Enhancing Shrimp Aquaculture Sustainability Through Water Reuse and Biological Treatment

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ABSTRACT

Overfishing of natural fisheries is a global issue that is becoming more urgent as the human population increases exponentially. According to the Food and Agriculture Organization of the United Nations, over 70% of the world's seafood species are fully exploited or depleted. This high demand for seafood protein is not going away; and, in fact, an astonishing one out of five people in this world depend on this source of protein. Traditional aquaculture practices use pond and flow-through systems which are often responsible for discharging pollutants into the environment. Furthermore, aquacultural feeds often contain high levels of fish protein, so the demand on wild fisheries is not completely eased. Even though traditional aquaculture has these drawbacks, there is a significant movement towards more sustainable practices. For example, implementing recirculating aquaculture systems (RAS) maximizes the reuse of culture water which decreases water demand and minimizes the levels of pollutants being discharged to the environment. And, alternative proteins (e.g., soy bean) are replacing the fish and seafood proteins in aquaculture diets.

Accordingly, the research described in this dissertation focused on maximizing the reuse of freshwater fish effluent to culture marine shrimp. More specifically, by using suspended-growth biological reactors to treat a tilapia effluent waste stream and to generate microbial flocs that could be used to support shrimp culture. This RAS technology will decrease water consumption by increasing the amount of recycled water and will also improve effluent water quality. The biomass generated in the bioreactors could be used to feed shrimp with an alternative source of protein. Treating fish effluent to be reused to culture shrimp while producing this alternative feed, could significantly decrease operational costs and make these operations more sustainable.

Understanding which ions are critical for the survival and normal growth of marine shrimp in freshwater effluents is essential. It is also very important to understand how to convert an effluent's organic matter into food for shrimp. Results from studies revealed that the marine shrimp, *Litopenaeus vannamei*, can be raised in freshwater effluent when supplemented with specific ions and wet microbial flocs fed directly to shrimp can enhance growth in shrimp fed a restricted ration of commercial feed. The treatability of the tilapia effluent using suspended-growth, biological reactors and nutritional analysis of the generated biomass were also reported. Carbon supplementation enhanced reactor performance and microbial floc generation. These microbial flocs also proved to be a superior feed ingredient when dried and incorporated into a pellet feed.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

LIST OF TABLES

LIST OF EQUATIONS

Chapter 2

Chapter 3

Chapter 4

CHAPTER 1: LITERATURE REVIEW

Biological Treatment

Overview

Biological treatment of wastewater is accomplished by microorganisms. Traditional biological treatment includes the removal of organic wastes and nutrients (e.g. nitrogen and phosphorus). However, virtually any pollutant of interest can be removed from a waste stream if the proper treatment process is implemented in conjunction with favorable environmental conditions that select for the proper microorganism. This section discusses biological treatment in terms of (1) treatment processes, (2) manipulation of basic metabolic functions, (3) wastewater characterization, (4) kinetics and stoichiometry, and (5) biological treatment in aquaculture.

Treatment Processes

There are two major treatment processes that can be used for biological treatment. These include a fixed film process and a suspended growth process. These two processes, while fundamentally the same because they use microorganisms to achieve treatment, are very different.

In fixed film reactors, microorganisms attach themselves to surfaces and create a biofilm. This immobilized biofilm consists of microorganisms and their extracellular polymers (Rittmann and McCarty, 2001). Organic material and nutrients are removed from the wastewater as it flows by this biofilm. The performance of a fixed-film process is largely dependent on the surface area of the biofilm in a reactor. Often times, a packing material is used and the surface area can be maximized if a material with a high surface area to

volume ratio (specific surface area) is used. For example, this packing material can be stacked in a trickling filter, where wastewater cascades over the surfaces via gravity. Alternatively, this packing material can be submerged in agitated wastewater. Fixed film processes are often used to treat a wastewater for organic material and/or nutrients. Fixed-film reactors (fluidized beds, trickling filters, or rotating biological contactors) are commonly used for nitrification (conversion of NH_3+NH_4 to NO_3) in the recirculating aquaculture industry. Even though a complex community of autotrophic bacteria is responsible for nitrification, oxidation of NH_3 to NO_2 and NO_2 to NO_3 is often attributed to *Nitrosomonas spp.* and *Nitrobacter spp.*, respectively (Hagopian and Riley, 1998)

In suspended growth reactors, microorganisms are maintained in suspension by well-mixing the reactor using pneumatic or mechanical agitation. Microorganisms in suspended growth reactors form floc particles which are typically between 50 and 200 μm in diameter (Metcalf and Eddy, 2003). These microbial flocs are conglomerates of microorganisms that are bridged together by polysaccharides, proteins, and multivalent cations (Higgins and Novak, 1997). Organic material and nutrients are removed from the wastewater as the microbial flocs move through the wastewater. Suspended growth reactors are typically used to treat municipal wastewater (Rittmann and McCarty, 2001).

Manipulation of Basic Metabolic Functions

Environmental conditions can be manipulated by the wastewater operator as a means to achieve particular treatment objectives. Among the most common manipulations include pH adjustments, carbon/nutrient supplementation, and the addition/restriction of oxygen.

During biological treatment, the pH of the wastewater will change due to metabolic activity (see stoichiometry section below for more details). If the pH fluctuates drastically it may adversely affect the treatment operation. For example, acidic conditions are favorable to undesirable filamentous organisms which can adversely affect operations (Eckenfelder, 2000). In certain situations it may become necessary to control pH with the addition of an acid or a base. Carbon and/or nutrient supplementation is occasionally needed during operation to ensure that the wastewater is properly balanced stoichiometrically. The most common manipulation is whether or not oxygen is added to the biological treatment operation and is covered in more detail below. In particular, aerobic, anoxic, anaerobic, and combined conditions are discussed.

Aerobic conditions occur when dissolved oxygen is readily available for biological metabolism. Dissolved oxygen at levels greater than 1.0 mg/L is required, levels greater than 4.0 mg/L is preferred, especially in fixed-film processes (Rittmann and McCarty, 2001). Aerobic or facultative bacteria are often the dominant microorganism in aerobic conditions and they utilize oxygen as an electron acceptor. This results in the oxidation of organic matter to CO_2 and the assimilation of nutrients. Aerobic processes are typically characterized as having rapid removal rates and high biomass production.

Anoxic conditions occur in the absences of dissolved oxygen. Anaerobic or facultative bacteria are often dominant microorganisms in these systems. These conditions force the microorganisms to scavenge for oxygen that is bound (e.g. nitrate, NO_3). This anoxic condition is often selected by the operator to force denitrification, which is the removal of

NO_3 (Beeman and Reitberger, 2003). In addition to the formation of N_2 gas from NO_3, denitrification results in the oxidization of organic matter to CO_2 and the assimilation of nutrients. Compared to aerobic processes, anoxic processes are typically slower and produce less biomass. If maintained properly, they should not produce an odor.

Anaerobic conditions occur in absence of oxygen. This terminology is often used interchangeably with anoxic conditions. However, many people define anaerobic conditions as being completely absent of molecular oxygen, free (e.g. O_2) or bound (e.g. NO_3).

Aerobic/anaerobic/anoxic conditions are often employed in various combinations within a process to achieve a particular treatment objective. For example, aerobic/anaerobic processes are commonly used to remove organic matter, nutrients, and nitrate from a waste stream.

Wastewater Characterization

There are a vast amount of constituents in wastewater and it is nearly impossible to individually characterize them all. Fortunately, many of these parameters can be grouped together and characterized adequately, so modeling wastewater treatment can be satisfactorily performed. For example, there are various forms of organic molecules in wastewater; they can be generalized as biochemical oxygen demand (BOD) or chemical oxygen demand (COD). This section discusses the commonly measured wastewater constituents.

Organic material that can be readily degraded by microorganisms is a major concern if high levels are released into the environment. One common concern is high levels of organics being released into a natural body of water. This can create an algae bloom, which can result in oxygen deficits, which can adversely affect fish populations. Organic material is commonly measured as BOD, COD, and total organic carbon (TOC). These parameters can be fractionated as particulate (> 1.5 μm), colloidal (between 0.45 and 1.5 μm), or soluble (< 0.45 μm). The amount of oxygen that can be used by microorganisms to oxidize organic material is called BOD. On a chemical bases, COD is the amount of oxygen required to chemically oxidize organic material using a strong chemical oxidant. Total organic carbon is a measurement of organic carbon. Typically, these various measurements can be correlated with each other so one measurement can be taken to estimate the other. Furthermore, taken ratios of these different measurements can yield valuable information. For example, some wastewaters have high loads of organic material that cannot be biodegraded, such as a pharmaceutical waste (Eckenfelder, 2000). This would result in a high COD:BOD ratio. Conversely, as the COD: BOD ratio approaches one this would indicate that the organic material is readily degradable by microorganisms (Grady et al., 1999).

Nutrient determination is important because nutrients need to be stoichiometrically balanced with other constituents for sufficient biological treatment of a wastewater (see stoichiometry section below for more details). Numerous nutrients are directly toxic to aquatic life. Similarly to organic material, nutrients can also contribute to algae blooms if

high levels are discharged in a receiving body of water. Common nutrient measurements in wastewater (APHA, 2005) include total ammonia (TAN), total Kjeldhal nitrogen (TKN), nitrite (NO_2), nitrate (NO_3), orthophosphate (OP), and total phosphate (TP). Total ammonia measurements include the sum of ammonia (NH_3) and ammonium (NH_4^+) concentrations; the fraction of toxic NH_3 increases with an increase in pH (Boardman et al., 2004). Nitrite is an intermediate chemical that is formed during nitrification and denitrification, toxic nitrite (HNO_2) fractions increase as the pH decreases (Klaassen and Watkins, 2003). Nitrate is often a product of nitrification reactions and is toxic to aquatic life. However, NO_3 is significantly less toxic than NH_3 or NO_2. Total Kjeldhal nitrogen is the sum of organic nitrogen and TAN. Even though both forms can be utilized by microorganisms, the organic nitrogen fraction is not as readily available for biodegradation. Orthophosphate (PO_4^{3-}), also known reactive phosphorus is readily available for uptake by microorganisms. Total phosphorus includes OP and bound phosphorus.

Other important water quality parameters include temperature, pH, alkalinity, solids, and ion levels. The temperature and pH of a wastewater can influence the type of microorganisms that can inhabit and be generated during treatment. Temperature also largely influences kinetic rates. Alkalinity is often needed to drive certain reactions. For example, alkalinity is necessary to drive nitrification (Ebeling et al., 2006). Even though solids leach and contribute soluble organic matter and nutrients (Maillard et al., 2005), solids are not rapidly degradable by microorganisms. Solids can be classified as organic and inorganic fractions. Typically, bulk solids are settled out prior to treatment, unless a

digester is being used. Multivalent cations contribute to floc formation (Higgins and Novak, 1997; Eckenfelder, 2000). If the ratio of multivalent cations to monovalent cations is low then undesirable pin point flocs may be formed.

Kinetics and Stoichiometry

Determining kinetic coefficients and understanding stoichiometric relationships can significantly enhance design and operations of wastewater treatment systems. Examples of kinetic coefficients are listed in Table 1-1. This is only a fraction of kinetic parameters that can be collected and determined. However, these are among the common parameters reported.

Table 1-1. Commonly determined kinetic coefficients in wastewater engineering.

Kinetic paramater	Common Symbol	Definition
Biomass yield coeficient	Y	Mass of biomass generated per unit of substrate removed [e.g., gVSS/gCOD]
Endogenous decay rate	k_d	Mass of biomass lost during endogenous respiration per unit of time [e.g., 1/day]
Specific growth rate	μ	Biomass growth rate per unit time [e.g., 1/day]
Uptake rate	r	Mass of substrate/nutrients removed by biomass per unit time [e.g. units vary based on n^{th} order rate]
Specific uptake rate	r_b	Uptake rate normalized to biomass concentration

Understanding stoichiometric relationships is important to estimate biochemical reactions. For example, if readily available nitrogen is limiting (not available for cell synthesis) then nitrogen may be supplemented as ammonia to help drive the reaction. Understanding stoichiometry can also help an operator select what water parameter they would like to treat. For example, if a wastewater operator limits oxygen addition to the treatment process they can select for nitrate uptake (Sharrer et al., 2007).

Biological Treatment in Aquaculture

Biological treatment processes are often used in the aquaculture industry. Fixed film processes have been well developed and are typically implemented in recirculating aquaculture systems to facilitate the oxidation of ammonia to nitrate. Nitrate is usually the limiting factor for water reuse in recirculating systems. Once levels accumulate to unsafe levels, water exchanges are performed to decrease the concentration of nitrate (Timmons et al., 2002). Alternative biological treatment methods have been employed in highly stocked shrimp ponds. A shrimp farmer can create an algae-based heterotrophic system by supplementing a pond with a carbon source (e.g. molasses) to increase the carbon-nitrogen ratio to 10:1 (Burford et al., 2003, Samocha et al., 2007). Alternatively, a low protein feed can be used to minimize or eliminate the need for an external carbon source. The majority of microorganisms in these types of systems assimilate nitrogen instead of oxidizing ammonia to nitrate (Ebeling et al., 2006). However, this method increases oxygen demand and solids production significantly.

Shrimp in Aquaculture

Overview

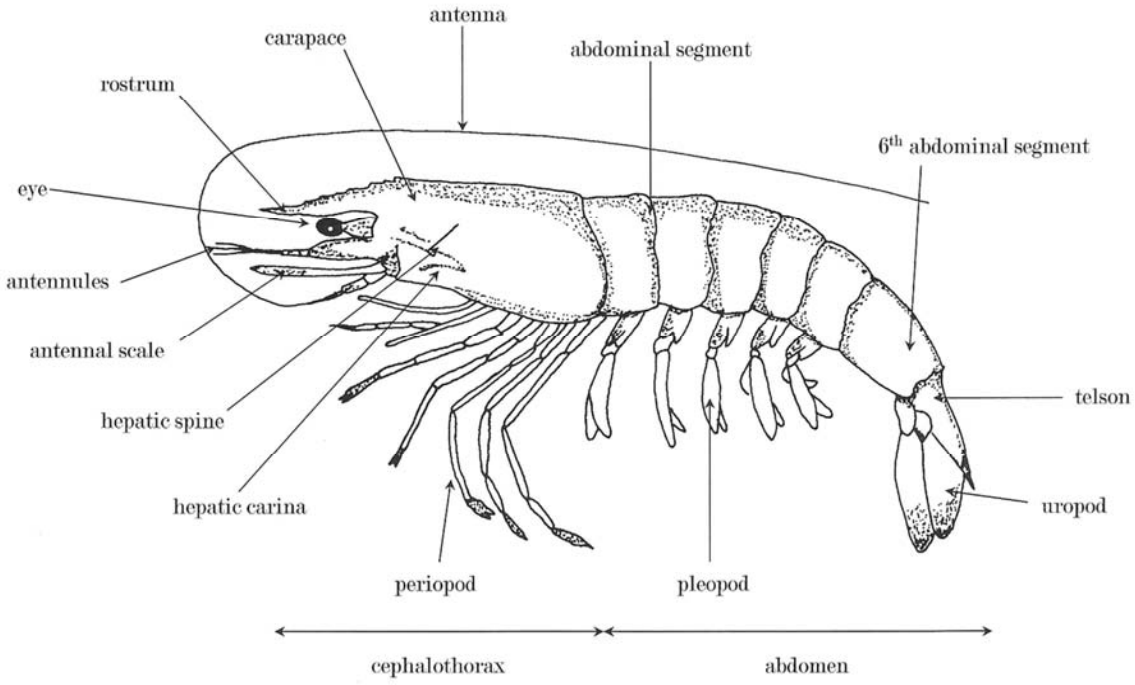
Shrimp are commonly farmed around the world as food and the majority of shrimp available in US markets are imported farmed animals. In order to produce shrimp domestically while stimulating local economies, food safety, and environmental sustainability, it is important to understand many aspects of shrimp and its culture. For this reason, this section includes a review of (1) *Litopenaeus vannamei*, (2) osmoregulation of penaeid shrimp, (3) influence of postlarvae age on salinity tolerance, (4) acclimation rate of postlarval shrimp to lower salinities, (5) toxicity of nitrogenous waste to shrimp, (6) growout techniques, and (7) microbial flocs in shrimp culture.

