$$\Delta = \delta_{SOURCE} - \delta_{PRODUCT}$$

Where the source is the pelleted shrimp feed and the product is the shrimp; $\Delta$ values are expressed in positive permil (‰) units.

In the biofloc treatment of Study 1 the $\delta^{13}$C and $\delta^{15}$N fractionation factors calculated above were subtracted from the raw $\delta^{13}$C and $\delta^{15}$N values for shrimp to account for isotopic fractionation between food source and shrimp. The following 2 equations were then used to estimate the relative amount of C and N contributed to shrimp by potential food sources (Fry 2006).

$$f_1 = (\delta_{SAMPLE} - \delta_{SOURCE2})/(\delta_{SOURCE1} - \delta_{SOURCE2})$$

$$f_2 = 1 - f_1$$

Where $f_1$ is the fraction of C or N contributed by source 1, and $f_2$ is the fraction contributed by source 2. The sample is shrimp and source 1 was feed and source 2 was biofloc. The use of these equations assumes that there are only 2 sources of C and N available to shrimp; feed and suspended matter (biofloc).

In Study 2, two sample t-tests were used to test for differences between the mean C and N isotope levels in whole shrimp versus shrimp tails. Percent C and N data did not fit normal distributions; therefore, Kolmogorov-Smirnov non-parametric tests were used to test for differences in %C and %N between whole shrimp and shrimp tails, following an arc sine transformation of the data.
Results

Study 1

Temperature, DO, pH, and salinity (Table 5) all remained within acceptable ranges for the growth of *L. vannamei* (Clifford 1985). The concentration of DO was high at times in the BF tanks due to pure oxygen injection.

Table 5.

*The general water quality parameters (mean ± SEM) measured twice daily during Study 1.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CW</th>
<th>BF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>29.9 ± 0.1 (25.5-32.8)</td>
<td>29.6 ± 0.1 (26.7-30.7)</td>
</tr>
<tr>
<td>PM</td>
<td>29.2 ± 0.1 (26.7-31.6)</td>
<td>29.6 ± 0.1 (27.1-30.6)</td>
</tr>
<tr>
<td>Dissolved Oxygen (mg L⁻¹)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>7.5 ± 0.1 (5.8-8.9)</td>
<td>8.2 ± 0.3 (5.5-21.0)</td>
</tr>
<tr>
<td>PM</td>
<td>7.5 ± 0.1 (3.5-8.8)</td>
<td>7.4 ± 0.1 (5.0-13.1)</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>7.8 ± 0.0 (7.1-8.1)</td>
<td>8.1 ± 0.0 (7.5-8.5)</td>
</tr>
<tr>
<td>PM</td>
<td>7.7 ± 0.0 (7.1-8.1)</td>
<td>7.8 ± 0.1 (3.9-8.5)</td>
</tr>
<tr>
<td>Salinity (‰)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20.4 ± 0.0 (19.4-21.3)</td>
<td>20.6 ± 0.1 (19.3-22.7)</td>
</tr>
</tbody>
</table>

The concentration of ammonia became elevated in the BF tanks during the last several weeks of the study, but was never a concern in the CW tanks (Figure 9a). During week 5 the ammonia concentration was measured at 1.7 mg TAN L⁻¹ in one of the BF tanks, during week 6 ammonia reached 1.8 mg TAN L⁻¹ in the other BF tank, and ammonia concentration was again over 1 mg TAN L⁻¹ in both tanks week 7. During
week 2 the concentration of nitrite rose in the BF tanks, it was still high in week 3 and had subsided to low concentrations by week 4 (Figure 9b). Nitrite concentrations never became elevated to a concerning degree in the CW treatment. Nitrate concentrations continually increased in the CW treatment (Figure 9c), but declined in the BF treatment and nitrate was below detection (< 0.01 mg L\(^{-1}\)) in 1 of the BF tanks during the last 2 sample dates (Table 6).

*Figure 9.* The concentrations of ammonia (a), nitrite (b), and nitrate (c) during Study 1. Data points are treatment means, and error bars are 1 SEM around the mean.
Table 6.

The water quality parameters during Study 1. Data are reported as mean ± SEM.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CW</th>
<th>BF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia (mg TAN L(^{-1}))</td>
<td>0.2 ± 0.0 (0.0-0.4)</td>
<td>0.6 ± 0.2 (0.0-1.8)</td>
</tr>
<tr>
<td>Nitrite (mg NO(_2)-N L(^{-1}))</td>
<td>0.1 ± 0.0 (0.0-0.7)</td>
<td>6.1 ± 2.2 (0.0-26.7)</td>
</tr>
<tr>
<td>Nitrate (mg NO(_3)-N L(^{-1}))</td>
<td>96 ± 14 (45-166)</td>
<td>38 ± 12 (0-80)</td>
</tr>
<tr>
<td>TSS (mg TSS L(^{-1}))</td>
<td>105 ± 13 (60-190)</td>
<td>288 ± 50 (185-560)</td>
</tr>
<tr>
<td>VSS (mg VSS L(^{-1}))</td>
<td>20 ± 5 (0-50)</td>
<td>216 ± 51 (80-490)</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO(_3) L(^{-1}))</td>
<td>87 ± 12 (61-135)</td>
<td>330 ± 61 (128-482)</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>0.7 ± 0.2 (0.1-1.4)</td>
<td>106.8 ± 48.0 (19.1-301.3)</td>
</tr>
</tbody>
</table>

The concentrations of TSS, VSS, and alkalinity were substantially lower in the CW treatment than the BF treatment (Table 6). Turbidity was much lower in the CW treatment than the BF treatment (Table 6).

There was no significant difference (P = 0.460) in the concentration of carbon between shrimp in the CW and BF treatments (Table 7). However, the concentration of nitrogen, and both the \(\delta^{13}C\) and \(\delta^{15}N\) values of shrimp (uncorrected) were significantly greater (P ≤ 0.02) in the BF treatment versus the CW treatment (Table 7). Based on data from the CW treatment, the fractionation value (\(\Delta\)) for C was calculated to be 1.3‰, and that of N was calculated to be 2.1‰. These were subtracted from the \(\delta^{13}C\) and \(\delta^{15}N\) values for shrimp in the BF treatment to account for trophic fractionation. Mean corrected \(\delta^{13}C\) and \(\delta^{15}N\) values for shrimp in the BF treatment were -21.5 and 9.1‰, respectively (Table 7).
Table 7.

Carbon and nitrogen dynamics during Study 1. Different superscript letters in a column indicate significant differences (P ≤ 0.02) between shrimp from the CW treatment versus the BF treatment.

<table>
<thead>
<tr>
<th></th>
<th>%C</th>
<th>δ^{13}C</th>
<th>Δδ^{13}C</th>
<th>δ^{13}C - ∆</th>
<th>%N</th>
<th>δ^{15}N</th>
<th>Δδ^{15}N</th>
<th>δ^{15}N - ∆</th>
</tr>
</thead>
<tbody>
<tr>
<td>CW Shrimp</td>
<td>49.9 ± 0.6</td>
<td>-21.8 ± 0.1</td>
<td>1.3</td>
<td>11.4 ± 0.1a</td>
<td>9.1 ± 0.0a</td>
<td>2.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BF Shrimp</td>
<td>49.4 ± 0.3</td>
<td>-20.2 ± 0.1b</td>
<td>-21.5 ± 0.1</td>
<td>11.9 ± 0.1b</td>
<td>11.2 ± 0.3b</td>
<td>9.1 ± 0.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feed</td>
<td>40.3 ± 1.9</td>
<td>-23.1 ± 0.1</td>
<td></td>
<td>5.6 ± 0.3</td>
<td>7.1 ± 0.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biofloc</td>
<td>44.2 ± 0.3</td>
<td>-17.4 ± 0.3</td>
<td></td>
<td>7.7 ± 0.1</td>
<td>10.6 ± 0.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sucrose</td>
<td>41.0 ± 0.2</td>
<td>-12.2 ± 0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The fractionation-corrected δ^{13}C and δ^{15}N values for shrimp fall between the isotope levels for feed and biofloc, indicating that shrimp may have received C and N from both sources (Figure 10). The C isotope level of the biofloc lies between that of the feed and the sucrose, indicating that biofloc may consist of C originating from both sources (Figure 10).

Figure 10. Isotope levels for shrimp (corrected for fractionation), biofloc, feed, and sucrose (vertical, dashed line) collected from the BF treatment of Study 1. Data points represent mean values and error bars are 1 SEM.

In shrimp from the BF treatment the fraction of C estimated to originate from the feed ($f_1$) was 72.3%, and 27.7% was estimated to originate from the biofloc ($f_2$) (Table 8). The estimated N contribution from feed was 41.5% and that from biofloc was 58.5%.
Table 8.

Estimated fractions ($f$) of C and N originating from the 2 potential food items; $f_1$ is the fraction originating from pelleted feed and $f_2$ is the fraction originating from biofloc.

<table>
<thead>
<tr>
<th></th>
<th>$f_1$</th>
<th>$f_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbon</td>
<td>72.3%</td>
<td>27.7%</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>41.5%</td>
<td>58.5%</td>
</tr>
</tbody>
</table>

Mean ± SEM weekly shrimp growth rate was 0.7 ± 0.1 g wk$^{-1}$ in both the CW treatment and the BF treatment. Survival was 61 ± 0.0% in the CW treatment and 43 ± 14% in the BF treatment (57% in a tank and 28% in the other). The feed conversion ratio (FCR) of shrimp in the CW treatment was 1.8 ± 0.2. The FCR was 3.0 ± 1.3 in the BF treatment (1.8 and 4.3 in the 2 BF tanks) because the tank with low survival was overfed.

Study 2

Shrimp abdomens (tails) were found to have significantly ($P = 0.000$) higher $\delta$ values for both $^{13}$C and $^{15}$N, indicating a relative enrichment of heavy isotopes in the tail muscle (Table 9). The $^{13}$C values were 5% higher in shrimp tails than in the whole body, and $^{15}$N values were 14% higher in tails. There was no significant difference ($P = 0.526$) between %C in whole shrimp versus tails. However, there was a significantly ($P = 0.000$) higher concentration of nitrogen in shrimp tails versus whole shrimp.
Table 9.

Percent C and N, and isotope levels of whole shrimp and shrimp with the cephalothorax removed (shrimp tails). Different superscript letters in a column indicate significant ($P < 0.001$) differences.

<table>
<thead>
<tr>
<th></th>
<th>%C</th>
<th>$\delta^{13}$C</th>
<th>%N</th>
<th>$\delta^{15}$N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Shrimp</td>
<td>48.4 ± 0.4</td>
<td>-22.2 ± 0.1$^a$</td>
<td>11.5 ± 0.3$^a$</td>
<td>7.7 ± 0.1$^a$</td>
</tr>
<tr>
<td>Tails Only</td>
<td>46.8 ± 1.4</td>
<td>-21.1 ± 0.1$^b$</td>
<td>13.1 ± 0.4$^b$</td>
<td>8.8 ± 0.1$^b$</td>
</tr>
</tbody>
</table>

Discussion

Study 1

Although ammonia (Li et al. 2007) and nitrite (Lin and Chen 2003) LC50 values are reported to be substantially higher than the concentrations recorded during this study, the levels reported in the BF treatment may have reduced survival or growth rates in those tanks. The BF tanks had elevated ammonia concentrations for at least 14 days and elevated nitrite concentrations for at least 7 days (Figure 9), considerably longer exposure times than standard toxicity trials. The highest nitrite concentration of 26.7 mg NO$_2$-N L$^{-1}$ was recorded in the BF tank that had the lowest shrimp survival of 28%. In contrast, ammonia and nitrite concentrations were never high in the CW tanks (Figure 9), likely due to the early establishment of nitrifying bacteria on the plastic media of the MBBR.

The continued function of nitrifying bacteria in the CW tanks is evidenced by the consistent accumulation of nitrate in those systems (Figure 9). In the BF tanks nitrate concentration decreased substantially, possibly due to bacterial or algal assimilation. Nitrate reduction in the BF treatment may also have been due to denitrification occurring in the settling chambers. Ray et al. (2011b) indicated that denitrification may occur in the settling chambers of biofloc systems. Furthermore, alkalinity concentration was substantially higher in the BF tanks, another potential indicator of denitrification.
Although nitrate is much less toxic than ammonia or nitrite, continued nitrate accumulation as a result of nitrification can hinder shrimp growth and survival (Kuhn et al. 2010).

Some TSS was detected in the CW treatment, although the water was clear. However, because very little of this material was VSS (Table 6) this indicates that the TSS measured was likely composed mostly of minerals which do not combust during the VSS measurement process. Although the filtered material for these analyses was rinsed with deionized water, it seems that some minerals remained on the filter and were weighed during the measurement of TSS in the CW tanks. A more accurate evaluation of the optical clarity of the water in this treatment is provided by the low turbidity values (Table 6). The TSS, VSS, and turbidity values in the BF tanks reached levels much higher than those recommended Ray et al. (2011b) to facilitate optimal shrimp production. This may have been a contributing factor to the low shrimp production values obtained during the current study.

Low turbidity values and observations that water was clear, with no obvious detritus or feces present, in the CW treatment indicate that pelleted feed was the only source of nutrition to shrimp in these tanks. The fractionation value calculated for $^{15}$N during this study (2.1‰) is slightly lower than that reported by Parker et al. (1989) who estimated a value of 2.4‰. This may be because the authors analyzed only shrimp tails which, according to the results of Study 2 in the current document, tend to have higher $\delta^{15}$N values. Parker et al. (1989) measured $^{13}$C fractionation values in the range of 1.3 to 1.6‰; the value calculated in Study 1 of 1.3‰ falls on the lowest end of this range.
When fractionation values are used to estimate the amount of isotopic fractionation that occurs between a food item and a consumer they are typically used with the assumption that the amount of fractionation will be the same regardless of dietary items (Gannes et al. 1997). However, this may not be the case; Anderson et al. (1987) demonstrated that different ingredients in shrimp feeds not only had diverse $\delta^{13}$C values, but also resulted in different apparent fractionation values. This may be due to the disparate utilization of various feed ingredients by shrimp. This issue could be complicated when shrimp are exposed to microbial assemblages. Kent et al. (2011) demonstrated that shrimp exposed to various types of algae were only able to consume certain types of cells, and of those cells consumed some were not digested. Nonetheless, a one source experiment as conducted for this study is likely the only practical way of estimating a fractionation factor for a study of this scale. Furthermore, this study shows what the values of shrimp isotopic levels are when shrimp consume a particular feed, comparing shrimp isotopic levels when shrimp are exposed to another potential food source in addition to that feed provides a useful comparison.

The significant differences between (uncorrected) C and N isotope levels in shrimp from the CW treatment versus the BF treatment indicate that shrimp from the two treatments likely utilized different sources of C and N.

If all of the C and N contained in shrimp tissues had originated from feed, it would be expected that the fractionation-corrected shrimp isotope values would be equal to those of the feed. However, as depicted in Figure 10, the corrected shrimp values fell between the two potential food sources. Based on estimates from a simple two source mixing model the feed contributed 72.3% of the C in shrimp and the biofloc contributed
27.7%. According to the model, 41.5% of the N in shrimp may have originated from the feed and 58.5% came from the biofloc. Differences in percent contributions between the two elements may be the result of disparity in the digestibility of components in the potential food sources (Schroeder 1983).

There is substantial variability among previous estimates of the contribution of microorganisms to shrimp nutrition using C and N isotopes in aquaculture systems. Most of this variability is likely associated with differences in system type and management (Epp et al. 2002). In a series of experiments Parker et al. (1989) calculated that between 44 and 86% of the C in shrimp came from natural biota in ponds stocked at 20 shrimp m$^{-2}$. These authors also estimated that pond biota contributed approximately 60% of the N in shrimp flesh. With shrimp stocked at 50 m$^{-2}$, Epp et al. (2002) estimated that shrimp attained 31% of N from the pond microbial community. In biofloc-based ponds, with shrimp stocked at 120 m$^{-2}$ Burford et al. (2004) estimated that shrimp attained 18 to 29% of their N from natural biota.

The estimates that biofloc microbes contributed 27.7% of the C and 58.5% of the N to shrimp in this study imply that operating biofloc systems in this manner can provide supplemental dietary items to shrimp, potentially reducing feed costs. However, shrimp production data do not reflect this. It is likely that the high ammonia and nitrite concentrations in the BF treatment led to the poor shrimp production results in that treatment. Low shrimp production in the CW treatment may have been the result of under-feeding. A strategy used in feeding shrimp for this study was to give both treatments the same amount of feed on a per shrimp stocked-basis. This was to help ensure that differences in water quality and shrimp production were not due to
differences in the amount of feed provided, but rather differences in the type of production system. However, uneaten feed was often observed in the BF tanks and the decision was made to lower the feeding rate, affecting shrimp in both treatments. Future studies similar to the current one should consider the contribution of suspended matter to shrimp nutrition in biofloc systems and lower feed additions to those tanks, while in clear water tanks with no additional nutrition available perhaps feed addition should increase.

Study 2

A higher concentration of N in shrimp tails is likely a result of higher muscle mass in that portion of the body. It is unclear why heavy isotope values were comparatively enriched in shrimp tails versus the entire body. However, Gamboa-Delgado et al. (2011) also found that $^{13}$C and $^{15}$N were relatively enriched in shrimp muscle tissue compared to whole bodies. These authors attributed their findings to differences in metabolic turnover rates between the two body segments, and preferential incorporation of amino acids in the muscle tissue.

When sampling small, larval or post-larval shrimp it is difficult to remove the head and isolate only abdominal segments. Conversely, processing large shrimp may pose logistical problems for some laboratories due to issues such as space limitations of drying apparatuses. The findings of this and previous studies suggest that consideration be given to whether whole shrimp or shrimp tails are used in isotope tracer projects.

The N fractionation estimated in this project was 14% lower than that estimated by Parker et al. (1989) (2.1 versus 2.4‰, respectively), the same percent difference found in δ$^{15}$N values between whole shrimp versus shrimp tails in this project. This suggests that sampling whole shrimp versus shrimp tails may explain the differences between the
current project in which whole shrimp were sampled, and that of Parker et al. (1989) during which tails were sampled.
CHAPTER IV
COMPARING CHEMOSYNTHETIC-BASED SYSTEMS AND THE USE OF THREE CARBOHYDRATES TO PROMOTE HETEROTROPHIC-DOMINATED BIOFLOC SHRIMP (*LITOPENAEUS VANNAMEI*) CULTURE SYSTEMS

Introduction

Biofloc aquaculture systems can be used for the intensive rearing of marine shrimp. These systems allow shrimp to be grown at high densities with little water exchange. High shrimp densities reduce the areal footprint of systems, but also necessitate high nutrient inputs through feed. Low water exchange reduces the need for salt water, conserves heat, and increases biosecurity. Unlike many recirculating systems, biofloc technology does not rely on external filters for biological filtration, but rather a dense microbial community, responsible for nutrient cycling, is allowed to develop in the water column. A portion of this community is contained on biofloc, small organic particles providing shelter, substrate, and access to nutrients for the microbes. It has been hypothesized that shrimp can consume these particles, thereby recycling nutrients and potentially reducing feed costs (Burford et al. 2004; Moss 1995; Wasielesky et al. 2006). However, the concentration of these particles must be controlled to optimize shrimp production (Ray et al. 2010a); often the only external filtration used in biofloc systems is a means of solids removal.