Litopenaeus vannamei

The Pacific White Shrimp, *Penaeus vannamei*, are native to the Pacific Ocean from northern Mexico to northern Peru (Perez-Farfante and Kensley, 1997) and recently have become the most commonly cultured species of Penaeid shrimp (Bonami et al. 1990). The general anatomy of *P. vannamei* can be observed in Figure 1-1. Species of Penaeid shrimp are commonly differentiated from each other by identifying features of the rostrum. Treece and Yates (1990) report that *P. vannamei* typically have between eight and nine (mode of eight) dorsal teeth and between one and two (mode of two) ventral teeth. Furthermore, they are typically described as having a transparent carapace with white legs. Female *P. vannamei* weighing between 30 and 45 grams typically spawn 100,000 to 250,000 eggs and hatching occurs about 16 hours after spawning and fertilization. *Penaeus vannamei* have the following production cycle: nauplii (for 2 days),

zoea (4 to 5 days), mysis (3 to 4 days), postlarvae (20 to 30 days), and subadult/adult (months). Typical growout of *P. vannamei* can take 4 to 5 months (for 15 to 25 g shrimp) or 6 to 8 months (for 30 to 40 g shrimp). *Penaeus vannamei* are often selected as the cultured specie of shrimp due to their availability and ability to be cultured in low salinity conditions.

Figure 1-1: Anatomy diagram of the Pacific White Shrimp, *L. vannamei*.



(Sketch, courtesy of Dr. Ewen McLean)

Osmoregulation of Penaeid Shrimp

Crustaceans and their ability to osmoregulate fall into two major categories, stenohaline and euryhaline (Pequeux, 1995). Stenohaline crustaceans cannot cope with changing salinities while euryhaline crustaceans can. Euryhaline crustaceans have the ability to osmoregulate ion concentrations at fairly stable levels despite the changes in ambient

salinity. Decapod crustaceans of the genus *Penaeus* have demonstrated in countless studies that they can survive in low salinity waters and have thus proved they are hyperosmotic regulators. A small sample of studies demonstrating the wide range of species that are hyperosmotic regulators within the genus *Penaeus* include; Mair (1980), Castille and Lawrence (1981), Cawthorne (1983), Bouaricha et al. (1994), Bray et al. (1994), Kumulu and Jones (1995), and Rosas et al. (1999). It was reported by Castille and Lawrence (1981) that *P.* setiferus, *P. stylirostris*, and *P. vannamei* are stronger osmoregulators at low salinities than *P. aztecus* and *P. duorarum*.

There are many inorganic ions present in the hemolymph of shrimp, but the primary ions present are Na^+ and Cl^- and the levels of these ions are maintained and regulated by what Pequeux (1995) refers to as (1) "limiting processes", and (2) "compensatory processes".

The "limiting processes" as described by Pequeux (1995) maintains the ionic levels in the hemolymph by minimizing diffusion through and by tissues. The epithelial structures that may be involved in salt transportation include the body wall, the gastrointestinal tract, and the excretory organs. The body wall accounts for a relatively large surface area of shrimp, and not much is known about the diffusivity of ions through this surface. However, the diffusivity through the walls for shrimp of the genus *Penaeus* is most likely minimal, except, perhaps during molting. Molting frequency from every few days to weekly intervals, varies by species and by age. The gastrointestinal tract may play a role in osmoregulation because shrimp will end up ingesting a lot of the water medium in which they live. The excretory glands are not suspected in playing much of a role in

hyperosmotic regulators. However, some evidence suggests that these organs may regulate divalent ions such as Mg^{2+}, Ca^{2+}, and SO_4^{2-}.

The "compensatory processes" is considered to play a far more prominent role in osmoregulation than the "limiting processes" as reported by Pequeux (1995). The gills basically comprise of anterior and posterior structures and are responsible for respiration and osmoregulation of ions. As a general point by Lucu (1990), Pequeux (1995), and more specifically for *P. vannamei* (Palacios et al., 2004b), most of the osmoregulation takes place in the posterior region of the gill. Pequeux (1995) outlines the posterior gill as comprising of both a basolateral membrane and an apical membrane. The basolateral membrane consists of (1) the Na^+/K^+ exchange pump, (2) the K^+ leak pathway, (3) the Cl^- channel, and (4) the Na^+-K^+-Cl^- cotransport. The apical membrane consists of (1) the Na^+/NH_4^+ exchange mechanism, (2) the Na/H exchange mechanism, and (3) the Cl^-/HCO_3^- exchange mechanism. These leak pathways, cotransports, exchange pumps, and exchange mechanisms all play a specific role in osmoregulation and more detail can be extracted from Pequeux (1995).

Influence of Postlarvae Age on Salinity Tolerance

Numerous studies have reported that salinity tolerance increases with age for postlarval *P. vannamei*. Using salinity shock methods, Aquacop (1991) demonstrated that 2 day PL were not as salinity tolerant as 20 day PL. Ogle et al. (1992) reported that 8 day PL were more sensitive to changes in salinity than 20 day PL. For younger PL, between 1 and 7 days old, Samocha et al. (1998) demonstrated an increase in salinity tolerance with age

over this age range. A recent study performed by McGraw et al. (2002) further strengthened the claim that PL tolerance to low levels of salinity increases with PL age. Demonstrating that salinity tolerance was significantly less for 10 day PL compared to 15 and 20 day PL. There was no significant difference between 15 and 20 day PL tolerance. Similar work by Saoud et al. (2003) evaluated salinity tolerance in inland saline well waters as opposed to synthetic seawater. This study also demonstrated that 10 day PL were less tolerant compared to 15 and 20 day PL.

Due to poor survival, McGraw et al. (2002) suggested that PL younger than 15 days of age should not be acclimated to salinities below 4 ppt. Based on 48 hour survival testing, PL older than 15 days should be able to acclimate and survive at 1 ppt salinity.

Salinity tolerance of postlarval *P. japonicus*, as reported by Charmantier et al. (1988), demonstrated similar results to those found for postlarval *P. vannamei*. Work performed by Bouaricha et al. (1994) investigated the osmoregulation structure and development by which *P. japonicus* postlarvae can effectively osmoregulate to tolerate low levels and changes in salinity. By the postlarval stage, salt transporting epithelium will be developed in the branchiostegites, pleura, gills and epipodites. As the shrimp matures from the postlarvae to the juvenile stage, only the gills and epipodites continue to develop osmoregulatory capacity. It was concluded for *P. japonicus* that the maximum osmoregulation capacity occurs at 5 and 6 day PL stages.

Furthermore, Palacios et al. (2004a) reported that gill area corrected to the postlarvae length, was significantly greatest for 14 day postlarvae. This was proven to be the case for both the anterior and posterior gills which were noted to be primarily responsible for respiration and osmoregulation respectively. Based on all the literature to date for *P. vannamei*, the peak osmoregulation capacity probably occurs neat PL15.

Acclimation Rate of Postlarval Shrimp to Lower Salinities

Postlarval *P. vannamei* are ready for low salinity/freshwater conditions near 15 days old (Van Wyk et al., 1999; McGraw et al., 2002) because their gills have developed proper osmoregulation ability due to extensive filament branching of this epithelium. This can be checked under the microscope. Acclimation of postlarvae to low salinities is crucial for high survival rates and Van Wyk et al. (1999) suggested that the salinity should be reduced by 50% over 8 hour periods until the target salinity is achieved. For example reducing the salinity from 16 ppt to 8 ppt should be done over 8 hours at a salinity reduction rate of 1 ppt per hour. Reduction from 1 ppt to 0.5 ppt should also take 8 hours but at a much slower reduction rate of 0.063 ppt per hour. This reduced salinity acclimation rate at lower salinity levels is crucial for survival of postlarvae at very low salinities as supported by McGraw and Scarpa (2004).

A study by McGraw et al. (2002) investigated and compared 10, 15, and 20 day old postlarvae survival rates for three different salinity acclimation rates down to a target salinity endpoint as low as 1 ppt. The rates tested were 19, 25, and 47 percent salinity reduction per hour. Survival was not significantly different for the various salinity

reduction levels at any of the postlarvae age groups. Survival rates were "good" for these relatively rapid salinity reduction rates, this implies that the slower rates suggested by Van Wyk et al. (1999) may be on the conservative side. However, these slower rates have demonstrated for many years it works for numerous farmers, so these acclimation rates should still be the rule of thumb until more evidence is provided demonstrating faster rates are consistently "good" and result in long term survival.

Toxicity of Nitrogenous Waste to Shrimp

Ammonia is the dominant form of nitrogen waste excreted by aquatic organisms as a by-product of protein metabolism. Ammonia is excreted directly across the gills via diffusion and ion exchange mechanisms (Kormanik and Cameron, 1981; Regnault, 1987). Ammonia is also leached out of feces and uneaten feeds. There are two forms of ammonia, ionized (NH_4^+) and unionized (NH_3). Unionized ammonia is very toxic to freshwater and marine organisms and the fraction of unionized ammonia is a function of pH, temperature and salinity (Boardman et al., 2004). Because the unionized ammonia can easily pass through the gills of an aquatic organism, it is thought that this is the main route of entry (Hampson, 1976). Shrimp exposed to high levels of ammonia will accumulate ammonia in the hemolymph until death (Chen and Kou, 1993). As a general rule of thumb, the chronic unionized ammonia concentration should be maintained below 0.1 mg/L. The 24 hour, 50% lethal concentration (LC50) was determined for 16-30 day old PL *P. vannamei* in freshwater (Scarpa et al., 2000). The LC50 was approximately 12.5 mg/L total ammonia nitrogen and 1.5 mg/L unionized ammonia. In seawater, Frias-Espericueta et al. (2000) reported for 12 day old postlarvae a 24, 48, 72, and 96 hour

LC50 of 17.9, 12.5, 12.2, and 12.2 mg/L total ammonia nitrogen respectively. Furthermore, comparing ammonia toxicity results using postlarvae in the Frias-Espericueta et al. (2000) study to the results found using juvenile in Frias-Espericueta et al. (1999), it was apparent that tolerance to ammonia increases with age.

It was reported that lower salinities increased the toxic effect of ammonia on juvenile *P. vannamei* (Lin and Chen, 2001). The same salinity effects on the toxicity of ammonia were also observed for both *P. penicillatus* and *P. chinensis* (Chen and Lin, 1991; Chen and Lin, 1992). Similar effects of salinity were reported by Liu and Chen (2003) for the toxicity of nitrite on juvenile *P. vannamei.* As the salinity was decreased the toxicity of nitrite increased significantly.

In addition to ammonia being directly toxic to shrimp it also has indirect negative impacts. The presence of ammonia, or any other toxic substance, induces stress on the shrimp and consequently the immune system is suppressed. A study by Liu and Chen (2004) investigated the effect of high ammonia concentrations on the immune response of juvenile *P. vannamei* during the intermolt stage while being exposed to the infectious *Vibrio alginolyticus*. Exposure to high levels of ammonia during the intermolt demonstrated a significant suppression of the immune system and increased susceptibility to *V. alginolyticus* which resulted in high levels of mortality.

Growout Techniques

There are numerous growout methods used for shrimp farming around the world. They include extensive, semi-intensive, intensive, and super-intensive growout techniques (FAO, 2007)

Extensive growout systems are commonly implemented in tidal ponds and often do not require aeration or pumping of water. These ponds are typically stocked with four to ten postlarvae (PLs) per meter squared and may yield 150 to 500 kilograms of shrimp per hectare (2.47 acres). These shrimp typically feed on natural biota (e.g. algae and algae grazers) enhanced through fertilization. This natural diet is often supplemented with a low cost/protein feed once a day. Usually, one to two crops of slow growing shrimp (11 to 12 gram shrimp) are produced a year.

Semi-intensive growout systems are commonly implemented in ponds. These ponds are stocked at higher rates (10 to 30 PL per meter squared) compared to extensive techniques, therefore they require aeration and pumping of water. Semi-intensive farming often produce 500 to 2,000 kilograms of shrimp per hectare. Fertilization is often used to enhance natural food production. Formulated diets, typically higher in protein compared to extensive-based feeds, are often offered to shrimp two to three times a day. Two to three crops a year can be produced per pond.

Intensive growout techniques have been developed to produce high quantities of shrimp, especially in inland low-salinity ponds. Stocking densities can vary from 60 to 300 PLs

per meter squared and production yields from 7,000 to 20,000 kilograms per hectare have been observed (claims as high as 50,000 kg/ha have been made). Two to three crops can be produced a year. Heavy aeration and water movement is implemented due to the relatively high biological loading from the shrimp and natural biota. Carbon supplementation is often used (e.g. carbon: nitrogen of 10:1) to promote the assimilation of nitrogen to generate algae-based microbial flocs (that largely consist of heterotrophic bacteria and algae). Shrimp are often fed a high quality formulated feed 4 to 5 times a day. Two to three crops a year can be produced per system.

Research of super-intensive growout techniques have been conducted in the United States using recirculating aquaculture systems. Among other benefits, this approach can conserve water, is more biosecure, and is environmentally friendly compared to pond production. This approach relies heavily on aeration and water movement and requires an educated staff to manage these systems. Carbon is often supplemented to these systems to assimilate nitrogen while producing algae-based microbial flocs in greenhouses. Rapid growth rates of 1.5 grams per week have been observed over 3 to 5 months. Over this period of time, production levels as great as 28,000 to 68,000 kilograms of shrimp per hectare have been reported.

Microbial Flocs in Shrimp Culture

Previous studies have demonstrated that *L. vannamei* can feed on and utilize nutrients of natural biota and bacterial supplements, such as algae-based microbial flocs (Avnimelech, 1999; Moss et al., 2000; Moss, 2002; Cuzon et al., 2004; Hari et al., 2004;

Izquierdo et al., 2006). If these alternative sources of nutrients are used, it often reduces the amount of protein required in the feed and consequently reduces the operation expenses associated with purchasing commercial feed. Nearly 50% of the operation costs are attributed to feed (Wyk et al., 1999). Understanding how to produce microbial flocs using bioreactors, while treating the tilapia effluent waste stream, is a new approach for producing a potential microbial floc that can be used for feed replacement. Furthermore, understanding the optimum microbial supplementation feed replacement rate is crucial for the production of a consistently healthy shrimp crop. Even though algae-based microbial floc systems have been extensively studied, these systems have an extremely high oxygen demand which increases the operation costs considerably (Tacon et al. 2002, Burford et al. 2003). They also have very little control on how much of the algae-based microbial floc is consumed. Treating a waste stream to produce microbial flocs externally from the shrimp culture systems can minimize the oxygen requirements because aerobic suspended solids processes only require 1 mg/L of oxygen (Grady et al., 1999). Since tilapia wastewater contains relatively high levels of organic matter and nutrients, using aerobic bioreactors to produce algae-absent microbial flocs while treating the water can be accomplished using the SBR process (Grady et al., 1999; Metcalf and Eddy, 2003).

Dietary Requirements of Shrimp

Carbohydrates

Carbohydrates include, but are not limited to sugar, starches, and fiber. Since carbohydrates are inexpensive, they are often maximized as an ingredient in commercial feeds. If carbohydrates are sufficient in a diet, protein requirements can be reduced. This is called protein sparing. One of the most effective carbohydrates used in shrimp feeds are wheat starches. These starches are highly digestible (90%) under amylase and glycosidase actions (Cousin et al., 1993). Interestingly, suppressed growth ($P < 0.05$) of shrimp were observed for juvenile shrimp fed simple sugars compared to shrimp fed dextrin or starches (Shiau and Peng, 1992).

Lipids

Even though lipids are technically fat-soluble (hydrophobic) molecules, the term lipid in the aquaculture industry is often used interchangeably with total fat. Lipids (fats) include animal or plant fats, fatty acids, phospholipids, oils, and sterols (e.g. cholesterol). Similar to protein requirements, lipid level requirements decrease with shrimp maturity. For example, shrimp less than 0.25 g require approximately 15% lipids in their diet while shrimp greater than 3 g require about 6.5% lipids in their diet. Essential fatty acids, that cannot be synthesized by shrimp, include linoleic acid (LOA, 18:2n6), linolenic acid (LNA, 18:3n3), eicosapentaenoic acid (EPA; 20:5n3), and decosahexaenoic acid (DHA; 22:6n3). Studies by Gonzalez-Felix et al. (2003a, 2003b) reported that polyunsaturated fatty acid (LOA and LNA) were inferior to highly unsaturated fatty acid (EPA and DHA). Even though both n3 and n6 highly unsaturated fats are required in shrimp diets,

n3 fatty acids promote faster growth of shrimp over n6 fatty acids. Fish oil is typically used to supply required fatty acids to shrimp feed (Lim et al., 1997; Zhou et al., 2007). Phospholipids are compounds that are required to build cell membranes. Cholesterol is a required in shrimp diets. De-oiled soy lecithin is also reported to be beneficial as a dietary ingredient because it aids in the transport and dispersion of cholesterol to shrimp tissue (D'Abramo, 1997; Gong et al., 2000).

Vitamins

Vitamins are organic molecules that are required by all living organisms because they are crucial for various biochemical functions, such as hormones, antioxidants, mediators of cell signaling, precursors for coenzyme, and immune system boosters. Most vitamins are water soluble except for vitamins A, D, E, and K. Young shrimp require higher levels of vitamins. Stable forms of vitamin C (Moreau et al., 1998) and vitamin E (He and Lawrence, 1993b) are often added to diets to improve shrimp survival and growth. Minimum levels of stable vitamin C were reported as 30 mg/kg (He and Lawrence, 1993a). Levels of 99 mg/kg of vitamin E are sufficient (He and Lawrence, 1993b).

Amino acids

Amino acids are the building blocks for peptides and polypeptides (proteins). Protein requirements of shrimp decrease as shrimp mature. For example, shrimp less than 0.25 g should be fed a 45 to 50% crude protein feed while shrimp greater than 3 g can be fed a 30 to 35% crude protein feed. Of the 20 standard amino acids only 10 are considered essential in a shrimp diet. The non-essential amino acids can be synthesized by the

shrimp from the essential ones. The indispensable amino acid requirements are approximated in Table 1 after Akiyama et al. (1991) and Fox et al. (1995).

Table 1-2. Target levels of essential amino acids for diet formulation and percent amino acid as measured in shrimp flesh on a protein basis.

Indispensable amino acid	% of dry diets[1]	% of shrimp protein[2]
Arginine	2.0	5.8
Histidine	0.9	2.1
Isoleucine	0.9	3.5
Leucine	1.8	5.4
Lysine	1.9	5.3
Methionine	0.9	2.4
Phenylalanine	1.3	4.0
Threonine	1.1	3.6
Tryptophan	0.3	0.8
Valine	1.0	4.0

Minerals

Minerals are inorganic compounds that include simple or complex forms of ions such as calcium, magnesium, potassium, chloride, and sulfur. Trace minerals include iron, iodine, manganese, copper, cobalt, zinc, selenium, and zinc ions (Davis et al., 1992). The calcium to phosphorus ratio in a diet is important because too high of a ratio may interfere with phosphorus absorption. Typically, in low salinity waters, approximately 2.5% calcium should be incorporated into a feed (Van Wyk et al., 1999). Calcium,

magnesium, and phosphorus are vital to shrimp because they represent major components of the exoskeleton (Vijayan and Diwan, 1996) and are important for normal physiological processes (Endo et al. 2002; Xie et al. 2004).

Influence of Diet on Low Salinity Tolerance

Diet plays a significant role in the ability of shrimp to osmoregulate at low salinities. It has been demonstrated that fatty acids influence the activity of Na^+/K^+-ATPase in the gills of shrimp (Morohashi et al., 1991; Palacios et al., 2004b). For *P. vannamei* postlarvae (Palacios et al., 2004b), 85% of the Na^+/K^+-ATPase activity was in the posterior gills as opposed to the anterior gills. When starved, the Na^+/K^+-ATPase activity decreased in both the anterior and posterior regions of the gills, but the ratio of activity remained unchanged.

A study by Palacios et al. (2004b) reported that highly unsaturated fatty acids influenced the survival rates of postlarval *P. vannamei* exposed to low salinities. It was demonstrated that the survival rates were significantly greatest for the medium highly unsaturated fatty acid levels. This corresponded to optimum fatty acid composition of the gills and to development of the largest gill surface area. This resulted in enhanced osmoregulation for the postlarvae fed medium highly unsaturated fatty acid levels by optimizing the Na^+/K^+–ATPase and carbonic anhydrase activities. Furthermore, Rees et al. (1994) reported that *Litopenaeus monodon* postlarvae that were fed high highly

unsaturated fatty acid diets developed more elaborate and “branchlike” gill structure. This would increase the surface area of the gills, resulting in better osmoregulation ability.

Literature Cited

Akiyama, D.M., Dominy, W.G., Lawrence, A.L. 1991. Penaeid shrimp nutrition for the commercial feed industry — revised. American Soybean Association. AQ32 (1991), p. 35.

APHA. 2005. Standard Methods for the Examination of Water and Wastewater, 21st edition. Edited by Clesceri, Greenberg and Trussell. Washington DC, US.

Aquacop. 1991. Modeling of resistance to salinity shocks of *Penaeus vannamei* postlarvae. Aquatic Living Resources 4: 169-174.

Avnimelech, Y. 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176:227-235.

Beeman, R.E., Reitberger, J.H. 2003. An integrated industrial management facility for biological treatment of high nitrate and carbonaceous wastewater. Environmental Progress 22: 37-45.

Boardman, G.D., Starbuck, S.M., Hudgins, D.B., Li, X., Kuhn, D.D. 2004. Toxicity of ammonia to three marine fish and three marine invertebrates. Environmental Toxicology 19: 134-142.

Bonami, J.R., Brehelin, M., Mari, J., Trumper, B., Lightner, D.V. 1990. Purification and characterization of IHHN virus of penaeid shrimps. Journal of General Virology 71: 2657-2664.

Bouaricha, N., Charmantier-Daures, M., Thuet, P., Trilles, J.P., Charmantier, G. 1994. Ontogeny of osmoregulatory structures in the shrimp *Penaeus japonicus* (Decapoda Penaeidae). Biological Bulletin 186: 29-40.

Bray, W.A., Lawrence, A.L., Leung-Trujillo, J.R. 1994. The effect of salinity on growth and survival of *Penaeus vannamei*, with observations on the interaction of IHHN virus and salinity. Aquaculture 122: 133-146.

Burford, M.A., Thompson, P.J., McIntosh, R.P., Bauman, R.H., Pearson, D.C. 2003. Nutrient and microbial dynamics in high-intensity zero-exchange shrimp ponds in Belize. Aquaculture 219: 393-411.

Castille, F.L., Lawrence, A.L. 1981. The effect of salinity on the osmotic, sodium, chloride, concentrations in the hemolymph of euryhaline shrimp of the genus *Penaeus*. Comparative Biochemistry and Physiology 68A: 75-80.

Cawthorne, D.F., Beard, T., Davenport, J., Wickins, J.F. 1983. Responses of juvenile *Penaeus monodon* Fabricius to natural and artificial sea waters of low salinity. Aquaculture 32: 165-174.

Charmantier, G., Charmantier-Daures, M., Bouaricha, N., Thuet, P., Eiken, D.E., Trilles, J.P. 1988. Ontogeny of osmoregulation and salinity tolerance in two decapod crustaceans: *Homarus americanus* and *Penaeus japonicus*. Biological Bulletin 175: 102-110.

Chen C.C., Lin, C.Y. 1991. Lethal effects of ammonia and nitrite on *Penaeus penicillatus* juveniles at two salinity levels. Comparative Biochemistry and Physiology 100C: 477-482.

Chen C.C., Lin, C.Y. 1992. Lethal effects of ammonia on *Penaeus chinensis* Osbeck juveniles at different salinity levels. Journal of Experimental Marine Biology and Ecology 156: 139-148.

Chen, J.C., Kou, Y.Z. 1993. Accumulation of ammonia in the hemolymph of *Penaeus monodon* exposed to ambient ammonia. Aquaculture 109: 177-185.

Cousin, M., Cuzon, G., Blanchet, E. and Ruelle, F. 1993. Protein requirements following an optimum dietary energy to protein ration for *Penaeus vannamei* juveniles. In: Kaushik and Luquet, P., Editors, 1993. *4.* Int. Symp. Fish Nutrition and Feeding, Biarritz (France), 24–27 Jun 1991 Colloq.INRA vol. 6, pp. 599–606.

Cuzon, G., Lawrence, A., Gaxiola, G., Rosas, C., Guillaume, J. 2004. Nutrition of *Litopenaeus vannamei* reared in tanks or ponds. Aquaculture 235: 513-551.

Davis, D.A., Lawrence, A.L., Gatlin, D.M. 1992. Mineral requirements of *Penaeus vannamei*: a preliminary examination of the dietary essentiality for thirteen minerals. Journal of the World Aquaculture Society 23: 8–14

D'Abramo, L., 1997. Triacylglycerols and fatty acids. In: *Crustacean Nutrition* Editors, D'Abramo, L., Conklin, D. Akiyama, D. 1997. Advances in World Aquaculture vol. 6.

Ebeling, J.M., Timmons, M.B., Bisogni, J.J. 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia–nitrogen in aquaculture systems. Aquaculture 257: 346-358.

Endo, H., Takagi, Y. Watanabe, T. 2002. Crustocalcin: A study on potential function of a skeletal Ca2+-binding protein of kuruma prawn Penaeus japonicus. Zoological Science 19: 1480.

Eckenfelder, W.W. 2000. Industrial water pollution control. 3rd ed. Singapore: McGraw-Hill.

FAO (Food and Aqriculture Organization of the United Nations). 2007. Cultured aquaculture species information programme *Penaeus vannamei* (Boone, 1931). Food and Aqriculture Organization of the United Nations. Online,http://www.fao.org/fi/website/FIRetrieveAction.do?dom=culturespecies&xml=Litopenaeus_vannamei.xml

Fox, J.M., Lawrence, A.L., Li-Chan, E. 1995. Dietary requirement for lysine by juvenile *Penaeus vannamei* using intact and free amino acid sources. Aquaculture 131: 279-290.

Frias-Espericueta, M.G., Harfush-Melendez, M., Osuna-Lspez, J.I., Paez-Osuna, F. 1999. Acute toxicity of ammonia to juvenile shrimp *Litopenaeus vannamei* Boone. Bullentin of Environmental Contamination and Toxicology 62: 646-652.

Frias-Espericueta, M.G., Harfush-Melendez, M., Paez-Osuna, F. 2000. Effects of ammonia on mortality and feeding of postlarvae shrimp *Litopenaeus vannamei*. Bullentin of Environmental Contamination and Toxicology 65: 98-103.

Gong, H., Lawrence, A.L., Jiang, D.H., Castille, F.L. Gatlin, D.M. 2000. Lipid nutrition of juvenile *Litopenaeus vannamei*: Dietary cholesterol and de-oiled soy lecithin requirements and their interaction. Aquaculture 190: 305–324.

Gonzalez-Felix, M.L., Lawrence, A.L., Gatlin, D.M. Perez-Velazquez, M. 2003a. Nutritional evaluation of fatty acids for the open thelycum shrimp, *Litopenaeus vannamei*: I. Effect of dietary linoleic and linolenic acids at different concentrations and ratios on juvenile shrimp growth, survival and fatty acid composition. Aquaculture Nutrition 9: 105–113.

Gonzalez-Felix, M.L., Gatlin, D.M., Lawrence, A.L. Perez-Velazquez, M. 2003b. Nutritional evaluation of fatty acids for the open thelycum shrimp, *Litopenaeus vannamei*: II. Effect of dietary n-3 and n-6 polyunsaturated and highly unsaturated fatty acids on juvenile shrimp growth, survival, and fatty acid composition. Aquaculture Nutrition 9: 115–122.

Grady, C.P., Daigger, G.T., Lim, H.C. 1999. Biological wastewater treatment. 2nd ed. New York: Marcel Dekker.

Hagopian, D.S., Riley, J.G. 1998. A closer look at the bacteriology of nitrification. Aquacultural Engineering 18: 223-244.

Hampson, B.L. 1976. Ammonia concentration in relation to ammonia toxicity during a rainbow trout rearing experiment in a closed freshwater-seawater system. Aquaculture 9: 61-70.