There are three general pathways for the remediation of ammonia in biofloc systems, as in many aquatic systems: algal assimilation, chemoautotrophic bacteria oxidation, and heterotrophic bacteria assimilation. These processes, as they apply to aquaculture, are reviewed in detail by Ebeling et al. (2006). In the presence of light and
nutrients algae are typically present in aquatic environments. Algae and other phototrophic microbes build proteins through assimilation of inorganic nitrogen, largely ammonia, in the water column. Algae are limited in the amount of nutrients they can assimilate, therefore intensive biofloc systems are typically dominated by bacteria in terms of ammonia remediation (Brune et al. 2003; Browdy et al. 2001).

Chemoautotrophic bacteria perform nitrification during which ammonia is oxidized to form the slightly less toxic nitrite compound, which is then oxidized to form nitrate. Nitrate is much less toxic than either of the preceding compounds; however, at high concentrations nitrate can reduce shrimp growth and survival (Kuhn et al. 2010). Often the process of nitrification can function reliably once it is established, but establishment can be inconsistent especially with respect to the oxidation of nitrite, which can lead to an accumulation of this toxic compound (Browdy et al. 2012; Ray et al. 2011b). Systems with high rates of nitrification require the regular addition of a carbonate buffer, as nitrifying bacteria consume carbonate. An advantage of chemoautotrophic nitrification is that this process typically consumes less oxygen than heterotrophic bacterial processes (Browdy et al. 2012).

Heterotrophic bacteria utilize organic carbon as an energy source and assimilate ammonia nitrogen to build cellular proteins. By adding carbohydrates to the water, especially carbohydrates that dissolve easily, heterotrophic assimilation can rapidly remove ammonia from a biofloc system (De Schryver et al. 2008). Avnimelech (1999) suggested that this process can be stimulated by maintaining a C:N ratio of inputs to the system above approximately 10:1. However, Ray et al. (2011b) found that a C:N ratio of 12.4:1 resulted in some increases of ammonia and nitrite in intensive shrimp biofloc
systems. Heterotrophic bacterial assimilation can consume dissolved oxygen (DO) at a high rate, and the bacterial population must expand to continually assimilate ammonia, leading to an accumulation of solids in the water column.

In the case of heterotrophically-dominated biofloc systems, the carbon source that is added may affect system function or animal production (De Schryver et al. 2012). Crab et al. (2010a) conducted a laboratory experiment using water with added ammonia, phosphate, and aquaculture filter backwash as nutrient sources. The authors then added either glucose, glycerol, or acetate as carbon sources to raise the C:N; differences in carbon source defined the three treatments in their experiment. Post-larval *Macrobrachium rosenbergii* shrimp were then grown in the biofloc-rich water. The authors found that the different carbon sources led to differences in protein, lipid, carbohydrate, acetic acid, and fatty acid compositions of the bioflocs, as well as differences in shrimp survival, with the glucose treatment resulting in significantly greater survival over the others.

System managers may choose a particular carbon source based on cost, availability, the rate at which it dissolves, the purity (carbon concentration of the substance), the effects on nutritional components in the biofloc, the effects on system function, or the effects on shrimp production. Sucrose is readily available in many markets due to its use in food products, it dissolves quickly in water, and it has been shown to successfully facilitate bacterial assimilation of nitrogen (Kuhn et al. 2009; Ray et al. 2011b). Molasses is a byproduct of the sucrose manufacturing process and is therefore less expensive than sucrose; the carbon concentration of molasses is much lower than sucrose, yet some authors suggest that molasses is an appropriate carbon
source to stimulate heterotrophic assimilation (Burford et al. 2004; Emerenciano et al. 2012). Glycerol is a byproduct of the biodiesel manufacturing process and biodiesel producers often seek methods for disposal or reuse of it (Thompson and He 2006). Although its use is not currently popular in biofloc aquaculture, glycerol has been shown to help generate potentially nutritious biofloc (Crab et al. 2010a) that may help protect animals from Vibrio harveyi bacterial infections (Crab et al. 2010b).

The purpose of this study was to evaluate the differences in system function and shrimp production between chemoautotrophic, nitrifying bacteria-dominated systems and heterotrophic bacteria-dominated systems that were established using either sucrose, molasses, or glycerol.

Methods

Equipment

For this study, 16, 500-L, fiberglass tanks were placed under a greenhouse structure. The dome-shaped greenhouse was covered with two layers of clear plastic sheeting. Each shrimp culture tank was aerated using four, 15 x 5 x 5 cm ceramic air diffusers receiving air from a 746 W regenerative blower (Sweetwater®, Aquatic Ecosystems Inc., Apopka, Florida, USA). Each tank also had a fine pore diffuser with a diffusing surface area of 129 cm² connected to a central, pressurized tank of oxygen gas. If dissolved oxygen (DO) concentration in a tank was found to be below 3 mg L⁻¹, the fine pore diffuser was placed into the water and oxygen gas was injected. This study was conducted at the University of Southern Mississippi’s Thad Cochran Marine Aquaculture Center (CMAC), a part of the Gulf Coast Research Laboratory, located in Ocean Springs, Mississippi, USA during the months of October through December. Because the study
was conducted during these colder, winter months the use of heaters was necessary. Each of the shrimp culture tanks had two 300 W, electric, submersible heaters.

Adjacent to each experimental tank was a 15-L container used as a settling chamber. A 15 x 5 x 5 cm ceramic air diffuser was placed in a 5 cm diameter, 20 cm long piece of polyvinyl chloride (PVC) pipe covered on the bottom by plastic screen with 0.3 cm mesh size, and connected on the top to a 1.9 cm diameter piece of PVC. The pipe and diffuser were placed in an experimental tank, the diffuser received blown air from the regenerative blower described above; this air lift mechanism propelled water up the 1.9 cm diameter pipe and into the top of a settling chamber. Inside the settling chamber, water traveled down a 5 cm diameter pipe causing velocity to slow and particles to settle out on the bottom of the chamber; relatively clear water at the top of the chamber flowed out of another 1.9 cm diameter pipe and back into the shrimp culture tank.

_Shrimp and Water Source_

Shrimp (_L. vannamei_) were originally obtained from Shrimp Improvement Systems, LLC (Islamorada, Florida, USA) as 12 day post-larvae. The shrimp were grown in an approximately 10 m$^3$ nursery raceway for 30 days until they weighed approximately 1 g. The shrimp were then used to stock a 50 m$^3$ raceway at approximately 100 shrimp m$^{-3}$. Salinity in the raceway was maintained at 16‰ and water was propelled around a central wall using a combination of air lift mechanisms and a pump delivering water to 18 Venturi nozzles, described in detail by Ray et al. (2011b). Shrimp in the raceway were fed Ziegler Hyperintensive-35 (Zeigler™ Brothers Inc., Gardners, Pennsylvania, USA). Shrimp were grown in the raceway for approximately 45 days prior to beginning the current study. All of the water and the shrimp used for the
current study were obtained from this raceway. This water was used because it contained biofloc (total suspended solids = 326 mg L\(^{-1}\)), and it contained nitrate (6.5 mg NO\(_3\)-N L\(^{-1}\)), indicating that the process of nitrification had been occurring. Shrimp weights were sampled just prior to stocking the current study using the techniques described by Ray et al. (2011a). These authors suggested sampling shrimp weights until the confidence bounds were within 5% of the cumulative mean weight. The mean ± SEM weight of shrimp at the time of stocking this study was 6.8 ± 0.2 g.

*Shrimp Culture*

Each shrimp culture tank received 500 L of water from the source raceway and 150, hand counted shrimp for a stocking density of 300 shrimp m\(^{-3}\). The bottom area of each tank was 1 m\(^2\), making the stocking density per area 150 shrimp m\(^{-2}\). Shrimp were grown for 56 days and fed Zeigler Hyperintensive-35. This diet was analyzed by Clemson University’s Agricultural Services Laboratory (Clemson, South Carolina, USA) and found to contain 33.4% crude protein, 10.4% fat, 8.6% moisture, and 6.6% ash. Feeding was based on an estimated feed conversion ratio (weight of feed provided/shrimp population weight gain), which was calculated by estimating the shrimp population (assuming 10% stocking mortality then 1% mortality per week, along with routine dip net sampling to check for uneaten feed and dead shrimp) and sampling individual weights periodically. The feed conversion ratio (FCR) was multiplied by the expected weekly growth based on previous experiments, which was then multiplied by the estimated shrimp population, to arrive at a weekly feeding amount. The feed was dispersed evenly into the tanks three times per day at uniformly spaced times of approximately 0730, 1200,
and 1630 h. Each feed portion was weighed on a digital balance, and each tank received
the same amount of feed during the study.

Experimental Design and Management

Four treatments were created for this project. One treatment (chemoautotrophic, abbreviated CA) was intended to facilitate the function of chemoautotrophic, nitrifying bacteria by adding only feed to the tanks. In the other three treatments the regular addition of carbohydrates was intended to encourage the assimilation of ammonia by heterotrophic bacteria. Sucrose (Extra Fine Granulated Cane Sugar, Sysco® Corporation, Houston, TX, USA) was added to one heterotrophic treatment (abbreviated HS). Molasses (Steen’s Pure and Natural Dark Molasses, C.S. Steen’s Syrup Mill, Inc., Abbeville, LA, USA) was added to another heterotrophic treatment (abbreviated HM). Glycerol (Glycerin 99P, Custom Formulating & Blending, Inc., Bristol, IN, USA) was added to the third heterotrophic treatment (abbreviated HG). Each of the 4 treatments was randomly assigned to 4 replicate tanks.

Each carbohydrate type was added to the heterotrophic tanks twice per day, between feedings. Each addition was weighed on a digital balance and dissolved directly into the water of the tanks. Initially, the intended C:N ratio of inputs (feed and carbohydrate) was 25:1; however, this was reduced by 15% to 21.25:1 during week 3, and reduced by 25% to 18.75:1 during week 5 of the experiment due to concerns about the over abundance of biofloc in the heterotrophic tanks. These C:N ratios are similar to those used by Xu and Pan (2012). Prior to the experiment, a precise measurement of the percent carbon for the carbohydrate sources was not available. Sucrose was thought to contain 42% C, based on prior measurements, molasses was assumed to contain 24% C,
based on published information (Samocha et al. 2007), and the chemical formula of glycerol ($C_3H_8O_3$) was used to calculate a percent C value of 39%. As feed rations were calculated each week, the amount of each carbohydrate needed to arrive at the intended C:N ratio was also calculated. After the experiment, each carbohydrate type was analyzed to determine more precisely the percent carbon using a Costech Elemental Combustion System model 4010 (Costech Analytical Technologies Inc. Valencia, CA, USA).

Settling chambers were operated based on turbidity measurements, as turbidity can be a predictor of suspended solids concentration (Hannouche et al. 2011). Using turbidity to determine when settling chambers should be operated was shown to be effective at maintaining consistent suspended solids concentrations by Ray et al. (2010a). Every morning at approximately 0800 h, beginning week 2, turbidity was measured in each shrimp culture tank using a Micro 100 Turbidimeter (HF Scientific, Fort Myers, Florida, USA). Turbidity was recorded in Nephelometric Turbidity Units (NTU), and when it was found to be greater than 150 NTU in a tank, that tank’s settling chamber was operated until 1700 h. Beginning week 3, if the turbidity was greater than 225 NTU in a tank, the corresponding settling chamber was operated all day and night until the next morning when turbidity was measured again. This extra solids removal effort was initiated because the turbidity and solids concentrations were increasing substantially in some tanks. Settling chambers were emptied weekly or when full to the point of returning suspended solids back to the shrimp culture tanks. Unless they were full, settling chambers were turned off and allowed to sit for approximately 10 minutes. In an effort to conserve water, the clear water at the top of the chambers was decanted back
into the shrimp culture tanks, and the thick material near the bottom was discarded. If the chambers were full, the entire volume was discarded. The volume of material removed from each settling chamber was recorded.

Each tank was managed to maintain a salinity of 16‰ and a volume of 500 L. To replace evaporation fresh, municipal water was added as needed and to replace the volume of water removed with settling chambers clean, artificial seawater was added. Artificial seawater was made with municipal water and a mixture of Fritz Super Salt Concentrate (Fritz Pet Products, Mesquite, Texas, USA) and sodium chloride (Morton® Purex® Salt, Morton® Salt, Chicago, Illinois, USA)

**Water Quality**

Twice daily, at approximately 0730 and 1600 h, temperature, dissolved oxygen (DO), pH, and salinity were measured in each tank using a YSI Model 556 Handheld Instrument (YSI Incorporated, Yellow Springs, Ohio, USA). Dissolved ammonia (measured as total ammonia nitrogen: TAN), nitrite (NO₂-N), nitrate (NO₃-N), and phosphate (reactive PO₄) were measured in each tank once per week. Ammonia was assessed using Hach method 8155 (Hach Company 2003) and NO₂-N was measured using the spectrophotometric procedure outlined by Strickland and Parsons (1972). Absorbance was measured at 655 nm for ammonia and 543 nm for nitrite using a Hach DR 3800 spectrophotometer (Hach Company, Loveland, Colorado, USA). The concentration of nitrite plus nitrate was determined using the chemiluminescence detection method described by Braman and Hendrix (1989), and then nitrate was calculated by subtracting nitrite concentration. This method of nitrate analysis resulted in a minimum detection limit of 0.1 mg NO₃-N L⁻¹. The concentration of phosphate was
measured using the PhosVer 3 method outlined in Hach Method 8048 (Hach Company 2003) and absorbance was measured at 890 nm using the Hach DR 3800 spectrophotometer.

Once per week the concentrations of alkalinity (as CaCO$_3$), total suspended solids (TSS), and volatile suspended solids (VSS) were measured in each tank. Alkalinity was measured following the Potentiometric Titration to Preselected pH procedure outlined in section 2320 B by the APHA (2005). TSS and VSS were measured using the procedures described in sections 2540 D and 2540 E by the APHA (2005). Five-day biochemical oxygen demand (BOD$_5$) was measured during weeks 4, 6, and 7, and settleable solids was measured weekly. BOD$_5$ was measured using the procedure described in section 5210 B by the APHA (2005), which includes a 5 day incubation period at 20ºC. Settleable solids was measured by allowing solids to settle for one hour in an Imhoff cone as described in Section 2540 F (APHA 2005).

Beginning the second week of the experiment, DO reduction was measured in the heterotrophic tanks after adding carbohydrate sources. The YSI 556 Handheld Instrument probe was placed in the water of a heterotrophic tank and the instrument was set to record DO concentration every 5 minutes. Recording was initiated just prior to adding a carbohydrate source such that the first DO reading was not influenced by the added carbohydrate source. The YSI 556 was allowed to record DO for as long as possible, although the actual length of time varied as the instrument was needed for other applications. DO depletion was measured for at least 60 minutes and as long as 315 minutes, although only up to 140 minutes is reported so that each treatment had at least 6 representative measurements. This process was repeated almost every time
carbohydrates were added, each tank was measured at least 4 times and as many as 6 times throughout the experiment. Tanks were labeled 1 through 16 and those receiving carbohydrates were subjected to the process in numerical order; at most, two tanks were included each day, as there was only 1 YSI 556 available for use. If pure oxygen was injected into a tank following carbohydrate addition, DO depletion was not measured for that tank.

Data Management and Analysis

The data reported in this document are presented as mean ± SEM, and in many cases the range is given in parentheses. The statistical software used to analyze data from this study was Systat Version 13 (Systat Software, Inc., Chicago, Illinois, USA). Weekly turbidity data were analyzed from the same day of each week that TSS, VSS, and settleable solids were measured because each of these parameters was used to make inferences about the concentration of suspended particles in the tanks. Turbidity, ammonia, nitrite, phosphate, alkalinity, TSS, VSS, settleable solids, and BOD data were all organized by treatment means over time. These data were then analyzed using a one-way, fixed model, repeated measures (RM) ANOVA with 4 levels representing the 4 treatments. Following each analysis a pairwise comparisons test between levels was performed to test for differences among treatments. The ammonia and nitrite data were transformed to meet the normality assumption of the ANOVA prior to analysis. Ammonia data were transformed by calculating the hyperbolic tangent followed by the $\log_{10}$ then the sine of each datum. Nitrite data were transformed by calculating the tangent followed by the absolute value then the hyperbolic tangent of each datum. Because most of the time nitrate was found to be below detection in the heterotrophic
tanks it was not possible to analyze nitrate data for all sample dates. Instead, the nitrate data for only the final sample date were analyzed using a Kruskal-Wallis non-parametric test. A non-parametric test was chosen because the data could not be transformed to fit a normal distribution.

To examine the relationship between VSS and alkalinity, least squares linear regressions were conducted comparing the two parameters in each treatment. In each case, VSS was designated as the independent variable and alkalinity the dependent variable. Data from the CA and HM treatments were log_{10} transformed to meet the normality assumption of the regression analysis.

For the DO depletion data in the heterotrophic treatments the percent decline was calculated based on the initial DO reading and the lowest DO concentration measured. These data were compiled for each treatment, arc sine transformed, then analyzed using the one-way RM ANOVA. The amount of time taken to reach the lowest observed DO concentration was calculated for each DO depletion measurement and organized by treatment. These data could not be transformed to meet the normality assumptions of an ANOVA, therefore a Wilcoxon Signed-Rank Test was used to analyze them based on the recommendations of Zimmerman and Zumbo (1993).

Mean weekly shrimp growth rate and final survival data were analyzed using a one-way, fixed model ANOVA with 4 levels. Survival data were arcsine square root transformed.

Results

During week 4, many dead shrimp were found in a HM treatment tank. The tank was emptied and 4 living shrimp, weighing a total of 23.8 g (wet weight) were found.
Dead shrimp weighed a total of 214.1 g. It is unclear what caused the mortality in this tank; however, low DO concentrations (< 3 mg O$_2$ L$^{-1}$) had been detected on several occasions, especially surrounding the addition of molasses to that tank. Although pure oxygen had been injected when low DO concentrations were measured, it is possible that the DO concentration dropped to a point of hypoxia, thereby killing most of the shrimp. Three days before the tank was drained, the nitrite concentration was 6 mg NO$_2$-N L$^{-1}$, which may have contributed to stress in the shrimp. Also, for 3 weeks the TSS concentration in this tank was higher than any other tank, reaching up to 950 mg TSS L$^{-1}$. Any of these factors, or a combination of them may have contributed to the mortality. Because this tank was removed from the project in the middle of the experiment, water quality and stable isotope data generated from the tank are not provided in the results of this study; however, shrimp production data from the tank are included in the results.

**C:N Management**

Sucrose contained 41% C, molasses contained 24% C, and glycerol contained 35% C. These percentages describe the carbon content of the carbohydrate sources in the form that they were weighed and added to the tanks, not based on dry weights. The feed contained 44.4 % C, 5.3% N, and 8.6% moisture (as determined by the Clemson Agricultural Services Laboratory, Clemson, SC, USA). Each shrimp culture tank received 1854 g of feed throughout the study. Because the carbohydrate sources differed in carbon content from what they were thought to contain at the beginning of the experiment, the C:N ratios differed slightly between treatments. Considering all of the feed and carbohydrate inputs throughout the study, the resulting C:N ratio of the HS
treatment was 24.4:1, that of the HM treatment was 20.4:1, and that of the HG treatment was 23.4:1.

**Water Quality**

Low temperatures were recorded during this study, especially low nighttime temperatures as reflected in the low end of the morning ranges (Table 10). This was especially pronounced during the 7th week of the study when mean morning water temperatures were below 20°C for four consecutive days. The DO concentration, pH, and salinity all remained within an acceptable range for the growth of *L. vannamei* (Clifford 1985).

**Table 10.** The water quality parameters measured twice daily during this study.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chemoautotrophic</th>
<th>Heterotrophic Sucrose</th>
<th>Heterotrophic Molasses</th>
<th>Heterotrophic Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (⁰C) AM</td>
<td>24.2 ± 0.3 (14.6-31.2)</td>
<td>25.0 ± 0.3 (15.8-30.1)</td>
<td>24.4 ± 0.3 (15.7-30.5)</td>
<td>24.5 ± 0.3 (15.8-29.7)</td>
</tr>
<tr>
<td>PM</td>
<td>26.9 ± 0.2 (19.1-31.9)</td>
<td>27.3 ± 0.2 (20.2-31.3)</td>
<td>27.1 ± 0.2 (20.0-31.7)</td>
<td>27.2 ± 0.2 (20.1-31.2)</td>
</tr>
<tr>
<td>Dissolved Oxygen (mg L⁻¹) AM</td>
<td>8.9 ± 0.1 (6.8-13.1)</td>
<td>8.4 ± 0.1 (6.7-11.6)</td>
<td>8.7 ± 0.1 (6.8-12.3)</td>
<td>8.3 ± 0.1 (6.4-11.5)</td>
</tr>
<tr>
<td>PM</td>
<td>7.9 ± 0.1 (6.5-11.8)</td>
<td>7.3 ± 0.1 (5.9-11.8)</td>
<td>7.6 ± 0.1 (5.5-13.2)</td>
<td>6.7 ± 0.1 (3.3-12.6)</td>
</tr>
<tr>
<td>pH AM</td>
<td>8.1 ± 0.0 (7.8-8.5)</td>
<td>8.3 ± 0.0 (7.6-8.6)</td>
<td>8.4 ± 0.0 (8.0-8.7)</td>
<td>8.2 ± 0.0 (7.9-8.5)</td>
</tr>
<tr>
<td>PM</td>
<td>8.3 ± 0.0 (7.8-8.6)</td>
<td>8.1 ± 0.0 (7.8-8.6)</td>
<td>8.3 ± 0.0 (7.8-8.6)</td>
<td>8.1 ± 0.0 (7.7-8.5)</td>
</tr>
<tr>
<td>Salinity (g L⁻¹) AM</td>
<td>16.4 ± 0.0 (15.4-17.5)</td>
<td>16.3 ± 0.0 (15.4-17.7)</td>
<td>16.4 ± 0.0 (15.6-17.6)</td>
<td>16.3 ± 0.0 (15.5-17.5)</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SEM (range).

Ammonia was present in the water prior to the beginning of the experiment, but at a relatively low concentration of 0.5 mg TAN L⁻¹. Ammonia concentration fluctuated in each treatment throughout the study (Figure 11a), but was significantly (P ≤ 0.03) higher in the HM treatment compared to the other 3 treatments (Table 11). Nitrite was highest in all treatments during the first week when mean nitrite concentrations reached 8.6, 3.4, 3.3, and 2.8 mg NO₂-N L⁻¹ in the CA, HS, HM, and HG treatments, respectively (Figure 11b). Mean nitrite concentration in the HM treatment increased to 3.2 mg NO₂-N L⁻¹
during week 5 of the study. There were no significant differences between treatments in terms of nitrite concentration. During week 1 of the study nitrate decreased to 0.2, 1.9, and 0.6 mg NO$_3$-N L$^{-1}$ in the HS, HM, and HG treatments, respectively. In the following weeks, nitrate was always below detection (< 0.01 mg NO$_3$-N L$^{-1}$) in the HS and HG treatments (Figure 11c). Relatively small concentrations of nitrate were detected in the HM treatment, up to 3.2 mg NO$_3$-N L$^{-1}$ in the 6$^{th}$ week of the study. Final nitrate concentration was significantly (P ≤ 0.01) greater in the CA treatment compared to all other treatments (Table 11).
Figure 11. Ammonia (a), nitrite (b), and nitrate (c) concentrations in the shrimp culture tanks throughout the study. Data points represent treatment means and error bars are one standard error around the mean.
Table 11.

The water quality parameters in the shrimp culture tanks measured during the study, the amount of material removed through settling chambers, and heterotrophic dissolved oxygen (DO) reduction characteristics.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Chemoautotrophic</th>
<th>Heterotrophic-Sucrose</th>
<th>Heterotrophic-Molasses</th>
<th>Heterotrophic-Glycerol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia (mg TAN L(^{-1}))</td>
<td>0.2 ± 0.0 (0.1 - 1.1(^a))</td>
<td>0.4 ± 0.1 (0.1 - 1.4(^a))</td>
<td>1.1 ± 0.2 (0.1 - 3.1(^b))</td>
<td>0.3 ± 0.0 (0.1 - 0.8(^a))</td>
</tr>
<tr>
<td>Nitrite (mg NO(_2)-N L(^{-1}))</td>
<td>1.5 ± 0.6 (0.0 - 9.2)</td>
<td>0.3 ± 0.2 (0.0 - 3.6)</td>
<td>1.2 ± 0.3 (0.0 - 4.3)</td>
<td>0.2 ± 0.2 (0.0 - 3.3)</td>
</tr>
<tr>
<td>Nitrate (mg NO(_3)-N L(^{-1}))</td>
<td>90.8 ± 10.0 (7.4 - 190.5(^a))</td>
<td>0.0 ± 0.0 (0.0 - 0.7(^b))</td>
<td>1.4 ± 0.6 (0.0 - 9.7(^b))</td>
<td>0.0 ± 0.1 (0.0 - 2.0(^b))</td>
</tr>
<tr>
<td>Phosphate (mg PO(_4)-L(^{-1}))</td>
<td>62.4 ± 2.4 (34.9 - 81.2(^a))</td>
<td>31.9 ± 1.3 (16.7 - 44.8(^b))</td>
<td>35.5 ± 2.4 (21.6 - 65.8(^b))</td>
<td>30.0 ± 1.7 (11.9 - 47.0(^b))</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO(_3) L(^{-1}))</td>
<td>169 ± 16 (72 - 367(^a))</td>
<td>276 ± 14 (187 - 424(^b))</td>
<td>352 ± 25 (208 - 590(^b))</td>
<td>279 ± 13 (192 - 425(^b))</td>
</tr>
<tr>
<td>BOD (mg BOD L(^{-1}))</td>
<td>134 ± 11 (70 - 204(^a))</td>
<td>387 ± 37 (226 - 650(^b))</td>
<td>241 ± 19 (148 - 327(^b))</td>
<td>301 ± 25 (156 - 440(^b))</td>
</tr>
<tr>
<td>TSS (mg L(^{-1}))</td>
<td>450 ± 28 (245 - 850(^a))</td>
<td>631 ± 46 (125 - 1325(^b))</td>
<td>570 ± 44 (235 - 1000(^b))</td>
<td>591 ± 40 (100 - 925(^b))</td>
</tr>
<tr>
<td>VSS (mg L(^{-1}))</td>
<td>342 ± 22 (175 - 625(^a))</td>
<td>512 ± 39 (110 - 950(^b))</td>
<td>467 ± 37 (175 - 875(^b))</td>
<td>506 ± 39 (85 - 800(^b))</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>121 ± 4 (75 - 155(^a))</td>
<td>229 ± 18 (85 - 509(^b))</td>
<td>146 ± 14 (48 - 322(^b))</td>
<td>189 ± 14 (34 - 359(^b))</td>
</tr>
<tr>
<td>Settleable Solids (mL L(^{-1}))</td>
<td>37 ± 3 (3 - 80)</td>
<td>115 ± 30 (10 - 700)</td>
<td>47 ± 6 (14 - 120)</td>
<td>64 ± 16 (1 - 475)</td>
</tr>
<tr>
<td>Removed Material (L)</td>
<td>26 ± 4 (17 - 33(^a))</td>
<td>79 ± 13 (55 - 110(^b))</td>
<td>63 ± 4 (59 - 70(^b))</td>
<td>74 ± 10 (55 - 95(^b))</td>
</tr>
<tr>
<td>DO Decline (%)</td>
<td>N/A</td>
<td>28.5 ± 0.0 (1.9 - 49.9)</td>
<td>27.7 ± 0.0 (16.3 - 41.8)</td>
<td>28.4 ± 0.1 (1.6 - 65.9)</td>
</tr>
<tr>
<td>DO Decline Time (minutes)</td>
<td>N/A</td>
<td>47.1 ± 5.9 (15 - 105(^a))</td>
<td>26.9 ± 6.3 (10 - 125(^b))</td>
<td>75.0 ± 9.2 (25 - 135(^a))</td>
</tr>
</tbody>
</table>

Data are reported as mean ± SEM (range), different superscript letters within a row indicate significant (P ≤ 0.05) differences between treatments.

The initial concentration of phosphate was 29.6 mg PO\(_4\) L\(^{-1}\). Phosphate concentration fluctuated and increased overall in the CA treatment (Figure 12a).

Phosphate concentration also fluctuated in the heterotrophic treatments, but the mean concentration over time in those treatments was not substantially greater than the starting concentration (Table 11). Phosphate concentration was significantly greater in the CA treatment versus all other treatments (P ≤ 0.01).
The concentration of dissolved orthophosphate (a) and alkalinity (b) over time in the shrimp culture tanks during the study. Data points represent treatment means and error bars are one standard error around the mean.

The initial concentration of alkalinity was 301 mg CaCO$_3$ L$^{-1}$. Alkalinity decreased substantially in the CA treatment (Figure 12b). Because alkalinity concentrations were below 120 mg CaCO$_3$ L$^{-1}$, 50 g of sodium bicarbonate was added to each CA treatment tank during week 3 and 100 g was added during weeks 4 and 8. Alkalinity concentration was significantly greater in the HM treatment versus any other treatment ($P \leq 0.01$). There was no significant difference in alkalinity concentration between the HS and HG treatments, although both had significantly greater ($P \leq 0.01$) alkalinity concentration than the CA treatment (Table 11).

The initial TSS concentration was 326 mg TSS L$^{-1}$, and VSS began at 251 mg VSS L$^{-1}$. Both of these parameters fluctuated throughout the study (Figure 13), and both
were significantly (P ≤ 0.02) lower in the CA treatment compared to all other treatments (Table 11). This is according to the results of a RM ANOVA which considers all data points. The final concentrations of TSS and VSS in the CA treatment were not substantially different than those of the heterotrophic treatments aside from HS (Figure 13).
Figure 13. The concentration of total suspended solids (a), volatile suspended solids (b), settleable solids (c), and the turbidity (d) of water in the shrimp culture tanks during the study. Data points represent treatment means and error bars are 1 standard error around the mean.
The VSS concentrations were good predictors of alkalinity in each treatment. The concentration of VSS was inversely related to the concentration of alkalinity (Figure 14). This relationship was especially strong in the HM and HG treatments, as indicated by higher R-square values and lower probability values resulting from a linear regression analysis. The TSS, settleable solids, and turbidity values were not significant predictors of alkalinity concentration.
Figure 14. Linear regression plots of VSS versus alkalinity in the chemoautotrophic (a), heterotrophic-sucrose (b), heterotrophic-molasses (c), and heterotrophic-glycerol (d) treatments.
The initial settleable solids concentration was 4 ml L\(^{-1}\). Occasionally, water contained buoyant solids that would not settle in the Imhoff cones used to measure settleable solids. There was a pronounced increase of settleable solids in 2 of the HS treatment tanks and 1 of the HG tanks during the last 4 weeks of the study. There were no significant differences in settleable solids concentrations between any of the treatments (Table 11), likely due to high variability within treatments (Figure 13c).

The initial turbidity was 90 NTU. Turbidity then increased substantially in the heterotrophic treatments, and subsequently decreased after settling chambers began to be operated overnight in tanks with a morning turbidity of 225 NTU or more (Figure 13d). In the HS treatment mean turbidity rose substantially again during week 6 and, similar to settleable solids measurements, the variability between tanks increased at this time. However, turbidity decreased in the subsequent weeks and mean settleable solids concentration continued to rise.

The amount of material removed with the settling chambers, using turbidity measurements to guide their operation, was significantly greater (P ≤ 0.01) in the HS and HG treatments compared to the HM and CA treatments (Table 11).

The BOD\(_5\) was relatively consistent over time in each treatment (Figure 15). BOD\(_5\) was significantly greater (P ≤ 0.04) in the HS and HG treatments versus the other treatments, and BOD\(_5\) was significantly greater in the HM treatment versus the CA treatment (Table 11).
Figure 15. The five day biochemical oxygen demand (BOD$_5$) in each treatment over time. Data points represent treatment means and error bars are one standard error around the mean.

**Heterotrophic Oxygen Reduction**

The addition of each carbohydrate led to a substantial decline in DO concentration (Figure 16). The percent decline in DO concentration, based on the difference between the initial concentration prior to carbohydrate addition and the lowest concentration measured during 140 minutes, was similar among the heterotrophic treatments. There was no significant difference between treatments ($P \geq 0.476$) with respect to the percent decline in DO concentration (Table 11). However, the amount of time it took to reach the lowest measured DO concentration was significantly lower ($P \leq 0.03$) in the HM treatment versus the HS and HG treatments (Table 11).
Figure 16. Dissolved oxygen concentration in the heterotrophic tanks just before carbohydrate addition (time 0) and measured every 5 minutes after that addition, up to 140 minutes. Data points represent treatment means and error bars are 1 standard error around the mean. Each data point is representative of at least 6 observations.

Shrimp Production

Shrimp production was low, possibly due to the cold temperatures during this study. Weekly mean ± SEM shrimp growth rate was 0.7 ± 0.1 g wk$^{-1}$ in the CA treatment, 0.7 ± 0.0 g wk$^{-1}$ in the HS treatment, 0.2 ± 0.1 g wk$^{-1}$ in the HM treatment, and 0.6 g wk$^{-1}$ in the HG treatment. There were no significant differences (P > 0.05) in
growth rate between the CA, HS, or HG treatments, but growth rate in the HM treatment was significantly (P < 0.05) lower than the CA and HS treatments (Figure 17a).

Figure 17. Mean shrimp growth rate (a) and survival (b) for each treatment: chemoautotrophic (CA), heterotrophic-sucrose (HS), heterotrophic molasses (HM), and heterotrophic glycerol (HG). Error bars are 1 standard error around the mean; different letters signify significant (P < 0.05) differences between treatments.

Mean ± SEM shrimp survival was 45.2 ± 3.6% in the CA treatment, 53.2 ± 8.9% in the HS treatment, 16.8 ± 6.9% in the HM treatment, and 49.2 ± 7.3% in the HG treatment (Figure 17b). There were no significant differences (P > 0.05) in survival between the CA, HS, and HG treatments, but survival was significantly lower (P < 0.05) in the HM treatment compared to the CA treatment.
Discussion

*C:N Ratio*

The C:N ratios of the heterotrophic treatments were intentionally maintained at a level higher than that recommended by Avnimelech (1999). The high ratios were intended to ensure the domination of heterotrophic bacteria in those systems, and were comparable to that of Xu and Pan (2012) who used a C:N ratio of 20:1.

The C:N ratios of the HS and HG treatments were similar at 24.4:1 and 23.4:1, respectively. The HM treatment had a C:N ratio of inputs that was somewhat lower at 20.4:1. This is still well above the published minimum for heterotrophic domination (Avnimelech, 1999).

*Temperature*

Low temperature was a problem in this study. Clifford (1985) reported that temperature in the range of 12-15° C can be lethal to shrimp. During this study morning water temperatures of 15° C were recorded (Table 10). Nighttime temperatures may have been lower than this because temperature was not measured until after sunrise. These low temperatures likely contributed to the relatively low growth rates and the low survival during the study. The small, 500-L tanks were susceptible to diurnal temperature swings as well, perhaps further hindering shrimp growth.

*Chemoautotrophic (CA) Treatment*

There was a small increase in ammonia concentration during week 2 (0.8 mg TAN L⁻¹) in the CA treatment. This was no higher than increases found in any other treatment, but could have caused some mortality. The increase in ammonia followed a relatively high concentration of nitrite (8.6 mg NO₂-N L⁻¹) during week 1. This
concentration is above the safe level (6.1 mg NO$_2$-N L$^{-1}$ at 15% salinity and a pH of 8.0) recommended by Lin and Chen (2003). There may have been some mortality in CA tanks during the time that nitrite concentrations were elevated. A spike in nitrite such as this can be a common occurrence in aquaculture systems that rely on nitrification to cycle nitrogen (Cheng and Chen 2002; Wang et al. 2004); this is one of the disadvantages of managing a chemoautotrophically-driven system. Nitrite oxidizing bacteria may be slower to establish an effective population than ammonia oxidizing bacteria resulting in a nitrite spike (Browdy et al. 2012).

Nitrate increased substantially in the CA treatment to a peak mean concentration of 162 mg NO$_3$-N L$^{-1}$. This signifies that nitrification proceeded as intended in this treatment. Although nitrate is much less toxic to shrimp than either ammonia or nitrite, elevated nitrate concentrations have been shown to hinder *L. vannamei* survival and growth (Kuhn et al. 2010). Tsai and Chen (2002) recommended a safe concentration for the culture of *Penaeus monodon* to be 145 mg NO$_3$-N L$^{-1}$ at 15% salinity.