Hari, B., Madhusoodana Kurup, B., Varghese, J.T., Schrama, J.W., Verdegem, M.C.J. 2004. Effects of carbohydrate addition on production systems in extensive shrimp culture systems. Aquaculture 241: 179-194.

He, H. Lawrence, A.L. 1993a. Vitamin C requirements of the shrimp *Penaeus vannamei*. Aquaculture 114: 305–316.

He, H. Lawrence, A.L. 1993b. Vitamin E requirement of Penaeus vannamei. Aquaculture 118: 245–255.

Higgins, M.J., Novak, J.T. 1997. Characterization of exocellular protein and its role in bioflocculation. Journal of Environmental Engineering - American Society of Chemical Engineers 123: 479-485.

Izquierdo, M., Forster, I., Divakaran, S., Conquest, L., Decamp, O. 2006. Effect of green and clear water and lipid source on survival, growth and biochemical composition of Pacific white shrimp *Litopenaeus vannamei*. Aquaculture Nutrition 12: 192-202.

Klaassen, C.D., Watkins, J.B. 2003. Casarett and Doull's: Essentials of toxicology. New York: MGraw Hill.

Kormanik, G.A., Cameron, J.N. 1981. Ammonia excretion in the seawater blue crab (*Callinectes sapidus*) occurs by diffusion and not by Na^+/NH_4^+ exchange. Journal of Comparative Physiology 141B: 457-462.

Kumulu, M., Jones, D.A. 1995. Salinity tolerance of hatchery-reared postlarvae of *Penaeus indicus* H. Milne Edwards originating from India. Aquaculture 130: 287-296.

Lim, C., Ako, H., Brown, C.L. Hahn, K. 1997. Growth response and fatty acid composition of juvenile *Penaeus vannamei* fed different sources of dietary lipid. Aquaculture 151: 143–153.

Lin, Y.C., Chen, J.C. 2001. Acute toxicity of ammonia on *Litopenaeus vannamei* Boone juveniles at different salinity levels. Journal of Experimental Marine Biology and Ecology 259: 109-119.

Liu, C.H., Chen, J.C. 2003. Acute toxicity of nitrite on *Litopenaeus vannamei* Boone juveniles at different salinity levels. Aquaculture 224: 193-201.

Liu, C.H., Chen, J.C. 2004. Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus*. Fish and Shellfish Immunology 16: 321-334.

Lucu, C. 1990. Ionic regulatory mechanisms in crustacean gill epithelia. Comparative Biochemistry and Physiology 97A: 297-306.

Maillard, V.M., Boardman, G.D., Nyland, J.E., Kuhn, D.D. 2005. Water quality and sludge characterization at raceway-system trout farms. Aquacultural Engineering 33: 271-284.

Mair, J.McD. 1980. Salinity and water-type preferences of four species of postlarval shrimp (*Penaeus*) from west Mexico. Journal of Experimental Marine Biology and Ecology 45: 69-82.

McGraw, W.J., Davis, D.A., Teichert-Coddington, D., Rouse, D.B. 2002. Acclimation of *Litopenaeus vannamei* postlarvae to low salinity: influence of age, salinity endpoint, and rate of salinity reduction. Journal of the World Aquaculture Society 33: 78-84.

McGraw, W.J., Scarpa, J. 2004. Mortality of freshwater-acclimated *Litopenaeus vannamei* associated with acclimation rate, habituation period, and ionic challenge. Aquaculture 236: 285-296.

Metcalf and Eddy. 2003. Wastewater engineering: treatment and reuse. 4th ed. New York: McGraw-Hill.

Moreau, R., Cuzon, G. Gabaudan, J. 1998. Efficacy of silicone-coated ascorbic acid and ascorbyl-2-polyphosphate to fast-growing tiger shrimp (*Penaeus monodon*). Aquaculture Nutrition 4: 23–29.

Morohashi, M., Tsuchiya, K., Mita, T., Kawamura, M. 1991. Identification of (Na, K) ATPase inhibitor in brine shrimp, *Artemia salina*, as long-chain fatty acids. Comparative Biochemistry and Physiology 161B: 69-72.

Moss, S.M., LeaMaster, B.R., Sweeney, J.N. 2000. Relative abundance and species composition of gram-negative, anaerobic bacteria associated with the gut of juvenile white shrimp, Litopenaeus vannamei, reared in oligotrophic well water and eutrophic pond water. Journal of the World Aquaculture Society 31: 255-263.

Moss, S. 2002.. Dietary importance of microbes and detritus in penaeid shrimp aquaculture. Pages 1-18 *in* C. S. Lee, P. O'Bryen, editors. Microbial Approaches to Aquatic Nutrition within Environmentally Sound Aquaculture Productions Systems. The World Aquaculture Society, Baton Rouge, Louisiana, US.

Ogle, J.T., Beaugez, K., Lotz, J.M. 1992. Effects of salinity on survival and growth of postlarval *Penaeus vannamei*. Gulf Research Reports 8: 415-421.

Palacios, E., Bonilla, A., Luna, D., Racotta, I.S. 2004a. Survival, Na^+/K^+-ATPase and lipid responses to salinity challenge in fed and starved white pacific shrimp (*Litopenaeus vannamei*) postlarvae. Aquaculture 234: 497-511.

Palacios, E., Bonilla, A., Perez, A., Racotta, I.S., Civera, R. 2004b. Influence of highly unsaturated fatty acids on the response of the white shrimp (*Litopenaeus vannamei*) postlarvae to low salinity. Journal of Experimental Marine Biology and Ecology 299: 201-215.

Pequeux, A. 1995. Osmotic regulation in crustaceans. Journal of Crustacean Biology 15: 1-60.

Perez-Farfante, I., Kensley, B. 1997. Penaeoid and sergestoid shrimps and prawns of the world. Paris: Memories du Museum National D'Historie Naturelle.

Reese, J.F., Cure, K., Piyatiratitivorakul, S., Sorgeloos, P., Menasveta, P. 1994. Highly unsaturated fatty acid requirements of *Penaeus monodon* postlarvae: an experimental approach based on *Artemia* enrichment. Aquaculture 122: 193-207.

Regnault, M. 1987. Nitrogen excretion in marine and fresh-water Crustacea. Biological Review 62: 1-24.

Rittmann, B.E., McCarty, P.L. 2001. Environmental biotechnology: principles and applications. 1st ed. New York: McGraw-Hill.

Rosas, C.G., Ocampo, L., Gaxiola, G., Sanchez, A. 1999. Effect of salinity on survival, growth, and oxygen consumption of postlarvae (PL10-PL21) of *Litopenaeus setiferus*. Journal of Crustacean Biology 19: 244-251.

Samocha, T.M., Guajardo, H., Lawrence, A.L., Castille, F.L., Speed, M., McKee, D.A., Page, K.I. 1998. A simple stress test for *Penaeus vannamei* postlarvae. Aquaculture 165: 233-242.

Samocha, T.M., Patnaik, S., Speed, M., Ali, A.-M, Burger, J.M., Almeida, R.V., Margasanto Harisanto, Z.A., Horowitz A., Brock, D.L. 2007. Use of molasses as carbon source in limited discharge nursery and grow-out systems for Litopenaeus vannamei. Aquacultural Engineering 36, 184-191.

Saoud, I.P, Davis, D.A., Rouse, D.B. 2003. Suitability studies of inland well waters for *Litopenaeus vannamei* culture. Aquaculture 217: 373-383.

Scarpa, J., Ladson, J.L., Stodghill, N. 2000. Ammonia tolerance of freshwater-acclimated marine shrimp Penaeus vannamei. Proceedings In: Aquaculture America 2000: 298.

Sharrer, M.J., Tal, Y., Ferrier, D., Hankins, J.A., Summerfelt, S.T., 2007. Membrane biological reactor treatment of a saline backwash flow from a recirculating aquaculture system. Aquacultural Engineering 36, 159-176.

Shiau, S.Y., Peng, C.Y. 1992. Utilization of different carbohydrates at different dietary-protein levels in grass prawn, *Penaeus monodon*, reared in seawater. Aquaculture 101, 241-250.

Tacon, A., Cody, J.J., Conquest, L.D., Divakaran, S., Forster, I.P., Decamp, O.E. 2002. Effect of culture system on the nutrition and growth performance of Pacific white shrimp, Litopenaeus vannamei (Boone) fed different diets. Aquaculture Nutrition 8: 121-137.

Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J., 2002. Recirculating Aquaculture Systems, 2nd Ed. Cayuga Aqua Ventures, Ithaca, New York, US.

Treece, G.D., Yates, M.E. 1990. Laboratory manual for the culture of penaeid shrimp larvae. TAMU-SG-88-202(R). Marine Advisory Service Sea Grant College Program, Texas A&M University, College Station, Texas, US.

Van Wyk, P., M. Davis-Hodgkins, C. R. Laramore, K. Main, J. Mountain, Scarpa, J. 1999. Farming marine shrimp in recirculating freshwater production systems: a practical manual. FDACS Contract #4520. Florida Department of Agriculture Consumer Services, Tallahassee, Florida, US.

Vijayan, K.K., Diwan, A.D. 1996. Fluctuations in Ca, Mg, and P levels in the hemolymph, muscle, midgut gland, and exoskeleton during the molt cycle of the Indian white prawn, Penaeus indicus (Decapoda: *Penaeidae*). Comparative Biochemistry and Physiology 114A: 91-97.

Xie, X-L., Chen, Q-X., Lin, J-C., Wang, Y. 2004. Purification and some properties of β-N-acetyl-D-glucosaminidase from prawn (*Penaeus vannamei*). Marine Biology 146: 143-148.

Zhou, Q.C., Li, C.C., Liu, C.W., Chi, S.Y., Yang, Q.H. 2007. Effects of dietary lipid sources on growth and fatty acid composition of juvenile shrimp, *Litopenaeus vannamei*. Aquaculture Nutrition 13: 222-229.

Chapter 2:

Evaluation of Tilapia Effluent with Ion Supplementation for Marine Shrimp Production in a RAS

Published Manuscript:

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Abstract

Reuse of fish effluent for the culture of marine shrimp, such as Pacific white shrimp *Litopenaeus vannamei*, could provide an opportunity for the US shrimp farming industry to ease constraints (e.g. environmental concerns and high production costs) that have limited them in the past. In this study under laboratory-scale conditions, the feasibility of culturing *L. vannamei* in effluents derived from a commercial facility raising tilapia in recirculating aquaculture systems (RAS), supplemented with various salt combinations, was compared to the shrimp's survival and growth in well-water supplemented with 17.6 (control) and 0.6 (freshwater treatment) g/L synthetic sea salt. Three independent trials were conducted in RAS in which survival and growth in the control, the freshwater treatment, and two effluent treatments were compared. Water quality during this study was within safe levels and no differences ($P < 0.05$) between treatments were observed for dissolved oxygen, nitrite, pH, total ammonia nitrogen and temperature. However, average nitrate and orthophosphate levels were consistently more than an order of magnitude greater in the effluent treatments compared to the control and the freshwater treatments. Survival and growth of shrimp over 6 wk periods did not vary significantly between the control and the freshwater treatments; however, shrimp tested in the tilapia effluents often exhibited significant effects ($P < 0.05$) depending on the salts added. In the low salinity waters, correlations ($P < 0.05$) were observed between Ca^{2+}, Mg^{2+}, Ca^{2+} and Mg^{2+}, K^+, Na^+:K^+ and Ca^{2+}:K^+, and shrimp survival and growth. The results of this study revealed that *L. vannamei* can be raised in tilapia effluent when supplemented with synthetic sea salt (0.6 g/L), CaO (50 mg/L Ca^{2+}) and $MgSO_4$ (30 mg/L Mg^{2+}).

Introduction

In the United States (US) shrimp is a high value food that accounted for a $3.8 billion trade deficit in 2004 (Harvey 2005). The high level of shrimp importation is due to an inability to supply internal demands by national fisheries or aquaculture. Aquaculture of marine shrimp in the US has been severely restricted due to high production costs and the value of coastal real estate located close to market bases. In addition to the latter constraints, the US, as in other countries, have severely tightened and enforced their environmental regulations (Boyd 2003). These reasons have limited the development of sustainable shrimp farming in the US. Nevertheless, the latter constraints can be addressed to varying degrees. For example, labor costs and environmental quality could be managed through automation of production systems and application of water reuse facilities. Real estate costs could be reduced by moving production facilities away from the coast. However, since most of the larger markets in the US are in coastal areas, where by 2025, 75% of all Americans will reside (NOAA 1998), this will necessitate moving away from the marketplace as well as losing access to high quality marine waters. Providentially, many penaeid shrimp are hyperosmotic in low salinity waters (Castille and Lawrence 1981).

Because Pacific white shrimp (*Litopenaeus vannamei*) are excellent osmoregulators, research into this species has increased, especially as this relates to the feasibility of rearing these animals in low salinity waters (Laramore et al. 2001; Atwood et al. 2003; McGraw and Scarpa 2003; Saoud et al. 2003; Sowers et al. 2005). However, low saline water sources vary greatly in their ionic composition such that shrimp growth

and survival may be compromised. Consequently, when contemplating the culture of marine shrimp in low salinity waters, preliminary laboratory-scale tests must be undertaken upon rearing waters to assess their suitability, irrespective of facility type employed (e.g. ponds, recirculating aquaculture systems [RAS], etc.).

RAS has many advantages over pond or flow-through culture systems including: conservation of water and supplemented ions, tighter control of water quality and biosecurity which improves survival and growth, enhanced effluent handling and discharge, and reduction in the risks of introducing disease and pollutants (Skjølstrup et al. 2000; Menasveta 2002; Timmons et al. 2002). Additionally, maximization of spent water use, for example through polyculture (Tian et al. 2001), or use of RAS effluents for farming marine shrimp, might enhance water conservation, profitability and ease environmental impacts. Although several studies have examined the combination of RAS and polyculture (e.g. aquaponics), comparatively few (Schneider et al. 2005) have suggested the application of RAS waste water as a method for supplementing aquaculture income by producing a "catch crop". Accordingly, the present study considered the feasibility of rearing *L. vannamei* in effluents derived from an inland commercial Nile tilapia Oreochromis niloticus RAS production facility. The tilapia effluent was essentially considered as waste stream, but had been heated and contained supplemental salt (NaCl). An ability to raise shrimp in this waste stream would therefore represent increased exploitation not only of the heat energy but also maximize the use of added salt. Research described herein includes evaluations of water ion concentrations and ion supplementation. A zero-exchange RAS was employed for these studies.