Phosphate increased over time in the CA treatment (Figure 12a), which is a normal finding as phosphorus from feed is continually introduced to the water (ex. Ray et al. 2010a; Ray et al. 2011b). Alkalinity decreased over time in the CA treatment tanks (Figure 12b) likely due to the consumption of carbonate by chemoautotrophic bacteria (Ebeling et al. 2006). Although sodium bicarbonate was added to the CA tanks, alkalinity was significantly lower in this treatment compared to the heterotrophic treatments.

Both TSS and VSS were significantly lower in the CA treatment, indicating a lower concentration of biofloc suspended in the water column of this treatment versus the
heterotrophic treatments. The lower concentration of biofloc likely signifies that a lower abundance of the microbes associated with those particles would be found in CA tanks. Ray et al. (2010b) found that removing biofloc from culture systems reduced the abundance of bacteria, cyanobacteria, rotifers, and nematodes. A lower abundance of such organisms, all of which consume oxygen in the dark, may be a contributing factor to the lower BOD$_5$ in the CA treatment. The mean concentration of settleable solids was lower in the CA treatment than the other treatments, although there were no significant differences between any of the treatments (Table 11). The high level of variability in settleable solids measurements likely prevented the detection of significant differences between treatments. It is unclear what led to this variability; however, notable differences in the buoyancy of particles were observed. These differences in buoyancy did not seem to correspond to particular treatments, and may have contributed to the variability.

Turbidity was significantly lower in the CA treatment compared to the HS and HG treatments; however, no significant difference was found between the CA and HM treatments. Correlations between turbidity and suspended solids are rare because characteristics of particles such as shape and size affect the turbidity measurement (APHA 2005). Although there were no statistical relationships between treatments with respect to settleable solids concentrations, the CA and HM treatments had similar mean concentrations at 37 and 47 ml L$^{-1}$, respectively (Table 11). The weight of particles, measured as TSS, was different between CA and HM treatments, but the turbidity and settling solids concentrations were similar. This may indicate that although the weight of
solids was different, some physical characteristics of the solids in these two treatments were similar.

The fact that BOD₅ was significantly lower in the CA treatment compared to the other treatments indicates that microbial respiration was lower in the CA treatment. In intensive biofloc systems the oxygen demand of the microbial community contained in the water column can at times equal that of the cultured animals (Browdy et al. 2012). Injection of pure oxygen to overcome these oxygen demands can represent a substantial operating cost. Therefore, managing the microbial community in a fashion that consumes less oxygen, such as the CA treatment in this case, may present an economical advantage.

**Heterotrophic Treatments**

Each of the heterotrophic treatments had an increase of ammonia and nitrite concentrations during week 1 of the study (Figure 1). During this time ammonia concentration was greatest in the HS treatment, followed by the HM treatment, and lastly the HG treatment. The concentration of nitrite was similar among all of the heterotrophic treatments during this time. This increase in inorganic nitrogen compounds may be indicative of an establishment period for heterotrophic bacterial function. The water originally brought into the culture tanks had not received any carbohydrate additions to stimulate heterotrophic assimilation prior to the beginning of the study. Feed and carbohydrate additions began when shrimp were placed into the water. The bacterial community may have taken some time to become established, either in abundance or in function, when this shift in system management took place. This event may be similar in causation to that of the spike in nitrite concentration in the CA treatment, where nitrite oxidizing bacteria did not adequately respond to the increase of nutrient inputs.
In subsequent weeks, the concentrations of ammonia and nitrite increased substantially in the HM treatment (Figure 11). At times there were relatively high concentrations of ammonia and nitrite simultaneously, posing a potential synergistically detrimental effect on shrimp.

One of the most discernible differences between the CA treatment and the heterotrophic treatments was that of nitrate concentration (Figure 11c). Nitrate was either very low or below detection most of the time in the heterotrophic treatments. This signifies that the dominant nitrogen pathway in those treatments was not nitrification. It is possible that some photosynthetic assimilation of nitrogen occurred in the treatments designed to be heterotrophic. However, Brune et al. (2003) indicated that high-rate algal assimilation could account for 10-12 g C removal m$^{-2}$ day$^{-1}$, after which systems should shift to bacterial domination (Avnimelech 1999). The heterotrophic systems in this study received between 34 and 40 g of C m$^{-2}$ day$^{-1}$ on average. Furthermore, there were no signs of algal proliferation or function in the heterotrophic treatments. Water in the HM treatment appeared dark brown, and water in the HS and HG treatments had a light brown appearance. The DO concentrations and pH values were consistently lower in the afternoon than the morning in the heterotrophic treatments (Table 10), which suggests net cellular respiration.

While phosphate concentration increased over time in the CA treatment, it decreased slightly in the heterotrophic treatments (Figure 12a). Initial phosphate concentration was 29.6 mg PO$_4$ L$^{-1}$, and the final mean phosphate concentrations were 21.1, 25.5, and 20.9 mg PO$_4$ L$^{-1}$ in the HS, HM, and HG treatments, respectively. This may be due to assimilation of phosphate by heterotrophic bacteria (Longnecker et al.)
2010; Zubkov et al. 2007). Phosphate was measured during this study as dissolved orthophosphate which readily binds to particles such as biofloc (Teichert-Coddington et al. 1999). The heterotrophic treatments had significantly higher biofloc concentrations (Table 11), which may have helped to remove dissolved phosphate from the water. Furthermore, removing biofloc with settling chambers has been shown to lower dissolved phosphate concentrations (Ray et al. 2010a), and a greater amount of biofloc was removed from the heterotrophic tanks in this study (Table 11).

Typically heterotrophic nitrogen assimilation results in reduced alkalinity because bicarbonate is used as a carbon source during the process (Ebeling et al. 2006). However, in the current study alkalinity concentration increased in the heterotrophic treatments. During week 2, alkalinity concentrations generally leveled-off in the heterotrophic treatments, and shortly thereafter began to increase (Figure 12). Week 2 was also the time that settling chambers were first used to remove biofloc from the tanks. Regression analyses indicated that there was an inverse relationship between VSS and alkalinity in each treatment. Ray et al. (2011b) saw increased alkalinity being returned from settling chambers. In the current study alkalinity concentration in the water returning from settling chambers was not measured. One potential explanation for increased alkalinity in heterotrophic treatments is that denitrification, which typically generates alkalinity, may have been occurring in the settling chambers. However, in the heterotrophic treatments of the current study and in one of the treatments described by Ray et al. (2011b) there was little or no nitrate detected. Nitrate is the thermodynamically superior electron acceptor for the denitrification process (van Rijn et al. 2006). It is possible that
denitrification was occurring in the two studies but electron acceptors other than nitrate such as nitrite or nitrous oxide were being utilized by denitrifying bacteria.

The significantly higher concentrations of TSS and VSS in the heterotrophic treatments versus the CA treatment signify that biofloc concentration was higher in the heterotrophic treatments. Ebeling et al. (2006) indicated that heterotrophic bacterial assimilation can generate 40 times the amount of solids compared to chemoautotrophic nitrification for each unit of ammonia processed. Generating more solids can be a negative attribute of systems managed to be dominated by heterotrophic bacteria. The concentration of solids in the water must be managed to optimize shrimp production (Ray et al. 2010a; 2011b). Relatively more solids in a culture system should result in a greater amount of solids removal. In the current study both the HS and HG treatments had significantly more solids removed through settling chambers than the other treatments (Table 11). Although more material was also removed from the HM settling chambers than the CA chambers on average, this difference was not significant. However, a low probability value (P = 0.09) suggests that the difference between these two treatments may be substantially large.

The BOD$_5$ was significantly higher in the HS and HG treatments versus the HM treatment. The BOD$_5$ in HS, HG, and HM treatments were significantly greater than in the CA treatment (Table 11). This indicates that microbial respiration and oxidative processes consumed a greater amount of oxygen in the heterotrophic treatments versus the chemoautotrophic treatment. This may translate to higher operating costs for heterotrophic systems, especially if the injection of pure oxygen gas is required (Browdy et al. 2012). The HM treatment had a lower BOD$_5$ than the other heterotrophic
treatments, and the microbial community in the HM treatment seemed to respond to carbohydrate inputs differently than the HS and HG treatments. This was evident from the significantly faster drop in DO concentration in the HM treatment versus the HS and HG treatments (Table 11). Although the DO concentration dropped to its lowest level at a higher rate in the HM treatment, the amount of decline in the DO concentration was similar among each of the heterotrophic treatments. The addition of each carbohydrate caused an approximately 28% drop in DO concentration. Although DO concentration in the HM treatment dropped quickly, it seemed to recover somewhat during the 140 minute observation time (Figure 16b). DO concentrations in the HS treatment showed a slight increase after reaching the lowest levels following carbohydrate addition (Figure 16a), but the HG treatment showed no substantial signs of recovery in DO concentration (Figure 16c). When carbohydrates are added, microbial oxygen consumption can create dangerously low DO concentrations. Therefore, it is important to understand how carbohydrate additions can affect DO concentration.

**Shrimp Production**

Mean shrimp growth rate was 0.7 g wk$^{-1}$ in the CA and HS treatments, 0.6 g wk$^{-1}$ in the HG treatment, and 0.2 g wk$^{-1}$ in the HM treatment. Survival was 45, 53, 49, and 17% in the CA, HS, HG, and HM treatments, respectively. The relatively slow growth rates and low survival of the CA, HS, and HG treatments may have been caused by fluctuations of inorganic nitrogen concentrations (especially during the first 2 weeks) and the unfavorable temperatures recorded during the study. Venero et al. (2009) reported a study conducted in a commercial scale biofloc culture system during which shrimp growth rate was similar, at 0.9 g wk$^{-1}$ and survival was 60%. It is possible that shrimp
grown at high density in small tanks do not perform well. In 6,200-L tanks Ray et al. (2010a) reported growth rates between 0.6-0.9 g wk\(^{-1}\). Growing shrimp in 500-L tanks at the same stocking density as the current study but with 100% daily water exchange and temperature between 25 and 30\(^\circ\) C, Suárez et al. (2009) obtained a growth rate of 0.9 g wk\(^{-1}\).

Both growth rate and survival were unacceptably low in the HM treatment. High ammonia concentrations in the HM treatment may have contributed to the poor shrimp production. Also, the rapid drops in DO concentration after molasses addition may have led to some undetected lethal DO concentrations. The rapid DO concentration drop also may have stressed the shrimp, potentially contributing to poor production. Furthermore, the low concentration of carbon in molasses implies that there is a higher concentration of unusable components in this carbohydrate source. Components other than carbon may foul the water and give it the dark brown color observed.

Shrimp production was similar among the HS, HG, and CA treatments suggesting the potential equivalency of these management strategies. The addition of carbohydrates, increased oxygen demand, and larger volume of solids generated in the heterotrophic treatments represent costs of operation that were not factors in the chemoautotrophic treatment. The results of this study indicate that managing biofloc systems to function in a chemoautotrophic fashion may be superior to heterotrophic function.
CHAPTER V

COMPARING SALINITIES OF 10, 20, AND 30‰ IN MINIMAL-EXCHANGE, INTENSIVE, BIOFLOC-BASED SHRIMP (*LITOPENAEUS VANNAMEI*) CULTURE SYSTEMS

Introduction

Minimal-exchange, intensive, biofloc-based systems undergo low rates of water exchange. This feature greatly enhances biosecurity, reduces or eliminates pollution from effluent, and may facilitate inland culture of marine animals. These systems are stocked at high animal densities which reduces the area needed to culture animals and makes indoor, temperature controlled production possible. As a result of low water exchange, high stocking densities, and the use of only crude solids management, a dense microbial community develops in the water column. This microbial community is responsible for cycling nutrients, some of which are potentially toxic to shrimp. The microbial community, partially contained on flocculated (biofloc) particles may also provide nutrition to shrimp, thereby helping to recycle expensive nutrients from feed (Burford et al. 2004; Moss 1995; Wasielesky et al. 2006).

These systems can be sited away from the coast, using less expensive land and in close proximity to urban areas. Many urban areas have a high demand for quality, fresh shrimp which can be sold at premium prices (Browdy and Moss 2005). Even with minimal-exchange systems some water must be replaced as a result of solids concentration management and, with reuse between culture cycles, some water may need to be replaced due to the accumulation of contaminants such as metals. Considering this water use in addition to the initial filling of production systems, the cost of artificial sea
salts or imported seawater can be a substantial expense at inland facilities. Therefore, shrimp production systems should be operated at the lowest salinity possible to optimize financial returns.

The isosmotic point for *L. vannamei* is reported to be 24.7‰ salinity (Castile and Lawrence 1981). At this salinity shrimp should not have to spend energy osmoregulating to maintain a stable haemolymph osmolality. At lower salinities *L. vannamei* typically has to hyperosmoregulate, a function performed partially by the renal organ and antennal gland, but to the largest extent by the gills (Roy et al. 2010).

There are conflicting reports as to whether *L. vannamei* growth rates are compromised at salinities below the isosmotic point. Bray et al. (1994) found no significant difference in shrimp growth between animals grown in 5 and 15‰ salinity and no significant difference in growth between shrimp grown in 25 and 35‰ salinity. However, the shrimp cultured in the two lower salinities grew significantly faster than those at the two higher salinities. This is in contrast to a more recent study by Yan et al. (2007) who found that as salinity was increased from 11 to 21 to 31‰, there was a significant increasing trend in shrimp growth rate. Neither study found significant differences in shrimp survival among these salinities.

In the United States, *L. vannamei* are currently being produced at salinities of 1-15‰ in Alabama, Arizona, Florida, and Texas (Roy et al. 2010). However, these are relatively low intensity operations, stocking shrimp at roughly 10 to 40 m³ in outdoor ponds. There is a lack of information on low or moderate salinity marine shrimp culture using minimal-exchange, intensive biofloc technology. Operating low salinity minimal-exchange, intensive biofloc systems will likely pose unique challenges due to the
common fluctuations in concentration of inorganic nitrogen compounds (Browdy et al. 2012). The toxicity of inorganic nitrogen compounds, including ammonia, nitrite, and nitrate, have inverse relationships with salinity (Schuler et al. 2010; Kuhn et al. 2010).

The ionic composition of saltwater can vary based on its source (Roy et al. 2010). The concentration and ratios of a few major seawater ions are most important for *L. vannamei* culture. Saoud et al. (2003) demonstrated that potassium, magnesium, and sulfate were all positively correlated with shrimp survival. Roy et al. (2007) found that decreasing the sodium:potassium ratio increased shrimp growth and survival. They also demonstrated that low concentrations of magnesium resulted in higher shrimp respiration and low survival rates. Roy et al. (2007) recommended maintaining a Na:K ratio approximately equal to that of seawater (28:1) and a Mg:Ca ratio of 3.1:1.

The purpose of this study was to compare water quality, system function, and shrimp production dynamics in commercial-scale, biofloc-based culture systems operated at three different salinities (10, 20, and 30‰).

**Methods**

*Shrimp Source, Nursery, and Feeds*

Eight-day postlarvae (PL 8) *Litopenaeus vannamei* were obtained from Shrimp Improvement Systems, LLC (Islamorada, Florida, USA). These shrimp were stocked in a nursery raceway at a density of 4000 shrimp m$^{-3}$. The nursery raceway was 3.2 m x 30.1 m x 0.52 m (W x L x D), constructed of cement blocks filled with steel-reinforced cement, and lined with HDPE. All raceways used for this study were located under dome-shaped greenhouse structures which were covered in two layers of clear plastic. The nursery raceway contained a central wall made of plastic sheeting and suspended
from the greenhouse cross beams; water was propelled around this wall using 6 airlift mechanisms described by Ray et al. (2011b) and Ray (2012). Each airlift mechanism consisted of three, 15.2 cm long ceramic air diffusers oriented parallel to the water flow and receiving air from a 746 W regenerative blower (Sweetwater®, Aquatic Ecosystems Inc., Apopka, Florida, USA). The airlifts were constructed of a 2.5 cm diameter PVC frame which held the diffusers approximately 6 cm above the raceway floor. Above the diffusers was a sheet of EPDM rubber held by the PVC frame and oriented at an approximately 35° angle relative to the water movement. Air from the diffusers traveled vertically and contacted the EPDM, which served as a deflector to project the air and the water traveling with it horizontally forward.

The nursery raceway was operated at a salinity of 25‰ and shrimp were grown in the nursery for 54 days. Sucrose was added periodically to raise the C:N ratio and stimulate heterotrophic bacterial assimilation of ammonia. However, ammonia concentration was found to be dangerously high (> 5 mg TAN L⁻¹) and water exchanges were conducted on 4 occasions to offset these high levels. Throughout the nursery phase a total volume of water was exchanged equivalent to 140% of the system volume.

For the first 16 days of the nursery shrimp were fed freshly hatched Artemia sp. (INVE Aquaculture, Inc., Salt Lake City, Utah, USA) at a rate of 500,000 Artemia sp. L⁻¹ day⁻¹. Next, a series of Zeigler feeds (Zeigler™ Brothers Inc., Gardners, Pennsylvania, USA) were applied in the nurseries; the guaranteed analysis information for these diets was provided by the manufacturer. On the first day, 600 mL of Zeigler EZ Larva (250-400 µm) was added to each nursery, and 300 mL was added on the second day. The Zeigler EZ Larva diet had a guaranteed analysis of 11.0% crude protein, 6.0 % crude fat,
1.0% crude fiber, and 70.0% moisture. On days 1 through 22 Zeigler PL Raceway Plus 400-600 µm diet was added to the nurseries; on days 9 through 34 Zeigler PL Raceway Plus 600-850 µm diet was added; on days 15 through 54 Zeigler PL Raceway Plus 850-1200 µm diet was added. Each of the Zeigler PL Raceway Plus diets had a guaranteed analysis of 50.0% crude protein, 15.0% crude fat, 1.0% crude fiber, 10.0% moisture, and 7.5% ash. On days 23 through 54, Zeigler PL 40-9 Vpak 1.5 mm diet was provided to the nurseries. This diet had a guaranteed analysis of 40% crude protein, 9% crude fat, 3% fiber, 10% moisture, and 13% ash. On days 34 through 54, Zeigler Hyperintensive-35 Extra Short 2.4 mm diet was provided. This diet had a guaranteed analysis of 35% crude protein, 7% crude fat, 2% crude fiber, 12% moisture, and 15% ash. During the nurseries shrimp were fed based on a percent of the assumed shrimp biomass, starting at 15% of biomass and gradually decreasing to 8.8%.

Experimental Systems and Design

At the end of the nursery phase, shrimp were sampled using the methods described by Ray et al. (2011a) and found to weigh 1.22 ± 0.02 g (mean ± SEM). At this time, shrimp were enumerated by weight and 12,500 shrimp were placed into each of 9 raceways. These raceways were identical to that used for the nursery phase, except that only 4 airlift mechanisms were contained in each raceway, a 560 W water pump at each raceway helped to circulate and aerate the water, and the raceways were filled only to a depth of 0.26 m (25 m$^3$ volume). The water pump was connected to a 5 cm diameter pipe that circumvented each raceway. At 10 locations throughout each raceway (Figure 18) a 1.3 cm diameter pipe connected Venturi nozzles (Turbo-Venturi®, Kent Marine, Franklin, Wisconsin, USA) to the 5 cm diameter pipe. Each Venturi was located just below the 5
cm diameter pipe. Connected to the outflow of the Venturi was 1.3 cm diameter piping that extended down to approximately 3 cm above the bottom of the raceway before turning 90 degrees and extending 14 cm parallel to the bottom of the raceway. Each Venturi had tubing attached to the gas injection point which then attached to another pipe, 2.5 cm in diameter that circumvented the raceway. This pipe had two valves to allow ambient air to be drawn in and a point where pure oxygen gas could be injected, allowing air, pure oxygen, or a combination of the two to be injected into the raceway water through the Venturi nozzles.

![Diagram of raceway configuration](image)

*Figure 18.* The configuration of the 9 experimental raceways used for this study.

The 5 cm diameter water pipe that circumvented each raceway supplied water to 4 spray bars. Spray bars were 1.3 cm diameter pipes with 2, 2 mm diameter holes drilled in them, and the spray bars were located just behind the airlift mechanisms (Figure 18).
Water coming from the holes in the spray bars contacted the surface water of the raceway on either side of the airlifts because previous experiments had shown that foam and floating microbial mats tended to accumulate in these areas otherwise. The 5 cm diameter water pipe also supplied water to a 1.9 cm pipe that carried water to the settling chambers at each raceway.

This experiment was conducted during winter months at the University of Southern Mississippi’s Thad Cochran Marine Aquaculture Center (CMAC), a part of the Gulf Coast Research Laboratory, located in Ocean Springs, Mississippi, USA. To control temperature a centralized heating system, consisting of two hot water boilers connected to heat exchangers located in each raceway, was utilized. The two 500,000 BTU boilers (RBI, Division of Mestek Canada, Inc., Ontario, Canada) heated fresh, clean water to 66°C. This water was continuously pumped through a central line that passed near each raceway. Adjacent to each raceway was a digital controller that received a signal from a temperature probe submerged in the raceway. The controller operated a small pump that, when turned on, opened a check valve and sent hot water from the central line through a 3.7 m long, 8 bar, titanium heat exchanger located in each raceway. Digital controllers could be set to maintain the desired temperature in each raceway.

An 8-day “rest” period was initiated after moving the shrimp to the experimental raceways, allowing shrimp to recover from any stress incurred during stocking. During this time the shrimp were kept in 25 m³ of 25‰ salinity water, and temperature was maintained at 24°C initially, and gradually increased to 28°C. The 9 raceways were each randomly assigned to 1 of 3 treatments: a low salinity treatment at 10‰ salinity (LS), a medium salinity treatment at 20‰ (MS), and a high salinity treatment at 30‰ (HS).
After the 8-day rest period, clean water was slowly added to the raceways to bring them to the correct salinity and depth. Salinity was measured using a YSI Model 556 Handheld Instrument (YSI Incorporated, Yellow Springs, Ohio, USA) which measures conductivity and temperature to calculate salinity. Water at a salinity of approximately 20‰ was obtained from Davis Bayou, a tributary of The Mississippi Sound adjacent to the CMAC in Ocean Springs, Mississippi, USA. This water was bleached, aerated, and dechlorinated with sodium thiosulfate. Salinity was increased to approximately 35‰ using Fritz Super Salt Concentrate (Fritz Pet Products, Mesquite, Texas, USA) and sodium chloride (Morton® Purex® Salt, Morton® Salt, Chicago, Illinois, USA). Municipal water was used as a source of fresh water. Combinations of fresh and salt water were used to reach the desired salinity in each raceway based on assigned treatments. This process was carried out over an additional 8 days, after which 50 shrimp from each raceway were weighed in groups of 10 to estimate mean individual shrimp weight and the experiment began; this was considered time point 0.

**Water Quality**

At time point 0, when the experiment began, water samples were collected and filtered with 0.7 µm pore size filters for Na, Mg, K, and Ca concentration analysis. These samples were sent to the Clemson Agricultural Services Laboratory (Clemson, South Carolina, USA) for analysis using inductively coupled plasma mass spectrometry.

Twice per day, at approximately 0730 and 1600 h, temperature, dissolved oxygen (DO), pH, and salinity in the raceways were measured using the YSI Model 556 Handheld Instrument. Ammonia, nitrite, alkalinity, total suspended solids (TSS), volatile suspended solids (VSS), turbidity, and settleable solids were measured in each
experimental raceway once per week. At time point 0, week 4, and week 8 nitrate concentration was measured in each raceway. Phosphate (orthophosphate) concentration was measured at weeks 1, 2, 4, and 6. Five-day biochemical oxygen demand (BOD$_5$) was measured at weeks 2 through 6, and chlorophyll-a concentration was measured at weeks 0, 3, 4, 7, and 8. Raceway water samples were collected approximately 4 cm below the water surface, near the intake of the pumps that distributed water around the raceways and to the settling chambers.

Ammonia (TAN) was assessed using Hach method 8155 (Hach Company 2003) and nitrite (NO$_2$-N) was measured using the spectrophotometric procedure outlined by Strickland and Parsons (1972). Absorbance was measured at 655 nm for TAN and 543 nm for NO$_2$-N using a Hach DR 3800 spectrophotometer (Hach Company, Loveland, Colorado, USA). The concentration of NO$_2$-N plus NO$_3$-N was determined using the chemiluminescence detection method described by Braman and Hendrix (1989); NO$_3$-N was calculated by subtracting NO$_2$-N concentration. The concentration of PO$_4$ was measured using the PhosVer 3 (ascorbic acid) method outlined in Hach Method 8048 (Hach Company 2003) and absorbance was measured at 890 nm using the Hach DR 3800 spectrophotometer. Alkalinity was measured following the Potentiometric Titration to Preselected pH procedure outlined in section 2320 B by the APHA (2005). BOD$_5$ was measured using the procedure described in section 5210 B by the APHA (2005), which includes a 5 day incubation period at 20° C. Chlorophyll-a extraction was performed according to the methods described by DeLorenzo et al. (2004) using a combination of acetone submersion and freezing. Absorbance was measured using the Hach DR 3800 at 664, 665, and 750 nm. Absorbance at 665 and 750 nm was remeasured following
addition of 1.0 M hydrochloric acid. Concentrations of chlorophyll-a were calculated using equations given in section 10200 H by APHA (2005). TSS and VSS concentrations were measured following ESS Method 340.2 (ESS 1993). Turbidity was measured in Nephelometric Turbidity Units (NTU) using a Micro 100 Turbidimeter (HF Scientific, Fort Myers, Florida, USA). Settleable solids was measured by allowing solids to settle for one hour in an Imhoff cone, as described in Section 2540 F (APHA 2005).

Systems Management

After checking salinity in all raceways each morning, those raceways that were farthest above the intended salinity due to evaporation received fresh water. The objective was to keep the water in each raceway within 0.5‰ salinity of the target value for each treatment. A 2 m³ tank was filled with municipal water, dechlorinated using sodium thiosulfate, aerated, and tested for chlorine prior to use. Water was pumped to raceways that needed it; this process was repeated 1-4 times per day.

The morning pH readings were used to decide how much sodium bicarbonate to add to each raceway every day. If the pH in a raceway was above 7.9 no NaHCO₃ was added; if the pH was less than 7.9 but greater than 7.7, 300 g of NaHCO₃ was added; if the pH was less than 7.7 but greater than 7.5, 500 g of NaHCO₃ was added; if the pH was less than 7.5, 1,000 g of NaHCO₃ was added.

Sucrose (Extra Fine Granulated Cane Sugar, Sysco® Corporation, Houston, TX, USA) was added to each raceway three times per day between feedings and through the night on 12 hour belt feeders to facilitate heterotrophic bacterial assimilation of inorganic nitrogen. The wet weight of feed added daily was multiplied by 50% to determine the amount of sucrose to add each day based on the protocol applied in Chapter II. When
concentrations of ammonia and nitrite were high, additional sucrose was added. Overall, the amount of sucrose added was 57.4% of the wet weight of the feed resulting in a C:N ratio of inputs of 10.9:1.

Each raceway was equipped with a 760 L settling chamber described in Chapter II. The pump that helped to circulate water in each raceway also delivered water to the respective settling chamber. Water flowed back from each chamber by gravity. Based on the findings in Chapter II, the settling chambers in this study were operated at a flow rate of 15 LPM. Water flowed through the settling chambers continuously, with the exception of the time that settled material was removed from them. Once per week, water flow was terminated to each settling chamber for approximately one hour to allow thorough settling of particles in the chamber. A small submersible pump with a short hose attached was lowered into the top of the settling chambers to decant the water back to each raceway and leave the settled material. As the pump was lowered, the color of the water being pumped to the raceway was monitored. When the pump was lowered to the point of returning settled material, signified by a much darker color, the pump was lifted just above that point and allowed to pump all of the relatively clear water back to the raceway. The settled material on the bottom of the chamber was then drained.

*Shrimp Culture*

For the first 9 days after shrimp were moved to the growout raceways, both the Zeigler PL 40-9 Vpak diet and the Zeigler Hyperintensive-35 diet were provided, after which only Zeigler Hyperintensive-35 diet was given. Equal portions of feed were broadcast evenly through each raceway by hand 4 times per day at 0730, 1000, 1230, 1500 hrs. At 1630 hr. feed was placed on 2, 12 hour belt feeders at each raceway which
constantly delivered feed for 12 hours. Each day 70% of the feed ration was delivered by hand and 30% was placed on the belt feeders. During the 8 day rest period and the 8 day salinity adjustment period feed rations were 40% of what they were during the experiment. During the experiment feed rations were based on routine dip net sampling for shrimp and uneaten feed in each raceway. Feed rations were adjusted such that no uneaten feed could be found in the raceways 30 minutes prior to each feeding. Each raceway received the same amount of feed at every feeding, and each feed portion was weighed on a digital balance. Shrimp weights were measured once per week by weighing five groups of ten shrimp from each raceway; these shrimp were collected from various locations throughout the raceways using a dip net. During this experiment shrimp were grown for 8 weeks.

*Data Management and Analysis*

The data reported in this document are presented as mean ± SEM, and in many cases the range is given in parentheses. The statistical software used for this study was Systat Version 13 (Systat Software, Inc., Chicago, Illinois, USA).

To compare the concentrations of Na, Mg, K, and Ca, the LS treatment data were multiplied by 3 and the MS treatment data were multiplied by 1.5. The data were then compared using a one way ANOVA with salinity as the factor. The concentration of DO, ammonia, phosphate, alkalinity, BOD$_3$, VSS, settleable solids, and the turbidity data were analyzed using a fixed model, one-way, repeated measures (RM) ANOVA followed by pairwise comparisons. Ammonia, VSS, and settleable solids data were log$_{10}$ transformed prior to analysis to conform to the assumptions of the ANOVA. Turbidity data were cosine transformed because this transformation resulted in adherence to the ANOVA
assumptions. Morning and afternoon temperature and pH data, salinity, nitrite, nitrate, chlorophyll-a, and TSS data could not be transformed to fit the ANOVA assumptions, therefore a nonparametric Wilcoxon signed rank test followed by pairwise comparisons was used to analyze these data according to the recommendations of Zimmerman and Zumbo (1993). The percent water exchanged data were arc sine transformed and analyzed using a one way ANOVA. The amount of seawater used per kg of shrimp, seawater used per raceway, and the cost of artificial salt for each treatment were all analyzed using a one way ANOVA. Shrimp mean weekly growth rate, FCR, final weight, biomass, and survival were all analyzed using a one way ANOVA.

Results

During week 4 of this study a water pipe on one of the HS raceways broke which resulted in the raceway being drained, killing all the shrimp in that raceway. No data from this raceway are included in the results.

During the last week of the study, one of the vertical water pipes in a LS raceway was removed in an attempt to clear blockage at the last Venturi in the water line. When this occurred a large amount of thick, dark, sludge-like material poured out of the water line and into the raceway. Later that day many dead shrimp were found in this raceway, and a 5% water exchange was performed using clean 10‰ salinity artificial seawater made in the manner described above. The following day ammonia concentration was 2.0 mg TAN L$^{-1}$ and nitrite was 3.5 mg NO$_2$-N L$^{-1}$. This raceway was, therefore, harvested on the third day following the disturbance which was 3 days before the others in the study. A total of 1366 dead shrimp, weighing 17.40 kg, were removed from the raceway over the course of three days; the remainder of the shrimp were living at the time of
harvest. All water quality data from this raceway are included, aside from the last week. All shrimp production data are included in the reported results. Shrimp production data from this raceway include only those shrimp that were alive at the time of final harvest because these were the only shrimp fit for human consumption.

The initial concentrations of some major cations indicate that the salinities of 10, 20, and 30‰ were generally reflected proportionately in the concentration of these ions (Table 12). When the LS data were multiplied by 3 and the MS data were multiplied by 1.5, for equal comparison, there were significant differences (P = 0.04) between LS and HS potassium and calcium values. Multiplied in this fashion, the mean potassium value for the LS treatment was 354 mg L\(^{-1}\) and that of the HS treatment was 335 mg L\(^{-1}\). The mean calcium value for the LS treatment was 312 mg L\(^{-1}\) and that of the HS treatment was 331 mg L\(^{-1}\).

Table 12.

*The initial concentrations of some major cations. Concentrations are reported in mg L\(^{-1}\) as mean ± SEM. Different superscript letters indicate significant differences between treatments when low salinity values are multiplied by 3 and medium salinity data are multiplied by 1.5 for comparison.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Low Salinity</th>
<th>Medium Salinity</th>
<th>High Salinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na(^+)</td>
<td>2873 ± 61</td>
<td>5617 ± 122</td>
<td>8236 ± 82</td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>339 ± 3</td>
<td>673 ± 7</td>
<td>1006 ± 6</td>
</tr>
<tr>
<td>K(^+)</td>
<td>118 ± 1(^a)</td>
<td>230 ± 3(^a)</td>
<td>335 ± 3(^b)</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>104 ± 1(^a)</td>
<td>210 ± 2(^a)</td>
<td>331 ± 1(^b)</td>
</tr>
</tbody>
</table>

Temperature was maintained at approximately 29° C (Table 13) with little variability (Figure 19) using the centralized heating system. There were significant
differences in temperature between the treatments for both morning and afternoon measurements (P ≤ 0.01): MS > LS > HS.

Table 13.

The water quality parameters during the 8 week shrimp production experiment. Data are presented as mean ± SEM (range), and different superscript letters in a row indicate significant differences (P ≤ 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Low Salinity</th>
<th>Medium Salinity</th>
<th>High Salinity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Temperature (⁰C)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>29.0 ± 0.0 (26.5 - 29.6)\textsuperscript{a}</td>
<td>29.1 ± 0.0 (26.3 - 29.6)\textsuperscript{b}</td>
<td>28.8 ± 0.1 (26.1 - 29.5)\textsuperscript{c}</td>
</tr>
<tr>
<td>PM</td>
<td>29.1 ± 0.0 (27.6 - 30.1)\textsuperscript{a}</td>
<td>29.2 ± 0.0 (27.7 - 30.0)\textsuperscript{b}</td>
<td>29.0 ± 0.0 (27.5 - 30.0)\textsuperscript{c}</td>
</tr>
<tr>
<td><strong>Dissolved Oxygen (mg L\textsuperscript{-1})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>8.6 ± 0.1 (6.7 - 11.1)\textsuperscript{a}</td>
<td>8.7 ± 0.1 (6.6 - 12.5)\textsuperscript{b}</td>
<td>8.9 ± 0.1 (6.0 - 12.9)\textsuperscript{c}</td>
</tr>
<tr>
<td>PM</td>
<td>7.9 ± 0.1 (5.8 - 10.8)\textsuperscript{a}</td>
<td>7.7 ± 0.1 (5.2 - 10.0)\textsuperscript{b}</td>
<td>8.0 ± 0.1 (4.5 - 15.0)\textsuperscript{c}</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>7.9 ± 0.0 (7.7 - 8.2)\textsuperscript{a}</td>
<td>7.8 ± 0.0 (7.6 - 8.0)\textsuperscript{b}</td>
<td>7.7 ± 0.0 (7.4 - 8.0)\textsuperscript{c}</td>
</tr>
<tr>
<td>PM</td>
<td>7.9 ± 0.0 (7.1 - 8.3)\textsuperscript{a}</td>
<td>7.7 ± 0.0 (7.4 - 8.2)\textsuperscript{b}</td>
<td>7.7 ± 0.0 (7.3 - 8.1)\textsuperscript{c}</td>
</tr>
<tr>
<td><strong>Salinity (‰)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>10.3 ± 0.0 (9.2 - 11.0)\textsuperscript{a}</td>
<td>20.2 ± 0.0 (18.2 - 21.2)\textsuperscript{b}</td>
<td>30.2 ± 0.0 (27.1 - 31.9)\textsuperscript{c}</td>
</tr>
<tr>
<td>Nitrate (mg NO\textsubscript{3}-N L\textsuperscript{-1})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>0.8 ± 0.2 (0.0 - 4.0)</td>
<td>1.2 ± 0.4 (0.0 - 8.0)</td>
<td>0.7 ± 0.3 (0.0 - 4.4)</td>
</tr>
<tr>
<td>PM</td>
<td>0.3 ± 0.1 (0.0 - 3.4)\textsuperscript{a}</td>
<td>0.4 ± 0.2 (0.0 - 3.5)\textsuperscript{b}</td>
<td>0.6 ± 0.2 (0.0 - 3.3)\textsuperscript{c}</td>
</tr>
<tr>
<td><strong>Phosphate (mg PO\textsubscript{4}-P L\textsuperscript{-1})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>1.4 ± 1.0 (0.0 - 8.7)</td>
<td>0.3 ± 0.2 (0.0 - 2.0)</td>
<td>0.6 ± 0.3 (0.0 - 1.5)</td>
</tr>
<tr>
<td>PM</td>
<td>2.4 ± 0.3 (0.6 - 3.8)</td>
<td>2.6 ± 0.3 (1.4 - 4.4)</td>
<td>2.0 ± 0.2 (0.8 - 3.3)</td>
</tr>
<tr>
<td><strong>Alkalinity (mg CaCO\textsubscript{3} L\textsuperscript{-1})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>0.8 ± 0.2 (0.0 - 4.0)</td>
<td>1.2 ± 0.4 (0.0 - 8.0)</td>
<td>0.7 ± 0.3 (0.0 - 4.4)</td>
</tr>
<tr>
<td>PM</td>
<td>0.3 ± 0.1 (0.0 - 3.4)\textsuperscript{a}</td>
<td>0.4 ± 0.2 (0.0 - 3.5)\textsuperscript{b}</td>
<td>0.6 ± 0.2 (0.0 - 3.3)\textsuperscript{c}</td>
</tr>
<tr>
<td><strong>BOD\textsubscript{5} (mg BOD\textsubscript{5} L\textsuperscript{-1})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>180 ± 12 (72 - 243)</td>
<td>164 ± 9 (87 - 211)</td>
<td>171 ± 17 (65 - 238)</td>
</tr>
<tr>
<td>PM</td>
<td>80 ± 16 (24 - 200)</td>
<td>85 ± 18 (24 - 240)</td>
<td>84 ± 16 (40 - 240)</td>
</tr>
<tr>
<td><strong>Chlorophyll-a (µg L\textsuperscript{-1})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>310 ± 15 (207 - 442)</td>
<td>320 ± 19 (200 - 509)</td>
<td>322 ± 25 (205 - 500)</td>
</tr>
<tr>
<td>PM</td>
<td>180 ± 12 (72 - 243)</td>
<td>164 ± 9 (87 - 211)</td>
<td>171 ± 17 (65 - 238)</td>
</tr>
<tr>
<td><strong>TSS (mg L\textsuperscript{-1})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>80 ± 16 (24 - 200)</td>
<td>85 ± 18 (24 - 240)</td>
<td>84 ± 16 (40 - 240)</td>
</tr>
<tr>
<td>PM</td>
<td>263 ± 14 (185 - 500)\textsuperscript{a}</td>
<td>286 ± 19 (175 - 510)\textsuperscript{b}</td>
<td>330 ± 30 (210 - 645)\textsuperscript{c}</td>
</tr>
<tr>
<td><strong>VSS (mg L\textsuperscript{-1})</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>198 ± 14 (95 - 460)</td>
<td>189 ± 35 (37 - 100)</td>
<td>191 ± 24 (90 - 490)</td>
</tr>
<tr>
<td>PM</td>
<td>74 ± 7 (49 - 211)</td>
<td>64 ± 4 (35 - 126)</td>
<td>61 ± 6 (41 - 127)</td>
</tr>
<tr>
<td><strong>Turbidity (NTU)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>7 ± 1 (3 - 18)\textsuperscript{a}</td>
<td>9 ± 1 (4 - 21)\textsuperscript{b}</td>
<td>9 ± 1 (4 - 21)\textsuperscript{c}</td>
</tr>
</tbody>
</table>
Figure 19. The mean morning (a) and afternoon (b) water temperature in the raceways.