Materials and Methods

The culture of *L. vannamei*, initiated with > PL_{25} (e.g., PL_{25} = 25 day old post-larvae) in half-strength sea water (18 g/L salinity), was compared to salinity-challenged conditions over a 42 d period for three independent trials (trials A-C). Prior to experimental start, preliminary trials were undertaken in order to establish survival of shrimp in tilapia effluents with and without added synthetic sea salt. These studies determined no significant differences (one-way ANOVA) in juvenile shrimp survival between treatments in which synthetic sea salt was added at > 0.6 g/L. Subsequently, trials were undertaken to optimize shrimp survival and growth in tilapia effluents with minimum salt additions.

Shrimp suppliers and acclimation

Certified, specific pathogen-free (SPF), shrimp post-larvae (PL) were supplied by commercial and research hatcheries. For the aforementioned preliminary trials, PLs were obtained from a commercial hatchery (Harlingen Shrimp Farms Ltd., Los Fresnos, TX, US). For the trials presented in this study, PLs were acquired from The Oceanic Institute (Kailua-Kona, Hawaii, US). PL shrimp were air freighted overnight. Dissolved oxygen (DO) was above saturation, water temperature between 18 and 21 C, and salinity either 18 (Harlingen Shrimp Farms) or 25 (The Oceanic Institute) g/L . After arrival, shrimp were acclimated to well water supplemented with 22 g/L synthetic sea salt (Crystal Sea, Marineland, Baltimore, MD, US). Once acclimatized, shrimp were transferred to aquaria outfitted with mechanical and biological filtration units. System water quality was: DO >

5.75 mg/L, total ammonia nitrogen (TAN) < 0.30 mg/L and temperature was 28 ± 1.0 C. Animals were maintained under these holding conditions for a minimum of 72 h and until the all shrimp attained > PL_{25}.

The > PL_{25} shrimp were acclimated to lower salinities using freshwater from a local well source. Salinity was adjusted using the following scheme according to Van Wyk et al. (1999): 32 to 16, 16 to 8, 8 to 4, 4 to 2, and 2 to 1 g/L with salinity reductions in steps of 2.0, 1.0, 0.5, 0.25, 0.13 g/L per h, respectively. Once salinity levels in the acclimation tanks matched that of pre-selected experimental salinities (1.0-18.0 g/L), studies commenced.

Experimental systems and stocking densities

Figure 1 provides an overview of an experimental system used during salinity challenges. Three 38 L aquaria were used for each treatment and each aquarium was outfitted with a 50 W Whisper® submersible heater (Tetra, Blacksburg, VA, US) and two Lee's breeder nets (L. Schultz Inc., San Marcos, CA, US). Each 1.9 L breeder net was initially stocked with 15 shrimp (8 shrimp/L) totaling 30 shrimp per aquaria. The 125 L nitrification reactors (Fig. 1) contained 20 L of KMT media (Kaldnes Inc., Providence, RI, US) and were fluidized using forced air from a 1 horsepower Sweetwater® regenerative blower that supplies forced air to the entire building. Water was pumped with a 40 W Quiet One Pond Pump (Pentair Aquatics™) from the nitrification reactors into the three aquaria (at 200 L/h per aquarium) and the return was gravity fed back to the nitrification reactors.

Shrimp were fed a 35% protein, ground shrimp feed (Melick Aqua Feeds, Catawissa, PA, US) to excess. Accumulated feed was removed by siphoning at the start of each day (thereby allowing overnight feeding). Aquaria were painted black on the exterior walls to minimize visual stress. A photoperiod of 12 hours with an additional 15 minutes of low intensity light (used to simulate dawn and dusk) was implemented daily using a 24 h Intermatic model ET100C (Intermatic Inc., Spring Grove, IL, US) control system.

Water source and ionic supplementation

Table 1 summarizes the water treatments employed during the study for the three independent trials, designated A, B and C. In each trial, control water always consisted of well water (0.4 g/L salinity) supplemented with synthetic sea salt to 18 g/L salinity, whereas the freshwater treatment always consisted of well water supplemented with 0.6 g/L sea salt. Tilapia effluents, collected from the bottom of settling basins at the local commercial farm (Blue Ridge Aquaculture, Martinsville, VA, US), were supplemented with various salt combinations (Table 1) including synthetic sea salt, CaO (technical grade calcium oxide, Fisher Scientific, Fairlawn, NJ, US), $MgSO_4$ (epsom salt, Kroger Co., Cincinnati, OH, US), and NaCl (non-iodized table salt, Morton Salt, Louisville, KY, US). Calcium and Mg^{2+} were supplemented to increase the divalent cation concentrations in the tilapia effluent because these proved to be in deficit. The tilapia effluent was treated using nitrification to reduce elevated ammonia (typically 2.0 to 3.5 mg/L) levels prior to experimentation. The effluent was aerated with cycled KMT media in 166 L

drums until TAN was reduced to levels < 0.30 mg/L. This reduction in TAN was typically observed within a 24 h period.

Sampling and monitoring

Water quality was monitored using the methods and frequencies noted in Table 2. All sampling events were conducted at equal intervals during the trials. HACH samples were analyzed using a HACH DR/2400 spectrophotometer and a HACH digital titrator (Hach Co., Loveland, CO, US). A modification to the Nessler method (HACH 8038) was used for the high salinity treatments which included ten drops of mineral stabilizer instead of three which is typically used for freshwater samples. A standard comparison was conducted for 0.50 mg/L TAN between dionized water and 18 g/L sea water (dionized water with synthetic sea salt). Triplicate measurements for TAN resulted in the following means (95% confidence intervals) for the freshwater and 18 g/L sea water samples respectively, 0.490 (0.430-0.550) and 0.492 (0.480-0.503) mg/L. Most of the samples (Table 2) were analyzed immediately after sampling events. Samples that were not immediately analyzed (including analysis for anions and cations) were handled and stored in accordance with Standard Methods for the Examination of Water and Wastewater (APHA 1998). As an extra precaution, to ensure that water quality did not degrade over time, apparent color, calcium hardness, chloride (HACH method), salinity and total hardness also were monitored. However, these parameters were not an issue during this study. Unionized ammonia was determined using the following equilibrium equations (Emerson et al. 1975);

$$\%NH_3 = \frac{1}{(10^{pK_a - pH} + 1)} \quad \text{(Equation 2-1)}$$

$$pK_a = 0.09018 + \frac{2729.92}{T} \quad \text{(Equation 2-2)}$$

where, T = temperature in degrees Kelvin

Performance indicators

The impact of varying treatments (Table 1) was monitored by assessing survival, growth and specific growth rates (SGR). Survival was determined by counting shrimp every 24 h for the first week and thereafter at 48 h periods. On days 1 and 42, shrimp were patted dry using Kim Tech Wipes (Kimberly-Clark, Roswell, GA, US) and weighed using an A&D HM-202 analytical balance (A&D Engineering Inc., Milpitas, CA, US) to the nearest 0.0001 g. SGR (Equation 3) was determined using the following formula (Ricker 1975):

$$SGR\left(\frac{\%}{d}\right) = \frac{100 * [\log_e \text{shrimp final mass (g)} - \log_e \text{shrimp initial mass (g)}]}{\text{time (d)}}$$

(Equation 2-3)

Statistical analysis

Statistical analysis was performed using SAS v9.1 for Windows (SAS Institute Inc., Cary, NC, US). Differences in water quality were considered significant when P <

0.05. A one-way ANOVA was employed with a Duncan's Multiple Range Test (where appropriate) to test significant differences ($P < 0.05$) between treatments for 6 wk growth and survival. A two-tailed Pearson's correlation coefficient analysis was utilized to determine correlations between survival and growth with various ions in the low salinity treatments.

Results

Water quality results are presented in Table 3. There were no differences in DO, nitrite, pH, TAN, unionized ammonia, or temperature between treatments. Unionized NH_3-N concentrations were notably highest during trial A. In particular, the freshwater treatment and effluent 1 experienced a 24 h unionized NH_3-N spike of 0.20 mg/L between 4 and 5 d (data not shown). Nitrate and orthophosphate on average were consistently more than an order of magnitude greater in the effluent treatments compared to the control and the freshwater treatments. Alkalinity and turbidity did not differ significantly between treatments. The following unreported water quality parameters that were used to check water quality consistency did not deviate more than 75% (apparent color), 30% (calcium hardness), 19% (chloride, HACH method), 17% (total hardness) when all treatments during the entire study were considered.

Survival and growth over the 6 wk for Trials A-C are noted in Table 4. No differences in growth or survival were observed between the control and the freshwater treatment during any of the trials. However, growth and survival in the tilapia effluent

(effluents 1 and 2) did vary significantly ($P < 0.05$) depending on the ion supplementation.

More specifically, during trial A, effluent 1 exhibited lower ($P < 0.05$) growth and survival rates compared to the control and the freshwater treatment, whereas in effluent 2, survival was 0% (by day 37). In trial B, addition of sea salt, CaO and $MgSO_4$ (effluent 1) or NaCl, CaO and $MgSO_4$ (effluent 2) to tilapia effluent resulted in differences ($P < 0.05$) in survival and growth when compared to the control and the freshwater treatment (Table 4). With respect to growth, the following order was obtained, freshwater treatment = control > effluent 1 > effluent 2. Consideration of survival illustrated: control = freshwater treatment = effluent 1 > effluent 2. In trial C, addition of sea salt, CaO and $MgSO_4$ (levels noted in Table 1) to the tilapia effluents resulted in similar levels of survival to those observed in the control and the freshwater treatment. In terms of growth, no differences were observed between the control, the freshwater treatment and effluent 2. However, differences ($P < 0.05$) were recorded in growth for effluent 1 animals, which were larger than other treatments in this trial (Table 4).

Recorded mean ion levels (with range) observed for the different low salinity treatments are presented in Table 5. As might be anticipated, variations in water ion content occurred following additions of the various salts. Since growth and survival of shrimp maintained in the control and the freshwater treatment were similar (Table 4, trials A-C), the ion datasets indicate that appropriate ion levels were present in the freshwater treatments (Table 5, trials A-C). However, the ion composition in the effluent treatments (Table 5) demonstrated that the ion levels did not always support shrimp survival and growth (Table 4). Correlations between survival and growth with various

ions are presented in Table 6. Calcium, Mg^{2+}, and $Ca^{2+} + Mg^{2+}$ ($P < 0.01$) were significantly correlated with survival in the low salinity waters. Growth was significantly correlated with Mg^{2+} ($P < 0.05$), and an even stronger correlation was observed with Ca^{2+}, $Ca^{2+}+ Mg^{2+}$, Na^{+}: K^{+}, and Ca^{2+}: K^{+} ($P < 0. 01$). A negative correlation ($P < 0.05$) was observed between growth and K^{+}.

Discussion

Water quality (Table 3), except for ion composition (Table 5), was not considered a cause of poor shrimp performance observed in many of the effluent treatments (Table 4). The short term unionized ammonia spikes of 0.20 mg/L observed during this study were < 7% of the reported 24 h LC50 of 2.95 mg/L (Lin and Chen 2001). Nitrite concentrations during the entire study (< 0.38 mg/L) also were considered to be within acceptable levels, for example Lin and Chen (2003) determined that acceptable levels of nitrite to be 6.1 mg/L at 15 g/L salinity. Even though nitrate levels were consistently higher in the effluent treatments when compared to controls and freshwater treatments, the highest concentrations never exceeded 5% of the 48 h LC50 (3,400 mg/L) for shrimp as determined by Wickins (1976). Furthermore, temperatures were maintained within the recommended range (23-30 C) for favorable growth and survival of *L. vannamei* (Wyban et al. 1995).

Even though the initial mass of shrimp used in trial A were significantly smaller than shrimp used in trials B and C, these shrimp were > PL_{30} by the time the experiment

commenced. McGraw et al. (2002) noted that PL_{15} can be acclimated to low salinities (1.0 g/L) and PL at this age have developed proper osmoregulation ability due to extensive filament branching of the gills (Palacios et al. 2004).

Treatments varied in ion composition (Table 5) and it was these variations that affected shrimp performance (Table 6). Shrimp in both effluent treatments (effluent 1 and 2) during trial A demonstrated poor survival and growth. During this trial, Ca^{2+} levels were consistently lower (< 32%) in the effluent treatments than that measured in freshwater treatments. Magnesium was in deficit as well, especially in effluent 2. Magnesium levels were < 44% of those measured in freshwater treatments. This warranted supplementation of Ca^{2+} and Mg^{2+} in the remaining trials (trial B and C) which resulted in improved shrimp performance (Table 4). Calcium and Mg^{2+} are vital to shrimp because they represent major components of the exoskeleton (Ca^{2+} = 15.95%, Mg^{2+} = 1.19% in Vijayan and Diwan 1996) and are important for normal physiological processes. For example, Ca^{2+} is essential for binding many proteins (Endo et al. 2002) whereas both Ca^{2+} and Mg^{2+} are engaged in enzymatic reactions (Xie et al. 2004). The demand for calcium in crustaceans is highest during ecdysis (Greenaway 1985) and Vijayan and Diwan (1996) suggested that the Ca^{2+} needed for cuticular mineralization is directly absorbed from ambient water.

The results from this study are consistent with Atwood et al. (2003) and Sowers et al. (2005), in that *L. vannamei* were deemed to require sea salt for survival and growth when compared to mixed salt environments at salinities ≤ 2.0 g/L. This is likely due to the essential trace minerals that sea salt contains. Even though the ionic composition in the hemolymph of shrimp is predominately Na^+ and Cl^- (Chen and Chen 1996), penaeid

shrimp require additional ions to Na^+ and Cl^- in the culture medium for adequate survival and growth (Cawthorne et al. 1983). Furthermore, Atwood et al. (2003) challenged *L. vannamei* in waters supplemented with 0, 0.25, 1.0 g/L sea salt with and without CaCl and NaCl. These authors found that 0.25 g/L sea salt, even with CaCl and NaCl supplementation, was insufficient for adequate survival and growth, whereas 1.0 g/L sea salt supplementation supported growth. The present study demonstrates that 0.6 g/L sea salt supplementation is feasible for shrimp culture, which could potentially save upwards of 40% of the costs associated with the addition of synthetic sea salt.

Potassium is a principle intracellular ion (Shiau and Hshieh 2001) and often can be a limiting ion for *L. vannamei* performance under salinity-challenged conditions. Levels of K^+ in this study were more than an order of magnitude greater than the recommended minimum concentration of 1 mg/L required for the culture of *L. vannamei* at low salinities (McGraw and Scarpa 2003). Saoud et al. (2003) observed a positive correlation between K+ and shrimp survival while results from this study demonstrated the opposite effect for growth (Table 6). This negative correlation was partly due to the poor performance observed in the tilapia effluent, which has relatively high levels of K^+ (between 49 and 78 mg/L) without the necessary synthetic sea salt supplementation (Effluent 2 in Trial A and B). Zhu et al. (2004) reported that *L. vannamei* weight gain, SGR, and food conversion efficiencies (FCE) improved as the Na^+:K^+ ratio decreased over the range of 187.4 to 34.1 mmol/mmol. The Na^+:K^+ ratios in this study were equal to and below this range in the low salinity treatments and except for the positive correlation observed in Table 6 (in terms of growth), ratios lower than 34.1 mmol/mmol did not impact shrimp performance.