Dissolved oxygen (DO) was maintained at relatively consistent morning and afternoon concentrations (Figure 20). There were significant differences among treatments with respect to morning DO concentration (P ≤ 0.02): HS > MS > LS. With respect to afternoon DO concentration there were significant differences between the LS and MS treatments and between the HS and MS treatments (P ≤ 0.01): HS, LS > MS (Table 13).
The pH was significantly higher in the LS treatment, followed by the MS treatment, and then the HS treatment ($P \leq 0.01$). Morning pH was relatively consistent throughout the study; afternoon pH was less consistent (Figure 21).

Figure 20. The mean morning (a) and afternoon (b) dissolved oxygen concentrations in the raceways.
Figure 21. The mean morning (a) and afternoon (b) pH in the raceways.

Salinity in the raceways was maintained at a consistent level throughout the study (Figure 22) and was significantly different between treatments (P = 0.000).

Figure 22. The mean salinity throughout the study.
The concentration of ammonia increased substantially in all raceways with the exception of one raceway belonging to the HS treatment during week 5 of the study (Figure 23a) which never had a measured ammonia concentration above 0.4 mg TAN L\(^{-1}\). One of the MS raceways contained a high concentration of ammonia the following week (8.0 mg TAN L\(^{-1}\)). Ammonia concentration increased again in most raceways the last week of the experiment (Figure 23a). There were no significant differences (P > 0.05) in ammonia concentrations between treatments (Table 13). Nitrite concentration increased to 1.4 and 1.5 mg NO\(_2\)-N L\(^{-1}\) in the two HS raceways that remained during week 6. Nitrite rose again in all but 1 LS and 1 MS raceway during the last week of the study (Figure 23b). Nitrite concentration was significantly greater in the HS treatment versus the LS treatment (P = 0.04) and there were no significant differences between the MS treatment and the other treatments (Table 13). Nitrate concentration was generally low during this study; one notable exception was that an LS raceway had a concentration of 8.7 mg NO\(_3\)-N L\(^{-1}\) on the last sample date (Figure 23c). There were no significant differences (P > 0.05) in nitrate concentration between treatments.
Figure 23. The concentrations of ammonia (a), nitrite (b), and nitrate (c) during the 8 week study. Data points are treatment means and error bars are 1 standard error around the mean.

The concentration of TSS was significantly higher in the HS treatment versus the LS and MS treatments (P ≤ 0.05): HS > MS, LS (Table 13). Over the course of the first two weeks, TSS concentration decreased substantially in the raceways (Figure 24a). This time period likely affected the results of the RM ANOVA; however, after this time, there appeared to be very little difference in TSS concentration between treatments. There
were no significant differences between treatments with respect to the concentration of VSS (Figure 24b).
Figure 24. The concentrations of total suspended solids (a), volatile suspended solids (b), and settleable solids (c), as well as turbidity (d) over the 8 week experiment. Data points are treatment means and error bars are 1 standard error around the mean.
The concentration of settleable solids was significantly greater ($P = 0.03$) in the HS treatment compared to the LS treatment (Table 13) and there were no significant differences ($P > 0.05$) between the MS treatment and any other treatment (Figure 24c). There were no significant differences between treatments with respect to turbidity (Figure 24d).

There were no significant differences ($P > 0.05$) between treatments in terms of the BOD$_5$ (Table 13). The BOD$_5$ increased between weeks 3 and 5, followed by a sharp decrease in week 6 (Figure 25a). The concentration of chlorophyll-a was approximately 200 µg L$^{-1}$ just prior to the beginning of the study but decreased to approximately 50 µg L$^{-1}$ by week 3 (Figure 25b). There were no significant differences ($P > 0.05$) in chlorophyll-a concentration between treatments.

![Figure 25. The BOD$_5$ (a) and chlorophyll-a (b) concentrations during the study. Data points are treatment means and error bars are 1 standard error around the mean.](image)
The volume of material removed with the settling chambers was replaced with clean water, constituting the only water exchange implemented for this study; the only exception being the water exchanged just before harvesting the LS raceway that was harvested early. This water exchange was not considered in the following calculations because it was performed during the last two days of shrimp culture in 1 raceway.

The mean amount of full salinity seawater (35‰) used per kg of shrimp produced in the LS, MS, and HS treatments was 104, 159, and 235 L kg\(^{-1}\), respectively (Table 14); these amounts were significantly different between the three treatments (\(P \leq 0.02\)). There were no significant differences between treatments (\(P > 0.05\)) with respect to the volume of water exchanged. The estimated cost of artificial sea salts based on those currently used at the CMAC to make one full raceway of water was significantly higher (\(P = 0.000\)) in the HS treatment, followed by the MS treatment, and then the LS treatment (Table 14). These results suggest that the cost of salt would be 4.37, 6.91, and 10.26 USD kg\(^{-1}\) shrimp in the LS, MS, and HS treatments, respectively.

Table 14.

<table>
<thead>
<tr>
<th></th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LS</td>
</tr>
<tr>
<td><strong>Total 35‰ Seawater Used per Raceway (m(^3))</strong></td>
<td>15.0 ± 0.1(^a)</td>
</tr>
<tr>
<td><strong>35‰ Seawater Used per kg Shrimp (L kg(^{-1}))</strong></td>
<td>104 ± 14(^a)</td>
</tr>
<tr>
<td><strong>Total Water Exchange (%)</strong></td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td><strong>Cost of Artificial Sea Salt per Raceway (USD)</strong>*</td>
<td>653.8 ± 2.2(^a)</td>
</tr>
</tbody>
</table>

*Cost of artificial sea salt does not include the cost of fresh water. Cost is based on using one 36 kg bag of Morton brand NaCl (Morton\(^®\) Purex\(^®\) Salt, Morton\(^®\) Salt, Chicago, Illinois, USA) and one 19-L bucket of Fritz brand Super Salt Concentrate (Fritz Pet Products, Mesquite, Texas, USA) to make each 1,514-L of 35‰ water.
Because of the mortality event that occurred in one of the LS raceways, the shrimp production in that treatment was poor compared to the others (Table 15).

However, there were no significant differences (P > 0.05) in any shrimp production metrics between treatments. Growth rate (Figure 26) was high and feed conversion ratio (FCR) was low (Table 15). At the time of harvest, all shrimp, including those in the problematic raceway, were of high quality. Shrimp had full, long antennae, firm exoskeletons, and almost no lesions in the exoskeletons.

Table 15.

*Shrimp production in the three treatments. Data are presented as mean ± SEM, there were no significant differences between the treatments with respect to any of these parameters.*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>LS</th>
<th>MS</th>
<th>HS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Rate (g week⁻¹)</td>
<td>1.8 ± 0.1</td>
<td>2.0 ± 0.0</td>
<td>2.0 ± 0.1</td>
</tr>
<tr>
<td>FCR</td>
<td>1.6 ± 0.2</td>
<td>1.2 ± 0.0</td>
<td>1.2 ± 0.0</td>
</tr>
<tr>
<td>Mean Weight (g)</td>
<td>17.8 ± 0.9</td>
<td>19.3 ± 0.2</td>
<td>19.0 ± 0.5</td>
</tr>
<tr>
<td>Biomass (kg)</td>
<td>149.5 ± 19.2</td>
<td>188.2 ± 5.7</td>
<td>191.5 ± 3.5</td>
</tr>
<tr>
<td>Biomass volume⁻¹ (kg m⁻³)</td>
<td>3.0 ± 0.4</td>
<td>3.8 ± 0.1</td>
<td>3.8 ± 0.1</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>68 ± 10</td>
<td>78 ± 2</td>
<td>81 ± 1</td>
</tr>
</tbody>
</table>

*Figure 26. Shrimp weight throughout the 8 week production study. Data points are treatment means and error bars are 1 standard error around the mean.*
Discussion

Significant differences in initial potassium and calcium between the LS and HS treatments when the LS values were multiplied by 3 may indicate that the concentrations of these two elements were not proportionate to the intended salinities of 10 and 30‰. The LS treatment potassium value was 6% lower than the HS treatment, and the LS treatment calcium value was 6% higher than that of the HS treatment. A difference of 6% is likely not a biologically significant difference. Furthermore, the ratios that Roy et al. (2007) determined to be biologically important for shrimp were similar between treatments. Using mean values from Table 12, the Na:K ratios were 24.3:1, 24.4:1, and 24.6:1 in the LS, MS, and HS treatments, respectively. The Mg:Ca ratios were 3.3:1, 3.2:1, and 3.0:1 in the LS, MS, and HS treatments, respectively. The Na:K ratios were lower than the 28:1 ratio recommended by Roy et al. (2007). However, according to those authors a lower ratio is preferable to a higher ratio. The Mg:Ca ratios were similar to the ratio of 3.1:1 recommended by Roy et al. (2007).

The water temperature during this study was adequately regulated by the central heating system. This is in contrast to daily temperature fluctuations experienced during shrimp production in the summer months, as in Chapter II. The significant differences in temperature between treatments were likely caused by the location of raceways with respect to the hot water delivery system. Those raceways farthest from the boilers tended to be slightly cooler. However, the maximum mean differences of 0.3°C are likely not biologically significant to shrimp or the microbial community in the water column.

The concentration of DO during this study was also relatively consistent. The DO concentration was maintained at a high level to ensure that this was not a source of stress.
for shrimp. In commercial operations the high concentrations used for this study may not be desirable due to the cost associated with oxygen injection. Raceways probably received different amounts of oxygen. This may have contributed to the significant differences in DO concentrations between treatments. Although these differences existed, DO concentration was always sufficient to allow survival and growth in each treatment. Because no significant differences were found in BOD₃ concentrations, it is not likely that substantial differences in microbial respiration existed between treatments.

The intensive monitoring of pH and salinity and the regular inputs of sodium bicarbonate and fresh water resulted in consistent pH and salinity during this study. The consistency in temperature, DO, pH, and salinity during this study may have contributed to the high shrimp production values obtained, as a more constant physical environment can help to minimize stress for aquatic animals (Stickney 2005).

The cause of the inverse relationship between pH and salinity in this study is unclear. In seawater pH typically increases with higher salinity because more carbonate and bicarbonate ions are present with higher salinity, contributing to a higher pH buffering capacity (Libes 2009). Saraswat et al. (2011) found that in both laboratory and estuarine experiments, as salinity increased so did pH. However, in a study similar to the current project, Decamp et al. (2003) cultured L. vannamei in minimal-exchange biofloc systems with treatments of 9, 18, and 36‰ salinities, and found that pH was significantly lower as salinity increased. These authors attributed the relationship between salinity and pH to potentially increased photosynthesis in the lower salinity treatments. This may have been the case in the current study as well, although photosynthetic oxygen production was not measured. Because no significant difference in the chlorophyll-a
concentration was found, there probably was not a substantial difference in algal abundance; however, the taxonomic composition of algae may have differed among treatments. Higher concentrations of the weak acid CO$_2$ may have been present as salinity increased, possibly due to increased shrimp respiration. However, the DO and BOD$_5$ concentrations seem to contradict the idea of increased respiration.

There are chemical and physical relationships between temperature, pH, alkalinity, CO$_2$, and salinity (APHA 2005). Temperature and salinity both have inverse relationships with the concentration of CO$_2$, although temperature has a much greater impact. The pH of water has an inverse relationship with CO$_2$, and alkalinity has a positive relationship with CO$_2$ concentration. Each of these parameters, aside from salinity which has the least effect, is in favor of an increasing dissolved CO$_2$ concentration and decreasing pH with increasing salinity. Carbon dioxide dynamics may be further justified as a cause of decreased pH by examining the morning versus afternoon pH readings (Table 13 and Figure 21). The afternoon pH values are generally lower than morning values and much more variable over time. This may be an indication of the relatively higher CO$_2$ generation during the day when feed and sucrose inputs were greatest and respiration rates may have been higher. Also, the DO concentrations were substantially lower in the afternoon (Table 13), further indicating relatively higher respiration rates during the day.

The ammonia spike during week 5 (Figure 23a) of the study was followed by a sharp decline in BOD$_5$ concentration the following week (Figure 25a). It is unusual that the BOD$_5$ decline did not correspond to a drop in DO or an increase of pH, suggesting that the BOD$_5$ measurement during week 6 may have been incorrect. However, the
BOD₅ drop, if it is accurate, may have been an indication that a portion of the microbial community died, and the decomposition of those microbes could have contributed to increased ammonia concentrations. Nitrite and nitrate concentrations began to increase at approximately the same time as ammonia (Figure 23b, c), potentially signifying the oxidation of ammonia and nitrite by nitrifying bacteria. It is unclear why these increases of inorganic nitrogen compounds occurred, as sucrose was added multiple times per day to facilitate heterotrophic assimilation of nitrogen.

At week 0, chlorophyll-a concentration was much higher than after the study started (Figure 25b). This is likely a result of the shift from systems with relatively abundant algae to systems more dominated by heterotrophic bacteria after the intensive additions of feed and sucrose began. Such a shift in microbial dominance also was noted by Browdy et al. (2001). The mean chlorophyll-a concentrations reported in the current study are approximately 15% of what was reported by Ray et al. (In Press) and 20% of that reported by Venero et al. (2009); both groups of authors operated biofloc systems with no supplemental carbohydrate additions. However, the chlorophyll-a concentrations reported here are more than 5 times higher than what was reported by Moreno-Ostos et al. (2008) in oligotrophic waters.

The concentration of TSS was most divergent between treatments and highest at time point 0 (Figure 24a). It is unclear why this is the case, but TSS concentrations in general may have been influenced by the differences in salinity during this study. There were significant differences in TSS, but not in VSS. The VSS data represents the filtered organic matter that is burned at 550⁰ C and does not include the weight of minerals which do not combust. Therefore, in this case where salinity is different between treatments,
VSS concentration is likely the best indicator of the weight of biofloc particles in the water column. Furthermore, finding no significant differences between treatments in turbidity is an indication that optical clarity was not substantially different. Settleable solids was significantly higher in the HS treatment compared to the LS treatment; however, considering the large overlap in ranges between treatments (Table 13) this difference may not have been significant in terms of system function.

Table 14 helps to illustrate the substantial savings in seawater use by considering the culture of marine shrimp at lower salinities. At inland aquaculture facilities, these water savings translate directly to cost savings when considering the price of artificial sea salts. The low rate of water exchange during this study (Table 14) also helps to lower seawater use and justify inland shrimp aquaculture. Water was exchanged only as a result of solids removal. Several similar trials conducted at the Oceanic Institute (OI) in Hawaii, USA were reported to have used 187, 172, and 402 liters of seawater per kg of shrimp (Otoshi et al. 2007).

The stocking density of shrimp in this intensive production study is lower than those densities reported for superintensive shrimp production. Venero et al. (2009) reported production trials from OI, Texas Agrilife Research Center in Corpus Christi, TX, USA, and the Waddell Mariculture Center in Bluffton, SC, USA. In these trials, shrimp were in life stages similar to the current study, stocking density ranged from 430 shrimp m$^{-3}$ to 807 shrimp m$^{-3}$; growth rates ranged between 0.9 and 1.5 g wk$^{-1}$, and survival ranged from 60 to 91%. The shrimp production results in each of the current treatments are similar to those reported by Venero et al. (2009). Considering the entire production cycle, including the time period that temperature and feeding rates were
deliberately low, this study was carried out in approximately 10 weeks. The lower stocking density likely helped to facilitate fast shrimp growth, as shrimp grow faster at lower densities (Sookying et al. 2011).

Although there were no significant differences in shrimp production between treatments, the substantially lower production in the LS treatment would translate to less profit for a commercial shrimp operation. Biomass produced in the LS treatment was 20.6% lower than that of the MS treatment, meaning a proportionally similar decrease in profit assuming that a similar price is obtained for shrimp from the two treatments as the LS shrimp were also smaller (Table 15). The estimated cost of artificial salts was 49.7% lower in the LS treatment compared to the MS treatment. However, in a commercial shrimp production facility the salt water used to grow shrimp would be saved and used for multiple shrimp culture cycles.