Conclusion

Even though there are conflicting reports regarding the effects of low salinity waters on survival and growth of *L. vannamei* (Ogle 1992; Bray et al. 1994; Laramore et al. 2001; Atwood et al. 2003, Sowers et al. 2005), this study demonstrated that there were no differences between the high salinity treatments (18 g/L) and the lowest salinity treatments (1.0 g/L). Moreover, correlations between Ca^{2+}, Mg^{2+}, Ca^{2+}+ Mg^{2+}, K^{+}, Na^{+}:K^{+} and Ca^{2+}:K^{+} and shrimp performance were observed in the low salinity waters. This study also demonstrated that *L. vannamei* can be reared in effluents produced by an inland commercial tilapia RAS when the water is supplemented with synthetic sea salt (0.6 g/L), CaO (50 mg/L Ca^{2+}) and $MgSO_4$ (30 mg/L Mg^{2+}).

Fish effluent can be responsible for negative cash flows for producers, but using shrimp as a "catch crop" or "cash crop" in the effluent is a viable solution that can provide for a sustainable and profitable operation. Reuse of fish effluents for the culture of marine shrimp in RAS systems could provide the US shrimp farming industry an opportunity to overcome limitations that have previously prevented sustainability and economic success in the past.

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Literature Cited

APHA (American Public Health Association). 1998. Standard methods for the examination of water and wastewater, 20th edition. Washington, D.C., USA.

Atwood, H. L., S. P. Young, J. R. Tomasso, and C. L. Browdy. 2003. Survival and growth of Pacific shrimp *Litopenaeus vannamei* postlarvae in low-salinity and mixed salt environments. Journal of the World Aquaculture Society 34: 518-523.

Boyd, C. E. 2003. Guidelines for aquaculture effluent management at the farm-level. Aquaculture 226: 101-112.

Bray, W. A., A. L. Lawrence, and J. R. Leung-Trujillo. 1994. The effect of salinity on growth, and survival of *Penaeus vannamei*, with observations on the interaction of IHHN virus and salinity. Aquaculture 122: 133-146.

Castille, F. L. and A. L. Lawrence. 1981. The effect of salinity on the osmotic, sodium and chloride concentrations in the hemolymph of euryhaline shrimp of the genus *Penaeus*. Comparative Biochemistry and Physiology 68A: 75-80.

Cawthorne, D. F., T. Beard, J. Davenport, and J. F. Wickins. 1983. Response of juvenile *Penaeus monodon* Fabricius to natural and artificial sea waters of low salinity. Aquaculture 32: 165-174.

Chen, J-C. and C-T Chen. 1996. Changes of osmotic and electrolyte concentrations in the hemolymph of *Penaeus japonicus* exposed to ambient ammonia. Comparative Biochemistry and Physiology 114C: 35-38.

Emerson, K., R. C. Russo, R. E. Lund and R. V. Thurston. 1975. Aqueous ammonia equilibrium calculations: effect of pH and temperature. Journal of the Fisheries Research Board of Canada. 32: 2379-2383.

Endo, H., Y. Takagi, and T. Watanabe. 2002. Crustocalcin: A study on potential function of a skeletal Ca2+-binding protein of kuruma prawn *Penaeus japonicus*. Zoological Science 19: 1480.

Greenaway, P. 1985. Calcium balance and moulting in the crustacean. Biological Review 60: 425-454.

Harvey, D.J. 2005. Electronic Outlook Report for Economic Research Service. Aquaculture Outlook 2004. LDP-AQS-21. USDA, Washington, D.C., US.

Laramore, S., C. R. Laramore, and J. Scarpa. 2001. Effect of low salinity on growth and survival of postlarvae and juvenile *Litopenaeus vannamei*. Journal of the World Aquaculture Society 32: 385-392.

Lin, Y-C. and J-C Chen. 2001. Acute toxicity of ammonia on *Litopenaeus vannamei* Boone juveniles at different salinity levels. Journal of Experimental Marine Biology and Ecology. 259: 109-119.

Lin, Y-C. and J-C Chen. 2003. Acute toxicity of nitrite on *Litopenaeus vannamei* (Boone) juveniles at different salinity levels. Aquaculture 224: 193-201.

McGraw, W. J., D. A. Davis, D. Teichert-Coddington, and D. B Rouse. 2002. Acclimation of *Litopenaeus vannamei* postlarvae to low salinity: influence of age, salinity endpoint, and rate of salinity reduction. Journal of the World Aquaculture Society 33: 78-84.

McGraw, W. J. and J. Scarpa. 2003. Minimum environmental potassium for survival of Pacific white shrimp *Litopenaeus vannamei* (Boone) in freshwater. Journal of Shellfish Research 22: 263-267.

Menasveta, P. 2002. Improved shrimp growout systems for disease prevention and environmental sustainability in Asia. Reviews in Fisheries Science 10: 391-402.

NOAA (National Oceanic and Atmospheric Administration). 1998. Population: Distribution, density, and growth. Pages 1-31 in State of the Coast Report. Silver Springs, MD, USA.

Ogle, J. T. 1992. Effects of salinity on survival and growth of postlarval *Penaeus vannamei*. Gulf Research Reports 8: 415-421.

Palacios, E., A. Bonilla, D. Luna, and I. S. Racotta. 2004. Survival, Na+/K+-ATPase and lipid responses to salinity challenge in fed and starved white pacific shrimp (*Litopenaeus vannamei*) postlarvae. Aquaculture 234: 497-511.

Ricker, W. E. 1975. Computation and interpretation of biological statistics of fish population. Bulletin of the Fisheries Research Board of Canada 191: 1-382.

Saoud, I. P., D. A. Davis, and D. B. Rouse. 2003. Suitability studies of inland well waters for *Litopenaeus vannamei* culture. Aquaculture 217: 373-383.

Schneider, O., V. Sereti, E. H. Eding, and J. A. J. Varreth. 2005. Analysis of nutrient flows in integrated intensive aquaculture systems. Aquacult. Eng. 32: 379-401.

Shiau, S-Y. and J-F. Hshieh. 2001. Dietary potassium requirement of juvenile grass shrimp *Penaeus monodon*. Fisheries Science 67: 592-595.

Skjølstrup, J., E. McLean, P. H. Nielson, and J-O. Frier. 2000. The influence of dietary oxolinic acid on fluidised bed biofilter performance in a recrculation system for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 183: 255-268.

Sowers, A. D., D. M. Gatlin, S. P. Young, J. J. Isely, and C. L. Browdy. 2005. Response of *Litopenaeus vannamei* (Boone) in water containing low concentrations of total dissolved solids. Aquaculture Research 36, 819-823.

Tian, X., D. Li, S. Dong, X. Yan, Z. Qi., G. Liu, and J. Lu. 2001. An experimental study on closed-polyculture of penaeid shrimp with tilapia and constricted tagelus. Aquaculture 202: 51-71.

Timmons, M. B., J. M. Ebeling, F. W. Wheaton, S. T. Summerfelt and B. J. Vinci. 2002. Recirculating Aquaculture Systems, 2nd edition, Cayuga Aqua Ventures, Ithaca, New York, USA.

Van Wyk, P., M. Davis-Hodgkins, C. R. Laramore, K. Main, J. Mountain, and J. Scarpa. 1999. Farming marine shrimp in recirculating freshwater production systems: a practical manual. FDACS Contract #4520. Florida Department of Agriculture Consumer Services, Tallahassee, Florida, US.

Vijayan, K. K. and A. D. Diwan. 1996. Fluctuations in Ca, Mg, and P levels in the hemolymph, muscle, midgut gland, and exoskeleton during the molt cycle of the Indian white prawn, *Penaeus indicus* (Decapoda: *Penaeidae*). Comparative Biochemistry and Physiology 114A: 91-97.

Wickins, J. F. 1976. The tolerance of warm-water prawns to recirculated water. Aquaculture 9: 19–37.

Wyban, J., W. A. Walsh, and D. M. Godin. 1995. Temperature effects on growth, feeding rate and feed conversion of the Pacific white shrimp (*Penaeus vannamei*). Aquaculture 138: 267-279.

Xie, X-L., Q-X. Chen, J-C. Lin, and Y. Wang. 2004. Purification and some properties of β-N-acetyl-D-glucosaminidase from prawn (*Penaeus vannamei*). Mar. Biol. 146: 143-148.

Zhu, C., S. Doug, F. Wang, and G. Huang. 2004. Effects of Na/K ratio in seawater on growth and energy budget of juvenile *Litopenaeus vannamei*. Aquaculture 234: 485-496.

Table 2-1. Tilapia effluent treatments with respective ion supplementation. Each trial (A-C) also included a control (18.0 g/L) and freshwater treatment (1.0 g/L).

Trial	Treatment	Water Source (Corresponding Salinity)	Ion Supplementation (Concentration)
A	Effluent 1	Tilapia effluent (1.5 ppt)	Sea salt (0.6 g/L)
	Effluent 2	Tilapia effluent (1.5 ppt)	No supplementation
B	Effluent 1	Tilapia effluent (1.1 ppt)	Sea salt (0.6 g/L), Ca^{2+} (50 mg/L), Mg^{2+} (30 mg/L), SO_4^{2-} (25 mg/L),
	Effluent 2	Tilapia effluent (1.1 ppt)	Ca^{2+} (50 mg/L), Mg^{2+} (30 mg/L), Na^{+} (99 mg/L), SO_4^{2-} (25 mg/L), Cl^{-} (150 mg/L)
C	Effluent 1	Tilapia effluent (1.2 ppt)	Sea salt (0.6 g/L), Ca^{2+} (50 mg/L), Mg^{2+} (30 mg/L), SO_4^{2-} (25 mg/L)
	Effluent 2	Tilapia effluent (1.2 ppt)	Sea salt (1.0 g/L), Ca^{2+} (50 mg/L), Mg^{2+} (30 mg/L), SO_4^{2-} (25 mg/L)

Table 2-2. Methods and number of sampling events used to determine water quality constituents.

Parameter	Number of sampling events (n)			Method
	Trial A	Trial B	Trial C	
Ammonia-N, total	18	20	19	Nessler method, HACH method 8038[b,c,d]
Alkalinity	6	8	8	Sulfuric acid method, HACH method 8203[b,c]
Ions (Ba^{2+}, Ca^{2+}, Fe, K^{+}, Mg^{2+}, Mn, Na^{+}, Pb, Cl^{-}, SO_4^{2-})	2	3	3	Ion Chromatograhy[a,d,e], DIONEX 120, AS 40 autosampler, outfitted with AG 9HC and AS 9HC columns
Chloride	0	11	7	Mercuric thiocyanate method, HACH method 8113[b]
Color, apparent	8	10	7	Platinum-cobalt standard method, HACH method 8025[b]
Dissolved oxygen	20	21	18	YSI model 85 (Yellow Springs, OH, USA)
Hardness, calcium	0	10	7	Titration with EDTA, HACH method 8204[b,c]
Hardness, total	6	8	7	Titration with EDTA, HACH method 8213[b,c]
Nitrite-N	13	12	12	Diazotization method, HACH method 8507[b,d]
Nitrate-N	13	8	10	Cadmium reduction method, HACH method 8039[b,c]
Orthophosphate (OP)	6	8	9	Ascorbic acid method, HACH method 8048[b,c,d]
pH	16	15	12	HI 9024 pH meter (HANNA Instruments, Woonsocket, RI, USA
Salinity	22	24	18	YSI model 85 (Yellow Springs, OH, USA)
Temperature	20	29	21	YSI model 85 (Yellow Springs, OH, USA)
Turbidity	8	8	5	HF Scientific DRT-15CE turbidimeter (HF Scientific Inc., Fort Myers, FL)

[a] Standard Methods for the Examination of Water and Wastewater, APHA (1998)
[b] Hach company, Loveland, CO, US
[c] Method developed/adapted from Standard Methods for the Examination of Water and Wastewater, APHA (1998)
[d] USEPA approved for wastewater analysis
[e] DIONEX Corporation, Sunnyvale, CA, US

Table 2-3. Water quality results, mean values with 95% confidence intervals.

Parameter	Treatment	Trial A	Trial B	Trial C
Ammonia-N, total [mg/L]	Control	0.56 (0-1.6)	0.22 (0-0.49)	0.17 (0.098-0.24)
	Freshwater	0.58 (0-1.8)	0.084 (0-0.16)	0.079 (0-0.17)
	Effluent 1	0.36 (0-0.97)	0.22 (0.10-0.34)	0.17 (0-0.38)
	Effluent 2	0.31 (0-0.57)	0.20 (0.066-0.33)	0.18 (0.061-0.31)
Ammonia-N, unionized [mg/L]	Control	0.077 (0-0.19)	0.034 (0-0.075)	0.038 (0.026-0.050)
	Freshwater	0.087 (0-0.24)	0.014 (0-0.032)	0.018 (0.002-0.034)
	Effluent 1	0.074 (0-0.25)	0.052 (0.018-0.086)	0.042 (0-0.10)
	Effluent 2	0.058 (0-0.12)	0.050 (0.007-0.094)	0.045 (0.017-0.073)
Dissolved oxygen [mg/L]	Control	5.87 (5.13-6.61)	5.64 (5.02-6.27)	5.53 (4.79-6.27)
	Freshwater	6.04 (5.53-6.55)	6.00 (5.63-6.37)	6.23 (5.42-7.03)
	Effluent 1	6.61 (5.99-7.24)	5.99 (5.61-6.37)	6.00 (5.31-6.69)
	Effluent 2	6.69 (6.23-7.15)	6.19 (5.85-6.53)	6.05 (5.27-6.84)
Nitrite-N [mg/L]	Control	0.014 (0-0.40)	0.057 (0-0.13)	0.012 (0-0.037)
	Freshwater	0.026 (0-0.052)	0.013 (0-0.035)	0.003 (0-0.006)
	Effluent 1	0.066 (0-0.22)	0.046 (0-0.12)	0.007 (0-0.015)
	Effluent 2	0.027 (0.008-0.046)	0.033 (0-0.094)	0.010 (0-0.020)
Nitrate-N [mg/L]	Control	3.9 (1.7-6.0)	8.1 (0-18)	8.5 (0-17)
	Freshwater	5.7 (3.0-8.3)	18 (0-38)	4.5 (0-9.6)
	Effluent 1	63 (38-87)	100 (29-170)	61 (0-150)
	Effluent 2	67 (46-87)	94 (48-140)	74 (0-150)
Orthophosphate [mg/L]	Control	2.9 (0.19-5.6)	2.8 (0-5.9)	2.7 (2.0-3.5)
	Freshwater	1.9 (0.65-3.1)	1.9 (0.16-3.6)	0.44 (0.19-0.70)
	Effluent 1	7.3 (6.0-8.6)	4.3 (3.2-5.4)	4.7 (0-10)
	Effluent 2	6.9 (5.3-8.5)	4.2 (3.3-5.1)	5.0 (2.0-8.0)
pH	Control	8.42 (8.20-8.65)	8.41 (8.17-8.66)	8.57 (8.39-8.67)
	Freshwater	8.48 (8.18-8.77)	8.44 (8.24-8.64)	8.55 (8.16-8.94)
	Effluent 1	8.58 (8.32-8.85)	8.63 (8.41-8.85)	8.63 (8.32-8.95)
	Effluent 2	8.60 (8.32-8.88)	8.64 (8.38-8.90)	8.65 (8.36-8.95)
Salinity [g/L]	Control	18.5 (17.0-20.0)	18.2 (16.1-20.4)	18.0 (15.7-20.4)
	Freshwater	1.06 (0.91-1.21)	1.03 (0.94-1.12)	1.03 (0.80-1.27)
	Effluent 1	2.11 (2.02-2.20)	2.10 (2.03-2.18)	1.59 (1.3-1.9)
	Effluent 2	1.59 (1.41-1.76)	1.52 (1.38-1.66)	2.26 (2.10-2.41)
Temperature [C]	Control	25.6 (24.1-27.1)	28.0 (26.8-29.2)	29.3 (27.3-31.3)
	Freshwater	25.9 (24.4-27.5)	28.4 (27.8-29.0)	28.3 (26.9-29.8)
	Effluent 1	25.8 (25.0-26.6)	28.7 (27.6-29.8)	29.0 (26.9-31.2)
	Effluent 2	25.7 (24.7-26.7)	28.2 (27.1-29.3)	28.7 (26.9-30.5)

Table 2-4. Six wk Survival and growth results for Trials A-C, alphas denote significant differences between treatments within the respective trial.

Trial	Treatment	Initial mass [g]	Final mass [g]	SGR	Survival [%]
A	Control	0.03442	0.2978[a]	5.14	67[a]
	Freshwater	0.03442	0.2694[a]	4.90	73[a]
	Effluent 1	0.03442	0.1103[b]	2.77	33[b]
	Effluent 2	0.03442	*	*	0[c]
	Pooled error		0.01641		5.199
	P > F		< 0.0001		< 0.0001
B	Control	0.07422	0.6943[a]	5.32	50[a]
	Freshwater	0.07422	0.6995[a]	5.34	46[a]
	Effluent 1	0.07422	0.4977[b]	4.53	46[a]
	Effluent 2	0.07422	0.2750[c]	3.12	27[b]
	Pooled error		0.03905		4.517
	P > F		< 0.0001		0.0243
C	Control	0.07802	0.4945[b]	4.40	69[a]
	Freshwater	0.07802	0.4649[b]	4.25	50[a]
	Effluent 1	0.07802	0.6256[a]	4.96	61[a]
	Effluent 2	0.07802	0.5271[b]	4.55	74[a]
	Pooled error		0.03564		9.046
	P > F		0.0092		0.3261

* Growth at 6 wk not measured due to 100% mortality by day 37

Table 2-5. Mean ion concentration (range) observed in low salinity treatments; refer to Table 2-2 for number of sampling events.

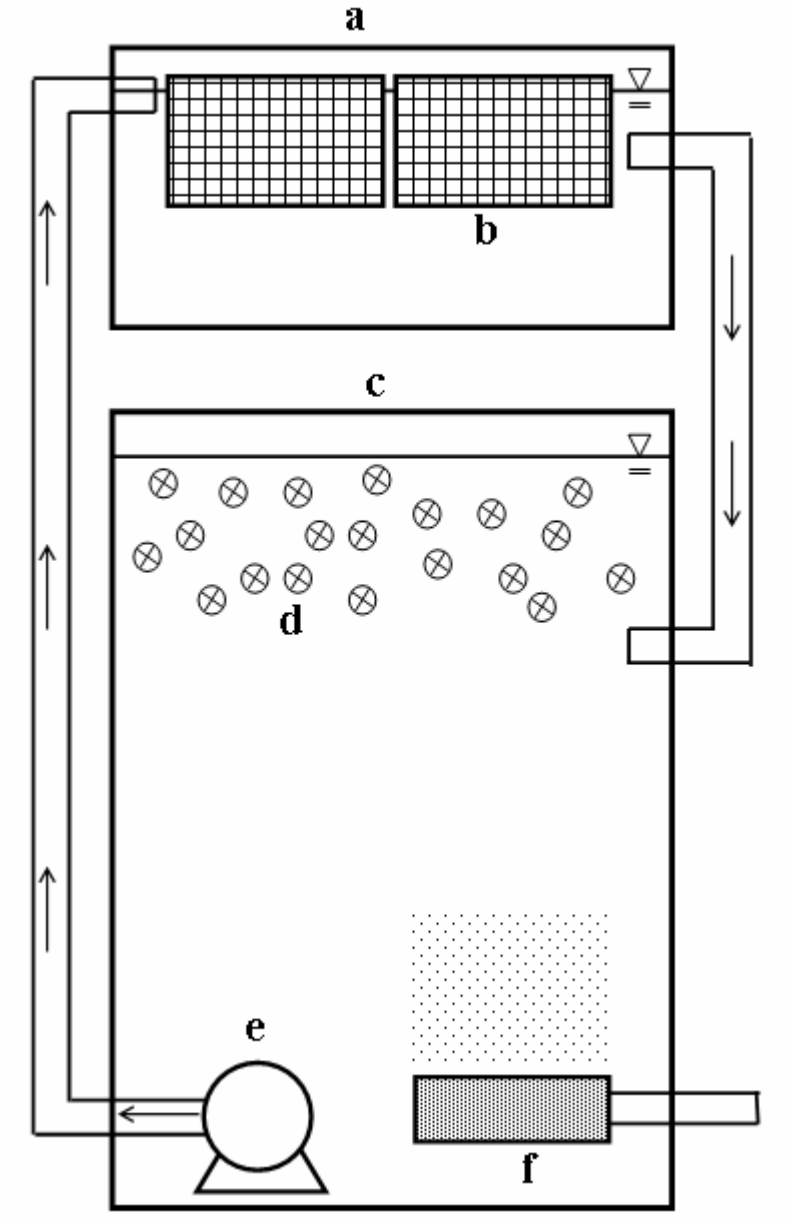
Trial	Treatment	Constituent										
		Ba^{2+} [mg/L]	Ca^{2+} [mg/L]	Fe [mg/L]	K^{+} [mg/L]	Mg^{2+} [mg/L]	Mn [mg/L]	Na^{+} [mg/L]	Pb [mg/L]	Cl^{-} [mg/L]	SO_4^{2-} [mg/L]	Na:K [mmol/mmol]
A	Freshwater	<0.05	89 (58-121)	<0.01	14 (13-14)	74 (70-78)	<0.01	268 (256-280)	<0.01	854 (830-878)	281 (275-286)	35.3
	Effluent 1	<0.05	28 (14-41)	<0.01	78 (73-83)	59 (56-63)	<0.01	558 (438-677)	<0.01	1420 (1370-1470)	369 (350-388)	12.2
	Efluent 2	<0.05	21 (11-32)	<0.01	76 (67-84)	32 (20-43)	<0.01	494 (473-515)	<0.01	668 (655-681)	241 (207-274)	11.0
B	Freshwater	<0.05	97 (57-137)	0.09	16 (11-19)	56 (49-60)	<0.01	242 (227-257)	<0.01	517 (488-569)	133 (120-151)	25.5
	Effluent 1	<0.05	80 (66-98)	0.10	76 (73-80)	58 (53-64)	<0.01	594 (579-606)	<0.01	703 (694-711)	279 (270-285)	13.2
	Effluent 2	<0.05	63 (59-67)	0.09	69 (65-74)	54 (48-59)	<0.01	434 (419-450)	<0.01	389 (353-440)	234 (215-263)	11.0
C	Freshwater	<0.05	63 (63-65)	<0.01	14 (11-17)	54 (44-67)	<0.01	227 (224-229)	<0.01	500 (492-509)	141 (133-148)	27.0
	Effluent 1	<0.05	91 (89-97)	<0.01	98 (97-99)	54 (53-57)	<0.01	576 (525-602)	<0.01	720 (714-723)	372 (344-388)	10.1
	Effluent 2	<0.05	96 (91-101)	<0.01	85 (82-89)	65 (62-69)	<0.01	642 (618-690)	<0.01	1000 (991-1020)	360 (342-373)	12.7

Table 2-6. Pearson's correlation coefficient (P-value) for 42 d survival and growth; data pooled from low salinity treatments.

	Ca^{2+}	K^{+}	Mg^{2+}	Na^{+}	Cl^{-}	SO_4^{2-}	$Na^{+}+K^{+}$	$Ca^{2+}+Mg^{2+}$	$Cl^{-}+SO_4^{2-}$	$Na^{+}:K^{+}$	$Ca^{2+}:K^{+}$	$Ca^{2+}:Cl^{-}$
Survival	0.521** (0.002)	-0.162 (0.367)	0.474** (0.005)	-0.043 (0.814)	-0.036 (0.844)	-0.002 (0.992)	-0.063 (0.727)	0.596** (<0.001)	-0.029 (0.875)	0.288 (0.105)	0.274 (0.123)	0.068 (0.705)
Growth	0.818** (<0.001)	-0.407* (0.049)	0.406* (0.049)	-0.337 (0.107)	-0.178 (0.405)	-0.179 (0.403)	-0.351 (0.092)	0.839** (<0.001)	-0.187 (0.381)	0.558** (0.005)	0.679** (<0.001)	0.207 (0.332)

* Correlation is significant at the 0.05 level (two-tailed)
** Correlation is significant at the 0.01 level (two-tailed)

Figure 2-1. Schematic of a system used to test shrimp performance: (a) three 38 L aquaria, (b) two 1.9 L breeder nets per aquarium, (c) 125 L nitrification reactor, (d) 20 L of KMT media, (e) water pump, and (f) 20 cm air diffuser.



Chapter 3:

Use of Microbial Flocs Generated from Tilapia Effluent as a Nutritional Supplement for Shrimp *Litopenaeus vannamei* in RAS

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Abstract

Recovering nutrients in a fish effluent to be used as a supplemental feed for shrimp culture could ease constraints (e.g. environmental issues and high production cost) that have limited the US shrimp farming industry in the past. In this study under laboratory-scale conditions, fish effluent was collected from a commercial tilapia farm and nutrients from the waste stream were offered as supplemental feed either as (1) untreated solids from tilapia effluent, or (2) microbial flocs generated from the biological treatment of the effluent by reducing soluble chemical oxygen demand >80%. The first feeding trial demonstrated that microbial flocs contributed significantly ($P<0.05$) to overall growth while untreated solids did not. Moreover, microbial flocs were larger and contained higher levels ($P<0.05$) of protein. The second feeding trial investigated different feeding rates of commercial diets with and without microbial floc supplementation. Weekly measurements of mass and specific growth rates demonstrated that microbial flocs significantly ($P<0.05$) contributed to shrimp performance. Weekly food conversion ratios were also reported. Water quality in shrimp systems during both studies were within safe levels and no differences ($P>0.05$) between treatments were observed for dissolved oxygen, nitrate-nitrogen, nitrite-nitrogen, pH, salinity, total ammonia nitrogen, and temperature.

Keywords: Bacteria, bioreactors, wastewater, sustainability, fish solids

Introduction

In 1999 the Food and Agriculture Organization of the United Nations estimated there were 375,913 shrimp farms globally (FAO 2000). Many of these shrimp farms, mostly located in tropical and subtropical countries, are moving from low to high intensity culture which increases the strain on the environment (Naylor et al. 1998, Boyd 2003). This has prompted a substantial movement towards more sustainable practices that lower environmental impacts (Avnimelech 1999; Hargreaves 2006), while maintaining and/or increasing shrimp health and growth (Moss et al. 2000; Moss et al. 2001; Tacon et al. 2002; Cuzon et al. 2004; Izquierdo et al. 2006). Shrimp is a high value food in the United States (US), which accounted for nearly half of the national seafood trade deficit, or $3.8 billion, in 2004 (Harvey 2005). The huge import of shrimp serves to highlight the numerous limitations for shrimp farming in the US. These limitations include the country's temperate climate, exacting environmental regulations, and the high costs of production associated with labor and feeds (Paez-Osuna 2001; Boyd 2003). These restrictions can be addressed to varying degrees through the implementation of recirculating aquaculture systems (RAS) and supplemental feeds such as microbial flocs.

RAS has numerous advantages over flow-through and pond culture systems, including: tighter control of water quality, temperature and biosecurity which improves survival and growth; enhanced effluent handling and discharge, water conservation, and reduction in the risks of introducing disease and pollutants (Skjølstrup et al. 2000; Menasveta 2002; Timmons et al. 2002). It has been noted that pond-raised shrimp appear healthier and have better growth rates in the presence of high microbial and algal counts

(Moss et al. 2000; Moss et al. 2001; Tacon et al. 2002; Cuzon et al. 2004). Therefore, it was hypothesized that feed supplementation with microbial flocs would enhance production characteristics of tank-reared animals. Moreover, generating microbial flocs from fish waste would help both shrimp and fish culture to become more sustainable. Tilapia wastewater contains valuable nutrients such as organic and suspended matter (Jiménez-Montealegre et al. 2002; Al-Hafedh et al. 2003) and converting these nutrients into microbial flocs can be accomplished in aerobic bioreactors (Metcalf and Eddy 2003).

The objective of this study was to determine whether microbial flocs generated from biological treatment of tilapia effluent could be used as a viable supplemental feed for shrimp culture in RAS.

Materials and Methods

Shrimp System and Husbandry

Aquatic Habitats™ (AHAB) benchtop systems (Aquatic Ecosystems, Apopka, FL, USA) were used to test shrimp performance. Six systems were used for the first feeding trial and three were used for the second feeding trial. Each benchtop system consisted of four 10 L aquaria, 38 L/min magnetic driven pump, 35 L aerated sump with Siporex™ Biomedia, 10 inch 50 micron canister filter, 10 inch canister carbon filter, 25 W ultraviolet sterilizer, and 250 W submersible heater. A 12 h photophase:scotophase was implemented using a 24 h Intermatic model ET100C (Intermatic Inc., Spring Grove, IL, USA) control system.

Certified, specific pathogen-free (SPF) postlarval (PL) shrimp, *Litopenaeus*

vannamei, were supplied by The Oceanic Institute (Kailua-Kona, Hawaii, USA) and were acclimated to the aforementioned AHAB benchtop systems. Salinity of the shipped samples was lowered from 25 g/L to approximately 2.0 g/L using protocols developed by Van Wyk et al. (1999). Both feeding trials were conducted using well water (0.4 g/L salinity) supplemented with 1.6 g/L synthetic sea salt (Crystal Sea, Marineland, Baltimore, MD, USA). The shrimp feed used during this study was a 35% crude protein commercial feed (first study, Melick Aqua Feeds, Catawissa, PA, USA; second study, Ziegler Bros. Inc., Gardners, PA, USA), untreated solids in tilapia effluent, and microbial flocs generated in bioreactors.