In summary, the production goals and availability of sea salt should be considered when deciding the salinity at which to culture shrimp. It is unclear whether the problems that arose in the low salinity raceway during this study would have occurred if a similar situation had taken place in a higher salinity system; however, the likelihood would have been lower. Because of the fluctuations in ammonia and nitrite that are common in intensive biofloc-based shrimp culture systems the medium salinity of 20‰ is likely the best choice of the three options explored here. This salinity represents considerable cost savings over higher salinity, but based on the current results, can result in excellent shrimp production.
CHAPTER VI
CARBON AND NITROGEN STABLE ISOTOPE DYNAMICS IN INTENSIVE, BIOFLOC-BASED SHRIMP (*LITOPENAEUS VANNAMEI*) NURSERIES

Introduction

Intensive, biofloc-based shrimp aquaculture systems provide distinct advantages over extensive systems. Because very little water is exchanged and specific pathogen free animals are used, the chance of viral pathogen introduction is virtually eliminated. Reduced water use provides enhanced environmental sustainability and the ability to culture marine shrimp at inland locations, potentially providing fresh shrimp to metropolitan markets (Browdy and Moss 2005).

Biofloc-based aquaculture systems do not have substantial mechanical filtration or external biofilters. Rather, as a result of intensive nutrient inputs through feeds, a dense microbial community develops in the water column. This microbial community is partially contained in flocculated, organic (biofloc) particles made up of detritus, microbial secretions, algae, bacteria, fungi, protists, and zooplankton. The microbial community is responsible for detoxifying nitrogen compounds, of which ammonia and nitrite are most toxic. Studies also have demonstrated the enhancement of shrimp growth in the presence of biofloc particles (Moss 1995; Otoshi et al. 2001; Xu and Pan 2012). Biofloc-based systems have proven to be effective for the nursery rearing of shrimp from several day post-larvae to over 1 g, juvenile shrimp (Cohen et al. 2005; Samocha et al. 2007).

The comparison of stable carbon and nitrogen isotope values between consumers and potential food items is a common ecological procedure to evaluate the contribution of
dietary items to animal nutrition (Fry 2006; Gannes et al. 1997). There is an increasing body of literature on the contributions of natural biota to shrimp in aquaculture systems using stable isotopes (Burford et al. 2004; Epp et al. 2002; Parker et al. 1989). However, information is lacking on the specific contributions of feed versus biofloc in shrimp nursery systems. The purpose of the current study was to evaluate the contribution of applied feeds and the suspended biofloc community to shrimp carbon and nitrogen needs during nursery production of juvenile shrimp. Also, the study aimed to investigate potential interactions between changes in feed, biofloc, and shrimp isotopic levels.

Methods

Shrimp Husbandry

Brood stock shrimp were obtained from the Oceanic Institute (Waimanalo, HI, USA) and placed into shrimp maturation systems described by Ogle (1992). Shrimp were matured and spawned according to the procedures described by Ogle (1991). Shrimp from a total of 6 spawning events were obtained and each of these sets of shrimp was staggered through larval rearing periods. Post-hatch larval shrimp were fed a combination of the diatom Chaetoceros gracilis and brine shrimp Artemia sp. during a 20 day larval rearing period. The larval rearing period was conducted in static-water fiberglass tanks receiving blown air through ceramic diffusers.

As the larval rearing periods ended, these 6 sets of shrimp were stocked into 3 nursery raceways over 14 days. Five-day postlarval shrimp (L. vannamei) were stocked into the nurseries at densities of 3000, 2700, and 3200 shrimp m$^{-3}$ in raceways 1, 2, and 3, respectively. Shrimp were grown in the nurseries for between 46 and 62 days, depending on when they were initially stocked.
The raceways were contained under clear plastic-covered greenhouses and measured 3.2 x 30.1 x 0.52 m (W x L x D). Approximately 75% of the water used to fill them was previously bleached water from Davis Bayou, a tributary of The Mississippi Sound adjacent to the Thad Cochran Marine Aquaculture Center (CMAC) in Ocean Springs, Mississippi, USA. The remaining 25% of the water in the 50 m³ nurseries was artificial seawater made with municipal water and a mixture of Fritz Super Salt Concentrate (Fritz Pet Products, Mesquite, Texas, USA) and sodium chloride (Morton® Purex® Salt, Morton® Salt, Chicago, Illinois, USA). Of the total water volume in each raceway, 20% was reused from the study described in Chapter II. Water was reused so that a microbial community would be present from the beginning to help cycle nutrients and provide nutrition to post-larval shrimp. Salinity was maintained at approximately 25‰ through the periodic addition of fresh municipal water. Water was propelled around a central wall in each raceway using airlift mechanisms described in Chapter II and by Ray (2012). Pure oxygen was injected through micro-pore diffusers if dissolved oxygen concentration was below 4 mg L⁻¹.

Shrimp were fed a combination of 3 Zeigler PL Raceway plus diets (Zeigler™ Brothers Inc., Gardners, Pennsylvania, USA) with size ranges 400 – 600, 600 – 850, and 850 – 1200 µm. The diets were guaranteed by the manufacturer to contain a minimum of 50% protein and 15% fat, and a maximum of 1% fiber, 12% moisture, and 7.5% ash. The next feed provided was 1.5 mm, Zeigler PL 40-9: Vpak, which had a manufacturer-provided guaranteed analysis of a minimum of 40% protein and 9% fat, and a maximum of 3% fiber, 10% moisture, and 13% ash. Each of the diets was provided to shrimp based on weekly observations of shrimp size and measurements of weight. Larger sized feeds
were applied as shrimp grew and application of different feeds overlapped one another to account for variability in the size of shrimp. The amount of feed provided was based on an assumed shrimp biomass calculated using shrimp weights and an estimated mortality rate of 2% wk\(^{-1}\). Feeding rate was originally 15% of assumed biomass and gradually decreased to 9.5% of biomass.

**Water Quality**

Twice per day, at approximately 0730 and 1600 h, temperature, dissolved oxygen (DO), pH, and salinity in the raceways were measured using a YSI Model 556 Handheld Instrument (YSI Incorporated, Yellow Springs, Ohio, USA). Ammonia, nitrite, nitrate, total suspended solids (TSS), volatile suspended solids (VSS), turbidity, and alkalinity were measured in each raceway once per week. Ammonia (total ammonia nitrogen, TAN) was assessed using Hach method 8155 (Hach Company 2003) and nitrite (NO\(_2\)-N) was measured using the spectrophotometric procedure outlined by Strickland and Parsons (1972). Ammonia and nitrite were measured more frequently when high concentrations of these toxic substances were detected. Absorbance was measured at 655 nm for ammonia and 543 nm for nitrite using a Hach DR 3800 spectrophotometer (Hach Company, Loveland, Colorado, USA). The concentration of NO\(_2\)-N plus NO\(_3\)-N was determined using the chemiluminescence detection method described by Braman and Hendrix (1989); NO\(_3\)-N was calculated by subtracting NO\(_2\)-N concentration. Both TSS and VSS were measured using ESS Method 340.2 (ESS 1993). Turbidity was measured in Nephelometric Turbidity Units (NTU) using a Micro 100 Turbidimeter (HF Scientific, Fort Myers, Florida, USA). Alkalinity (as CaCO\(_3\)) was measured using the
Potentiometric Titration to Preselected pH procedure outlined in section 2320 B by the APHA (2005).

When TSS concentrations were above 500 mg L\(^{-1}\) the 760 L settling chambers described in Chapter II were used to lower TSS. Submersible pumps contained in a wire frame cage, covered in fine mesh were placed in the raceways and used to pump water to the settling chambers at a flow rate of approximately 6 LPM. Water flowed back from the settling chambers to the raceways. Settling chambers were operated until the following week and their use was discontinued if TSS concentration was below 500 mg L\(^{-1}\).

*Stable Isotopes*

Beginning the fifth week after the first shrimp were stocked in the nurseries, 10 shrimp were collected randomly from each raceway. Beginning the fourth week after the first shrimp were stocked, 1 L of water was collected from each raceway. During the weeks that both shrimp and water were collected, the two items were gathered simultaneously. Both shrimp and water samples were collected during 4 consecutive weeks. Shrimp were rinsed with deionized water, dried, ground, acid washed with 10% HCl, rinsed, dried and finely ground and homogenized. Water was centrifuged and the pellet was acid washed with 10% HCl, rinsed, dried and finely ground and homogenized. Each of the feeds also were collected, dried and finely ground and homogenized. Triplicate samples were placed into a Costech Model 4010 Elemental Combustion System which combusted the samples and supplied gaseous C and N (as CO\(_2\) and N\(_2\)) to a Thermo, Delta V Advantage Isotope Ratio Mass Spectrometer (Thermo Scientific,
Bremen, Germany). The mass spectrometer generated $\delta^{13}$C and $\delta^{15}$N values, calculated using the following equation:

$$\delta = \left( \frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1000$$

where $R$ is the ratio of heavy over light isotopes, in this case $^{13}$C/$^{12}$C or $^{15}$N/$^{14}$N. The standard used for C was Pee Dee Belemnite and that used for N was atmospheric N.

Microbes can assimilate nutrients quickly, and isotope levels in those microbes can reflect those of the source within two days (Burford et al. 2004). However, more than one trophic level of organisms inhabit biofloc systems (Ray et al. 2010b), likely leading to dynamic temporal changes in biofloc isotope levels. Also, it has been shown that it can take approximately 14 days for post-larval shrimp to reach isotopic equilibrium with their diet (Al-Maslamani 2006). Considering these issues, the sampling regime depicted in Figure 27 was used. Data reported from a particular sample number for this study include the weighted mean of feed isotope values for a full week, biofloc isotope data collected at the end of that week, and shrimp isotope data from the following week. This is to allow time for the feed to affect isotope levels of the biofloc, and both the biofloc and feed to affect isotope levels of the shrimp.
The shrimp fractionation factors calculated in Chapter III (2.4‰ for δ\textsuperscript{15}N and 1.3‰ for δ\textsuperscript{13}C) were subtracted from the measured shrimp isotope values in this study to account for trophic fractionation between shrimp and their diet.

The following two equations were used to estimate the relative amount of C and N contributed to shrimp by potential food sources (Fry 2006).

\[ f_1 = \frac{(\delta_{\text{sample}} - \delta_{\text{source2}})}{(\delta_{\text{source1}} - \delta_{\text{source2}})} \]

\[ f_2 = 1 - f_1 \]

Where \( f_1 \) is the fraction of C or N contributed by source 1, and \( f_2 \) is the fraction contributed by source 2. The sample was shrimp; source 1 was feed and source 2 was biofloc. The use of these equations assumes that there are only 2 sources of C and N available to shrimp.
Data Analysis

To determine if relationships existed between parameters measured for this study, linear regression analysis was performed in multiple instances. The software utilized was Systat Version 13 (Systat Software, Inc., Chicago, Illinois, USA). The concentration of VSS was compared to C and N isotope levels of the biofloc using a natural polynomial regression; in the analysis of VSS versus $\delta^{15}$N values data were $\log_{10}$ transformed to satisfy the normality assumption of the test. Ammonia and nitrite concentrations were compared to $\delta^{15}$N values of the biofloc using a linear least squares analysis; nitrite comparison data were $\log_{10}$ and tangent transformed to make the data fit a normal distribution. Using linear least squares analyses, the concentration of VSS and the mean weight of shrimp were each compared to the calculated %N contribution from feed to shrimp, and the feed and biofloc isotope levels were compared to shrimp isotope levels.

Results

Water Quality

Temperature, DO, pH, and salinity (Table 16) were within acceptable ranges for the growth of *L. vannamei* (Clifford 1985). The afternoon DO concentration was occasionally high due to pure oxygen injection. However, the trend in higher mean DO concentrations and pH levels in the afternoon versus the morning measurements is likely indicative of algal productivity during the day. Also, higher mean afternoon temperatures are the result of solar heat input to the raceways throughout the day.
Table 16.

The water quality parameters measured in each of the three nursery raceways used for this study. Data are reported as mean ± SEM (range).

<table>
<thead>
<tr>
<th></th>
<th>Raceway 1</th>
<th>Raceway 2</th>
<th>Raceway 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>29.3 ± 0.1 (27.4 - 30.8)</td>
<td>29.0 ± 0.1 (24.2 - 30.5)</td>
<td>29.6 ± 0.1 (25.8 - 31.1)</td>
</tr>
<tr>
<td>PM</td>
<td>30.9 ± 0.1 (29.2 - 32.2)</td>
<td>30.8 ± 0.2 (25.9 - 33.3)</td>
<td>31.6 ± 0.1 (29.7 - 34.0)</td>
</tr>
<tr>
<td>Dissolved Oxygen (mg L⁻¹)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>7.3 ± 0.2 (5.8 - 12.5)</td>
<td>6.7 ± 0.1 (4.9 - 9.2)</td>
<td>6.5 ± 0.1 (4.8 - 9.2)</td>
</tr>
<tr>
<td>PM</td>
<td>7.7 ± 0.2 (5.3 - 10.4)</td>
<td>8.0 ± 0.2 (4.8 - 17.9)</td>
<td>7.6 ± 0.2 (5.2 - 14.9)</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AM</td>
<td>8.2 ± 0.1 (6.7 - 9.2)</td>
<td>8.3 ± 0.1 (7.0 - 9.5)</td>
<td>8.3 ± 0.1 (7.2 - 9.4)</td>
</tr>
<tr>
<td>PM</td>
<td>8.5 ± 0.1 (7.1 - 9.4)</td>
<td>8.5 ± 0.1 (7.3 - 9.8)</td>
<td>8.6 ± 0.1 (7.5 - 9.7)</td>
</tr>
<tr>
<td>Salinity (%)</td>
<td>26.5 ± 0.2 (24.7 - 28.0)</td>
<td>26.4 ± 0.1 (24.9 - 28.2)</td>
<td>27.1 ± 0.1 (25.8 - 28.5)</td>
</tr>
<tr>
<td>Ammonia (mg TAN L⁻¹)</td>
<td>2.9 ± 0.6 (0.0 - 9.2)</td>
<td>1.8 ± (0.0 - 6.9)</td>
<td>2.1 ± 0.5 (0.0 - 6.2)</td>
</tr>
<tr>
<td>Nitrite (mg NO₂-N L⁻¹)</td>
<td>1.6 ± 0.4 (0.0 - 8.6)</td>
<td>7.0 ± 1.2 (0.0 - 17.4)</td>
<td>5.4 ± 1.0 (0.0 - 12.2)</td>
</tr>
<tr>
<td>Nitrate (mg NO₃-N L⁻¹)</td>
<td>0.0 ± 0.0 (0.0 - 0.1)</td>
<td>0.5 ± 0.3 (0.0 - 1.2)</td>
<td>0.0 ± 0.0 (0.0 - 0.0)</td>
</tr>
<tr>
<td>TSS (mg TSS L⁻¹)</td>
<td>358 ± 56 (115 - 750)</td>
<td>520 ± 49 (220 - 710)</td>
<td>374 ± 38 (210 - 595)</td>
</tr>
<tr>
<td>VSS (mg VSS L⁻¹)</td>
<td>343 ± 61 (100 - 750)</td>
<td>439 ± 50 (200 - 665)</td>
<td>369 ± 42 (180 - 585)</td>
</tr>
<tr>
<td>Turbidity (NTU)</td>
<td>88 ± 22 (18 - 158)</td>
<td>139 ± 34 (34 - 242)</td>
<td>103 ± 23 (28 - 144)</td>
</tr>
<tr>
<td>Alkalinity (mg CaCO₃ L⁻¹)</td>
<td>219 ± 18 (153 - 302)</td>
<td>237 ± 11 (174 - 262)</td>
<td>241 ± 9 (216 - 286)</td>
</tr>
</tbody>
</table>

The concentration of ammonia was of concern during this study. When shrimp sample number 1 was collected ammonia was below detection (BD) (< 0.01 mg TAN L⁻¹), 2.1, and 2.5 mg TAN L⁻¹ in the 3 raceways, respectively. At the following sample point ammonia concentration was BD, 5.1, and 5.3 mg TAN L⁻¹, respectively; it was 6.4, 0.3, and 0.4 mg TAN L⁻¹, respectively at the following sample point. During the last sample time, ammonia was below 1 mg TAN L⁻¹ in all raceways. Table 16 provides values for ammonia concentrations as well as other water quality constituents over the entire nursery period, not limited to the time that samples were collected for stable
isotope analysis. This is to ensure a complete representation of the water quality conditions that shrimp were exposed to during the study.

Nitrite also became highly concentrated at times during this study. Nitrite reached the highest concentration in raceway (RW) 1 at the time the 4th shrimp sample was collected (8.6 mg NO$_2$-N L$^{-1}$) for this study. Nitrite remained between 7.7 and 8.6 mg NO$_2$-N L$^{-1}$ in that raceway for 7 days. Nitrite reached the highest concentration in RW 2 (17.4 mg NO$_2$-N L$^{-1}$) just after shrimp sample 3 was collected; nitrite was 7.9 mg NO$_2$-N L$^{-1}$ or higher for 19 days surrounding this time period. Dead shrimp were found in RW 2 on the day that nitrite reached the highest concentration; through dip net sampling it was estimated that approximately 5% of the population died at that time. Nitrite concentration was highest in RW 3 during the same time period that it was elevated in RW 2; however, no dead shrimp were found in this raceway. Nitrate did not accumulate appreciably in the nursery raceways (Table 16).

The concentrations of TSS, VSS, and turbidity levels (Table 16) were at times higher than what has been recommended for optimal shrimp growth in biofloc culture systems (Ray et al. 2011b). The concentration of VSS, used to estimate the amount of organic biofloc particles, tended to increase over time in each of the raceways (Figure 28). Alkalinity (Table 16) remained within an acceptable range for nitrification and buffering against swings in pH (Browdy et al. 2012).
Figure 28. Stable isotope levels in shrimp, biofloc (floc), and feed, as well as the concentration of volatile suspended solids (VSS) over the four weekly sample collections times in raceways 1 (a, b), 2 (c, d), and 3 (e, f). Graphs on the left represent $\delta^{13}$C values and graphs on the right represent $\delta^{15}$N values. Feed values are weighted means of the isotope values of all feeds added during one week, floc values are representative of the biofloc collected at the end of that week, and shrimp values represent shrimp collected one week later. VSS concentration was measured when the biofloc was collected, as a means of estimating the amount of biofloc particles. Shrimp isotope values are corrected to account for trophic fractionation.

**Stable Isotopes**

There was a significant inverse polynomial relationship ($P = 0.000$) between VSS concentration and the $\delta^{13}$C values of suspended matter (biofloc) (Figure 29a). There was
a significant positive polynomial relationship (P = 0.000) between VSS concentration and the δ¹⁵N values of biofloc (Figure 29b).

![Graph](image)

Figure 29. The VSS concentration versus biofloc δ¹³C values (a), and VSS concentration versus biofloc δ¹⁵N values (b)

There was no significant relationship between the concentration of ammonia and the δ¹⁵N values of biofloc (P > 0.05). However, there was a significant positive relationship (P = 0.000) between nitrite concentration and the δ¹⁵N biofloc values (Figure 30).
Figure 30. The dissolved nitrite concentration versus the $\delta^{15}$N values of biofloc.

There were no significant relationships between VSS concentration or shrimp weight versus the %N contribution from feed. There were positive relationships ($P = 0.000$) between both the feed $\delta^{13}$C values and the biofloc $\delta^{13}$C values versus the $\delta^{13}$C values of shrimp (Figure 31a, b). There was also a significant positive relationship ($P = 0.001$) between the $\delta^{15}$N of feed and that of the shrimp (Figure 31c). Conversely, there was a significant inverse relationship existed ($P = 0.001$) between biofloc $\delta^{15}$N values and those of the shrimp (Figure 31d).
Figure 31. Regression plots of feed δ\textsuperscript{13}C values (a) and biofloc δ\textsuperscript{13}C values (b) versus shrimp δ\textsuperscript{13}C values, as well as feed δ\textsuperscript{15}N values (c) and biofloc δ\textsuperscript{15}N values (d) versus shrimp δ\textsuperscript{15}N values. Shrimp isotope levels are corrected to account for trophic fractionation.

For each sample collected except RW 1 sample 1 and RW 3 sample 3, the δ\textsuperscript{13}C values for shrimp were above those for feed and biofloc (Figure 28). This made calculating estimated %C contributions to shrimp from the feed and biofloc impossible on all but those two dates because consumers must have isotope values between that of their potential food sources to perform this calculation. Two sample dates would not be representative of the study. Also, the fact that shrimp isotope values were higher than both of their potential food sources most of the time indicates that an error may have occurred. For these reasons, %C contributions to shrimp from feed and biofloc were not calculated for any sample date.
The $\delta^{13}$C values for biofloc decreased substantially throughout the study, and the feed $\delta^{13}$C values also decreased (Figure 28a, c, e). Aside from a relatively large increase of the $\delta^{13}$C value in RW 1 shrimp between sample number 1 and 2, the $\delta^{13}$C values of shrimp also tended to decrease throughout the study. Similar to the $\delta^{13}$C values for the feed, the $\delta^{15}$N values for the feed tended to decrease over time (Figure 28b, d, f). However, unlike $\delta^{13}$C values of the biofloc, the biofloc $\delta^{15}$N values had a tendency to increase over time. The $\delta^{15}$N values of shrimp were variable between raceways in terms of changes over time; however, in each instance shrimp $\delta^{15}$N levels fell between that of feed and biofloc, making an estimation of the %N contribution of those potential N sources possible.

The estimated proportion of N in shrimp originating from feed or biofloc was variable between sample dates and between raceways (Figure 32). On the first sample date shrimp in each raceway seemed to be obtaining a substantial portion of N from the biofloc. In raceways 2 and 3, that relatively high N contribution from biofloc continued until the last sample date, during which the N contribution from biofloc was low in each raceway. The overall mean ± SEM percent N contribution from feed to shrimp in RW 1 was 66 ± 10% and that of biofloc was 34 ± 10%. In RW 2 the mean contribution of feed to shrimp N was 50 ± 14% and that of biofloc was 50 ± 14%. In RW 3 the mean contribution of feed to shrimp N was 52 ± 17% and that of biofloc was 48 ± 17%.
Figure 32. The estimated N contribution to shrimp from the applied feed and the suspended matter (biofloc) in raceways 1 (a), 2 (b), and 3 (c).

**Shrimp Production**

Survival in RW 1 was 42.4%, survival in RW 2 was 21.8%, and survival in RW 3 was 18.4%. The final mean shrimp weight was 1.27g.

**Discussion**

The high concentrations of ammonia during this study likely contributed to the relatively low shrimp survival. Lin and Chen (2001) recommended a 3.55 mg L\(^{-1}\) safe concentration of TAN at a salinity of 25‰. The ammonia concentration was above this value in RW 1 for 10 days and for 6 days in both raceway 2 and 3. Nitrite concentration...
also may have contributed to low shrimp survival. Nitrite was lowest in RW 1 (Table 16), the raceway with highest shrimp survival.

Similarly, Epp et al. (2002) found that $\delta^{13}$C values of particulate matter in *L. vannamei* culture tanks decreased over time and that $\delta^{15}$N values in the particulate matter increased over time (Figure 28). Two likely carbon sources for microbes in these systems are feed and atmospheric carbon (~8‰ $\delta^{13}$C) incorporated into sugars and lipids by algae. However, in the current study the $\delta^{13}$C values of biofloc seem to generally track those of feed (Figure 28). In contrast, the $\delta^{15}$N values of the biofloc tended to increase while those of the feed decreased. The increase of $\delta^{15}$N values in biofloc may be the result of a changing microbial community, possibly with an increasing number of higher trophic-level organisms. As trophic level increases so increases the enrichment of heavy isotopes. Checkley and Entzeroth (1985) found that copepods can have $\delta^{15}$N values enriched by as much as 5.8‰ over a diet of suspended particulate matter. If a higher abundance of zooplankton such as these had occurred in the biofloc, relative to other organisms, this may have led to an overall enrichment of $^{15}$N in the biofloc. However, the decrease in $\delta^{13}$C biofloc values does not support the occurrence of trophic enrichment.

The concentration of biofloc tended to increase over the course of the study, as indicated by increasing VSS concentrations (Figure 28). It is unclear whether or not this caused the lower biofloc $\delta^{13}$C and higher $\delta^{15}$N values, but there was a relationship between VSS and isotope values (Figure 29). It may be more likely that increased VSS concentration and the observed changes in biofloc isotope levels are both characteristics of abundant and dynamic microbial communities. As the systems aged shrimp feeding
rates increased and added an increasing amount of C and N. With no water exchange, a portion of C and N that has been added accumulates in microbes. Biofloc systems are typically dominated by algae in the beginning and transition to bacterial, heterotrophic-dominated systems as nutrients accumulate (Browdy et al. 2001).

One of the shifts in microbial composition that may have occurred is the proliferation of ammonia oxidizing bacteria which oxidize ammonia to nitrite. The accumulation of nitrite is evidence that these bacteria may have increased in abundance. The positive relationship between nitrite concentration and the $\delta^{15}$N values of biofloc (Figure 30) supports the idea that ammonia oxidizers may have been associated with changes in biofloc N isotope levels.

There was no detectable relationship between shrimp weight and the %N contribution from feed or biofloc. This is in contrast to a study by Burford et al. (2004) who found that of 1, 5, and 9 g shrimp, the 1 g shrimp attained the highest proportion of nitrogen from suspended particulate matter. However, shrimp in the current study were much smaller, in the weight range of approximately 1 to 1270 mg. Burford et al. (2004) also found that 9 g shrimp attained a higher portion of N than 1 or 5 g shrimp when exposed to an approximately 20% greater biofloc concentration. In the current study no relationship was found between the VSS concentration and the %N contribution from feed or biofloc to shrimp.

Although it was not possible to calculate %C contributions of feed and biofloc to shrimp, the feed and biofloc $\delta^{13}$C values both have positive relationships with $\delta^{13}$C values in shrimp (Figure 31a, b). This may be an indication that both feed and biofloc contributed to the C contained in shrimp. Likewise, the positive relationship that feed
\( \delta^{15}N \) values have with shrimp \( \delta^{15}N \) values may indicate that feed contributed a substantial portion of N to shrimp. This is in agreement with the estimates made by calculating \( \%N \) contribution to shrimp from feed.

The inverse relationship between biofloc \( \delta^{15}N \) values and shrimp \( \delta^{15}N \) (Figure 31d) values seems to contradict the estimated contribution of biofloc to shrimp N. The relatively low \( R^2 \) values of the regression analyses between feed and biofloc \( \delta^{15}N \) values and those of shrimp indicate that feed and biofloc N isotope levels do not explain as much of the variability in shrimp N isotopes as compared to C contributions to shrimp. It appears that the strength of the inverse relationship between biofloc \( \delta^{15}N \) and shrimp \( \delta^{15}N \) values may be disproportionately affected by 6 observations with biofloc \( \delta^{15}N \) values above 10‰ (Figure 31d). In fact, when these observations are deleted from the analysis, there is no relationship between biofloc and shrimp \( \delta^{15}N \) values (\( P = 0.208 \)). These 6 observations were made during the last sample date (Figure 28b, c, e), the time when estimated shrimp \( \%N \) contribution from biofloc was lowest in RWs 2 and 3, and among the lowest in RW 1 (Figure 32). Therefore, it is not surprising that the observations contribute to a poor relationship between biofloc and shrimp \( \delta^{15}N \) values. The fact that shrimp \( \delta^{15}N \) values were between those of the feed and biofloc on every sample date seems to be a good indication that both feed and biofloc contributed to shrimp N content.

It is unclear why shrimp \( \delta^{13}C \) values were consistently higher than those of feed and biofloc (Figure 28). It is possible that the degree of trophic \( ^{13}C \) fractionation between shrimp and their diet was higher in this study than in other studies (Chapter III; Parker et al. 1989). The \( ^{13}C \) fractionation value of 1.3‰ was used because of the findings in
Chapter III. The diets used during this study were similar to those used in Chapter III, although they contain higher protein levels, and are said by the manufacturer to contain specialized ingredients for the nutrition of young post-larval and juvenile shrimp. Anderson et al. (1987) demonstrated that different feed ingredients can result in different apparent levels of fractionation in the trophic transfer to shrimp. This is largely due to varying assimilation of feed ingredients by shrimp, and the same holds true for varying microbial assemblages (Gannes et al. 1997).

The variability of %N contribution from biofloc to shrimp may be somewhat concerning from a management perspective. When shrimp culture managers decide how much feed to apply in nursery systems, ideally that feed rate should be linear over time. However, if shrimp receive varying contributions of nutrients from biofloc, feed rates may need to be adjusted accordingly.

The estimated contributions of biofloc to the nitrogen contained in shrimp during this study are relatively high. Because protein is typically the most expensive ingredient in shrimp diets this could contribute to a substantial savings in feed costs for shrimp nurseries. In this study shrimp from RW 1 were estimated to have received 34% of N from biofloc, while RWs 2 and 3 received 52 and 48% respectively. These values fall in the range of what other researchers have estimated. Parker et al. (1989) estimated that pond biota contributed approximately 60% of the N found in shrimp. Epp et al. (2002) estimated that shrimp obtained as much as 31% of N from pond biota, and Burford et al. (2004) estimated that natural biota contributed between 18 and 29% of shrimp N.

In summary, the results of this study support the use of biofloc-based systems for the nursery culture of *L. vannamei*. Post-larval and juvenile shrimp may utilize biofloc as
a substantial source of C and N, suggesting that feeding can be reduced in biofloc systems compared to clear water systems while maintaining superior biosecurity over extensive systems. The isotopic composition of biofloc was related to the amount of biofloc, and the N isotopic composition was related to the concentration of dissolved nitrite. These relationships may be indications of changes in the microbial community. Due to high concentrations of ammonia and nitrite during this study, alternative biofloc management strategies should be considered. One such strategy is to elevate the C:N ratio of inputs to favor heterotrophic bacterial N assimilation over nitrification (Avnimelech 1999), as nitrification did not function reliably in this case.
CHAPTER VII

SUMMARY

The results of Chapter II indicate that controlling the flow rate delivered to simple settling chambers may be an effective means of regulating the concentration of biofloc particles. Continuous operation of these devices appears to be advantageous due to the increased alkalinity and decreased solids and nitrate concentrations returned to the raceways from them. The two sizes of settling chambers used for this study helped to maintain similar retention times in those chambers.

The treatment with higher flow rate to larger settling chambers resulted in lower nitrate and solids concentrations, and higher alkalinity; this treatment also had significantly improved shrimp production. It seems that this management regime is more appropriate than one with lower flow rate to smaller sized settling chambers. However, removing more material will necessitate the disposal or on-site remediation of that material. The results of this study may be important to consider for shrimp production results, chemical dynamics, and operational expenses for intensive, minimal-exchange, biofloc-based shrimp culture systems.

Based on the results of Chapter II, the study described in Chapter III compared recirculating systems with no biofloc to those with biofloc. Particle concentration was significantly higher in the biofloc tanks, while solids were removed with external filters in the clear water tanks. Nitrate was substantially higher in the clear water tanks because of external nitrifying biofilters, while heterotrophic bacteria apparently assimilated inorganic nitrogen in the biofloc systems. The external biofilters of the clear water systems maintained low ammonia and nitrite concentrations in those tanks while these
compounds became dangerously concentrated in the biofloc tanks at times, likely contributing to poor shrimp production in one of those tanks.

Although shrimp production was generally low in this study, the use of clear water tanks allowed C and N stable isotope fractionation values to be estimated. Using these fractionation levels, the percent C and N in shrimp contributed from the feed and the biofloc in the biofloc-based tanks was estimated. The estimated C contribution to shrimp from feed was 72% and that from biofloc was 28%, while the estimated contribution of N from feed was 42% and that from biofloc was 59% (rounded values). These results indicate that biofloc may have contributed substantially to shrimp nutrition, especially with regard to N which enters the system primarily through expensive proteins in feed. It is unclear whether clear water or biofloc systems are best for shrimp culture. Based on this preliminary and somewhat narrow study, toxic nitrogen compounds may be better controlled in clear water RAS; however, biofloc may contribute to shrimp nutrition thereby potentially lowering feed costs.

Chapter III also provided a comparison of stable isotope levels in whole shrimp versus what was found only in the abdominal sections. It was found that both $^{13}$C and $^{15}$N were significantly enriched in shrimp abdomens, which may have implications for comparing the results in this document to those of other studies.

The fourth chapter of this document describes an experiment that evaluated the use of three carbohydrate sources to promote heterotrophic nitrogen assimilation, and compared these to systems without carbohydrate addition. Tanks that received molasses had relatively high concentrations of ammonia. In the chemoautotrophic systems nitrification apparently proceeded, as evidenced by an accumulation of nitrate; however,
this accumulation only occurred after a relatively high spike in nitrite. There was no appreciable amount of nitrate in the tanks intended to function as heterotrophic systems, indicating that assimilation was likely the dominant process.

A significantly greater amount of solids was generated in the heterotrophic systems, and biochemical oxygen demand was higher in those tanks indicating that microbial respiration was greater. In each of the heterotrophic treatments DO concentration declined by approximately 28% after the addition of each respective carbohydrate source. However, DO declined significantly faster in the molasses treatment, reaching its lowest mean point in 27 minutes while the lowest mean DO concentrations were measured 47 and 75 minutes after the additions of sucrose and glycerol, respectively. In all of the treatments there were significant inverse relationships between VSS concentration and alkalinity, indicating that higher VSS may have led to a decline in alkalinity.

Shrimp growth rate was slow in this study, likely due to low temperatures. Shrimp survival also was low; however, both growth rate and survival were much lower in the molasses treatment than any other treatment.

The results of this study indicate the need to consider solids accumulation and system oxygen demand when deciding on the management protocol for biofloc systems. Nitrification can result in spikes of toxic nitrogen compounds, whereas in this case, those compounds did not become highly concentrated in heterotrophic systems with the exception of the molasses treatment. The results indicate that molasses may not be a good choice of carbohydrate source for this type of system management, while sucrose and glycerol appear to yield similar results.
In Chapter V three salinities were used to culture shrimp in large raceways. Most water quality parameters were remarkably similar between the salinities of 10, 20, and 30‰. Temperature and DO concentration differed between the treatments, but it appears likely that these subtle differences were due to management issues and probably did not have any biologically significant effects on shrimp or system performance. The pH was significantly greater as salinity decreased. It is unclear why this was the case, but may be at least partly attributable to greater CO$_2$ concentrations at higher salinity. Nitrite concentrations were higher in the 30‰ salinity treatment compared to the 10‰ salinity treatment, but these differences were not great and, due to lower toxicity of nitrite at higher salinity, likely did not affect shrimp.

The cost differences associated with salinity are evident when the price of artificial sea salts is considered. These differences may have substantial impacts for inland shrimp farming. Shrimp production was not significantly different between the salinities used in this study. However, due to the introduction of what was likely anaerobic sludge into a low salinity raceway, the shrimp production was substantially lower in the low salinity treatment. This event would likely have been less detrimental in a higher salinity raceway because the toxicities of ammonia and nitrite are lower with increased salinity. In the 20‰ and 30‰ salinity treatments shrimp growth rate and FCR were excellent, likely due to the consistency and near optimal level of environmental parameters during this study. This study illustrates some of the important issues to consider when deciding what salinity to operate biofloc systems.

Chapter VI helps to illustrate the usefulness of operating shrimp nurseries as biofloc systems. The C isotope levels of biofloc were inversely related to VSS
concentration, and the N isotope levels of biofloc were positively correlated with VSS concentration. These results may indicate that the concentration of biofloc particles has an effect on the isotope ratios found in that material, or the results could indicate a change over time in isotope levels as the system progresses and biofloc becomes more concentrated. The concentration of nitrite also was positively correlated with the $\delta^{15}$N values of biofloc, possibly indicating a divergence in the microbial community associated with nitrite concentration.

The $\delta^{15}$C values of both feed and biofloc were positively related to $\delta^{15}$C values of shrimp, indicating that both feed and biofloc may have contributed C to shrimp. The $\delta^{15}$N values of feed were positively related to those of shrimp; however, the $\delta^{15}$N values of biofloc were inversely related to shrimp $\delta^{15}$N values. These results seem to indicate that feed was the main contributor of N to shrimp. However, when the N isotope values of shrimp, feed, and biofloc are placed into a simple two source mixing model, the estimated contribution of N to shrimp from biofloc ranged from 34 to 50%. The cause of an inverse correlation between biofloc and shrimp $\delta^{15}$N values is unclear, but the mixing model suggests a substantial contribution of N from biofloc.

Juvenile shrimp in nurseries may be especially well suited to take advantage of biofloc particles due to their smaller size. However, the estimated C and N contributions of biofloc to shrimp in this nursery study are similar to those calculated using much larger shrimp in Chapter III. It may be that shrimp size has little impact on biofloc utilization as a food source. Nonetheless, because biofloc appears to contribute to shrimp nutrition during the nursery phase, using this technology may help save on feed cost.
There are a wide variety of management issues to consider when operating intensive, minimal-exchange biofloc-based shrimp culture systems. This technology can result in greater shrimp production per unit area, limited water use, and enhanced biosecurity compared to extensive systems. However, the effects of biofloc concentration, carbohydrate sources used, and salinity are among the issues that must be considered to optimize production, and ultimately the economic competitiveness of both nursery and growout shrimp production systems. Considering the rapid expansion of the shrimp aquaculture industry in response to growing demand for shrimp, intensification will be necessary. Biofloc-based systems offer a potentially environmentally-sustainable means of accommodating this intensification. Advances in the reliable operation of biofloc-based culture systems may therefore contribute to responsible growth in shrimp aquaculture.
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