Untreated solids and Microbial flocs

Tilapia effluent was collected from a local commercial RAS tilapia facility (Blue Ridge Aquaculture, Martinsville, VA, USA). The effluent was collected as settling basins at the farm were drained as part of normal operations. Untreated solids were collected directly from the tilapia effluent without any treatment. Microbial flocs were generated from the tilapia effluent in twelve 38 L aquaria that were aerated with 12 cm air diffusers. Soluble chemical oxygen demand (sCOD) in the untreated effluent was between 86-97 mg/L and 80-135 mg/L for the first and second feeding trials, respectively. Biomass is generated as sCOD is consumed (Metcalf and Eddy 2003). First-order rates of biodegradation were determined for three random batch reactors to determine when sCOD removal would be > 80%. From these data, it was determined a 10 d reaction period would yield > 80% sCOD removal and this was verified by testing sCOD on day 10. Therefore, microbial flocs were not harvested as a feed supplement until a reaction

period of 10 d was achieved. Particle size distributions were also compared between untreated solids collected directly from the tilapia effluent and microbial flocs sampled from the batch reactors.

In the first feeding trial, untreated solids and microbial flocs produced in the batch reactors were collected with a 60 mL syringe after a 30 to 45 min settling period. Following complete removal of the untreated solids and microbial flocs, they were homogenized manually using a laboratory spatula. The untreated solids and microbial flocs were then preserved in a -20 C freezer until aliquots were required for feeding as part of diets two and three. Relative protein levels were determined on the untreated solids and microbial flocs for future comparison.

For the second feeding trial, microbial flocs were removed on a daily basis from the 38 L aquaria as a supplemental feed. Microbial flocs were often harvested for several consecutive days beyond the 10 d required to reduce sCOD by > 80%. Due to the possible influence of endogenous decay of the microbes, the relative protein levels of the microbial flocs were monitored on a weekly basis.

First Feeding Trial

The first study was conducted over a 40 d period with different treatment regimes. They included: (diet one) 100% shrimp feed, (diet two) 50% shrimp feed with 50% microbial flocs, (diet three) 50% shrimp feed with 50% untreated solids, and (diet four) 50% shrimp feed. All diets were offered ad libitum, and a surplus of shrimp feed, microbial flocs, and untreated solids were available for consumption during their respective 24 h period. As part of diets two and three, microbial flocs and untreated solids

were fed on alternating days substituting shrimp feed, making up 50% of the diet. For diet four, shrimp were fed shrimp feed on alternating days, thereby making up 50% of the shrimp feed available as compared to diet one. Every 24 h, all feed was completely removed from the shrimp aquaria by siphoning with a 60 mL syringe. Water flow rate was adjusted to 10 L/min to keep the untreated solids and microbial flocs in the aquaria.

The four dietary treatments were randomly distributed between the six AHAB benchtop systems. The treatments were conducted in sextuplicate for all diets. Each aquarium was initially stocked with five juvenile shrimp with a mean weight of 26.3 ± 1.3 mg.

Survival was recorded every 48 h, and final weights were determined individually at the end of the 40 day trial. The systems were also monitored daily for mortalities, and dead shrimp were immediately removed from the study.

Second Feeding Trial

The second study was conducted over a 35 d period and consisted of the following treatment regimes: (diet one) 8% body weight per day (8% BW/d), (diet two) 4% BW/d, (diet three) 6% BW/d + microbial flocs, and (diet four) 4% BW/d + microbial flocs. Since microbial flocs from the first feeding trial outperformed the untreated solids treatment, microbial flocs were chosen for further investigation in the second feeding trial. Settled microbial flocs were siphoned daily and randomly distributed in equal 8 mL aliquots to the aquaria dedicated to diets three and four. At 0900 h ± 30 min., water flow to the aquaria was turned off for a 1.0 h period to prevent the microbial flocs from being washed out during feeding. In most cases, apparent satiation was observed after shrimp

were fed microbial flocs for 30 to 45 min. At the end of the 1.0 h period, microbial flocs were still present. The tank was visually clear of microbial flocs shortly (typically within 15 min.) after the water flow rates were normalized (38 L/min). Approximately 1.0 h after renewing water circulation, shrimp were fed daily rations of commercial shrimp feed.

Three AHAB benchtop systems were dedicated to this study and each of the randomly distributed dietary treatments were conducted in triplicate. Each aquarium was initially stocked with four juvenile shrimp with a mean weight of 1.54 ± 0.01 g. Survival was recorded daily and dead shrimp were immediately removed from the aquaria. Individual shrimp weight was recorded on a weekly basis to monitor growth and to adjust feed rations.

Performance Indicators

Survival was recorded by visually counting shrimp. Individual shrimp were patted dry using Kim Tech Wipes (Kimberly-Clark, Roswell, GA, USA) and weighed using an A&D HM-202 analytical balance (A&D Engineering Inc., Milpitas, CA, USA) to the nearest 0.0001 g. Specific growth rates (SGRs) were determined using Equation 1 (Ricker 1975). Food conversion ratio (FCR) calculations took into account the commercial shrimp feed (dry matter of 87.9%, n = 3).

$$\mathrm{SGR}\left(\frac{1}{\mathrm{d}}\right) = \frac{100 * \left[\ln \text{shrimp final mass}\,(\mathrm{g}) - \ln \text{shrimp initial mass}\,(\mathrm{g})\right]}{\text{time}\,(\mathrm{d})}$$

(Equation 3-1)

Laboratory Analysis

Nitrite-nitrogen, nitrate-nitrogen, and total ammonia nitrogen (TAN) were measured in accordance with HACH spectrophometric methods 8507, 8039 and 8038, respectively. Salinity, temperature, and dissolved oxygen (DO) were determined with an YSI 85 probe (Yellow Springs Inc., Yellow Springs, OH, USA). A HI 9024 pH meter (HANNA Instruments, Woonsocket, RI, USA) was used to determine pH.

Soluble COD was determined using Method 5220D (APHA 1998) after samples were passed through a 0.45 µm filter. Samples were quantified against a 6 point calibration curve (0-150 mg COD/L) using the HACH DR/2400 spectrophotometer set at 440 nm. Based on the calibration curve, the detection limit was approximately 10-15 mg COD/L because standards measured within this range often varied greatly. Values observed below this concentration were more qualitative than quantitative. A comparison of particle size distribution between untreated solids and microbial flocs was made using a laser diffraction particle size distribution analyzer (HORIBA LA-300 analyzer, Horiba Instruments Inc., Irvine, CA, USA; Windows Ver. 3.57 with a R.R. Index of 1.20-0.00i).

Relative protein levels for the untreated solids and microbial flocs were determined using a modified Lowry protein method (Pierce, Rockford, IL, adapted from Lowry et al., 1951). A ten point calibration curve (between 0 and 1,500 µg/ml), using bovine serum albumin as the reference standard, was constructed using spectrophotometry at 750 nm (Spectronic® Genesys™ 8 UV/Visible spectrophotometer, Thermo Spectronic, Rochester, NY, USA). Homogenized untreated solids and microbial floc samples were extracted for Lowry protein levels using the method outlined in

Higgins and Novak (1997), except samples in this study were homogenized for 30 s instead of 3 s using a Waring blender. Bound protein levels were normalized to total suspended solids (TSS). TSS was determined using Method 2540D outlined in Standard Methods (APHA 1998).

Statistical Analysis

Statistical analysis was performed using SAS v9.1 for Windows (SAS Institute Inc., Cary, NC, USA). Differences in water quality were considered significant when $P < 0.05$. A one-way ANOVA was employed with a Duncan's Multiple Range Test (and Levine's Test for Homogeneity), where appropriate, to test significant differences ($P < 0.05$) between mean survival, mass, and SGR for the various dietary treatments. Pooled error (pooled estimate; Moore 2003) values are included in the results if significant differences ($P < 0.05$) were observed and were calculated by taking the square-root of the mean-square error from the ANOVA output.

Results

Bioreactor

Degradation of sCOD over time is shown in Fig. 1. For each of the reactors, first-order rates of 0.160, 0.248, and 0.247 1/d were observed for reactors 1, 2, and 3, respectively. After 8 d, sCOD levels were below the limits of detection in both reactors 2 and 3. A representative particle size distribution, comparing untreated solids and

microbial flocs, is provided in Fig. 2. The mean diameter for untreated solids and microbial flocs were respectively 78.5 and 250 μm.

First Feeding Trial

Table 1 summarizes analyses of water quality variables in the six systems during the first feeding trial. No differences ($P > 0.05$) were observed for any of the water quality parameters examined, thereby confirming system parity in terms of performance of water treatment components. Mean relative protein levels of the microbial flocs and untreated solids were respectively 40.2 ± 1.5 and 23.8 ± 2.5 mg Lowry protein/g TSS. Microbial floc protein levels were greater (by 68.7%, $P < 0.05$) than that observed for untreated solids.

Survival rates and final mass are presented in Table 2. In terms of final mass, shrimp receiving 100% of their nutrition via commercial diets had greater weight gain than all other groups ($P < 0.05$). Animals receiving dietary microbial floc supplementation were on average larger than shrimp fed untreated solids. However, no differences were discerned between the two groups. Similar observations were made when shrimp fed untreated solids were compared against animals maintained on 50% of commercial ration. In contrast, shrimp provided with microbial flocs outperformed ($P < 0.05$) groups fed the 50% ration (Table 2). As expected, growth responses of shrimp were matched by their respective SGRs of 6.68, 5.71, 5.35, and 4.80 for diets one, two, three, and four. No differences were observed in terms of survival between the treatments. A comparison of size between animals fed on 100 or 50% commercial rations indicated that the latter were 53% smaller than the former. However, they expressed significantly

higher survival rates (Table 2). These results justified the use of diet 4 as a direct comparison against diets 2 and 3.

The microbial flocs used in diet 2 contributed significantly to growth, whereas the untreated solids used in diet 3 did not. Shrimp fed diets 2 and 3 were, at the end of the 40 day trial, respectively, 44% and 25% larger these fed diet 4.

Second Feeding Trial

Table 1 summarizes observations with respect to water quality analyses during the second feed study. Again, water quality did not vary significantly between systems ($P > 0.05$; Table 1). Relative protein levels for the microbial flocs on day 0, 7, 14, 21, 28, and 35 were respectively 60.1 ± 9.6, 50.7 ± 1.5, 55.4 ± 10.0, 62.6 ± 5.8, 58.1 ± 6.3, and 59.6 ± 9.5 mg Lowry protein/g TSS; there were no significant differences observed between weekly composite samples.

Weekly shrimp growth, SGRs, and FCRs are provided in Figs. 3, 4, 5, respectively. Shrimp fed diet three (6% BW/d + microbial flocs) significantly ($P < 0.05$) outperformed all other dietary treatments during this study in terms of growth and SGRs. Shrimp fed diet three outperformed all other diets from day 21 onwards on a mass basis. SGR data mimicked that observed for growth performance observed for the different diets. Thus, SGRs for shrimp fed at 6% per day with floc supplementation expressed significantly ($P < 0.05$) higher SGRs than all other dietary groups. No differences for mass or SGRs were observed between diets 1, 2 and 4 throughout this trial. Shrimp fed diet one (8% BW/d without flocs) demonstrated significantly ($P < 0.05$) poorer FCRs during some of the trial. Also, no differences were observed in terms of survival rates

between groups, final survival rates for diet one, two, three, and four were respectively 58, 83, 58, and 83%.

Discussion

The quality of water in the benchtop systems during the two trials did not differ significantly and was optimal for shrimp growth (Van Wyk et al. 1999). Accordingly, any growth effects were a function of feed effects. Levels of TAN and nitrate during both feeding trials did not exceed 7 and 1%, respectively, of the reported 24 h LC50 levels reported as 2.95 and 3,400 mg/L (Wickins 1976; Lin and Chen 2001) while levels of nitrite did not exceed 2.5% of the 96 h LC50 of 8.4 mg/L (Sowers et al. 2004).

The microbial flocs generated as a result of oxidizing sCOD were larger and contained higher levels of protein than the untreated solids in the tilapia effluent. As shown in Fig. 1, degradation of sCOD was relatively slow; however, if the sequencing batch reactors (SBRs) were operated in a traditional manner (e.g. using fill, react, settle, and decant cycles), their efficiency would increase (Fortin and Deshusses 1999; Metcalf and Eddy 2003) and thereby establish a microbial population that would be acclimated to the wastewater and would be recycled within the SBR. To control sludge age, portions of the microbial flocs would have to be wasted, and these portions could be used to supplement shrimp feed. This study focused on whether or not shrimp performance would be enhanced using microbial flocs generated in SBRs, rather than SBR performance. However, future studies are needed to fully understand the treatability of

fish effluent and nutritional properties of the associated microbial flocs.

Numerous studies (Moss et al. 2000; Moss et al. 2001; Tacon et al. 2002; Cuzon et al. 2004; Hari et al. 2004; McLean et al. 2006) have demonstrated that shrimp growth is enhanced in pond systems that are productive in terms of microbial flocs, algae, and other natural biota. In many cases, carbon to nitrogen (C:N) ratios were adjusted by reducing protein in the feed or by adding a carbon source (e.g. molasses) to promote heterotrophic floc growth, which requires a minimum stoichiometric C:N ratio of 10:1 (Hargreaves 2006). The study herein differed in that microbial flocs were generated while treating a waste stream, but nevertheless, yielded similar results as microbial flocs contributed significantly to shrimp growth. Treating a waste stream, such as fish effluent, instead of manipulating C:N in ponds, clearly offers an alternative and sustainable approach to integrated shrimp farming.

The superior performance of shrimp fed with microbial flocs relative to animals fed at the 50% ration level, or supplemented with untreated solids, was expected given that protein levels were higher in the microbial flocs than the untreated solids. The microbial flocs also were larger than the solids in untreated waters, which may have made these more attractive to the shrimp. Even though survival during the second feeding study appeared to vary greatly, survival rates were not significantly different ($P > 0.05$) between any of the treatments. Furthermore, final stocking densities were identical between diet one (8% BW/d without flocs) and diet three (6% BW/d + microbial flocs) treatments and the latter exhibited superior performance ($P < 0.05$) in terms of growth and SGRs.

In addition to the oxygen demand of shrimp, pond systems have the additional

oxygen demands of microbial flocs and algae respiration (Tacon et al. 2002, Burford et al. 2003). This added oxygen demand requires shrimp producers to aerate at high levels, which increases aeration expenses above those that culture shrimp in clear water systems. Aerobic suspended growth processes, such as the batch reactors used in this study, require low levels of DO (approximately 1 mg/L). Shrimp require levels of DO > 3.0 mg/L (Seidman and Lawrence 1986) and preferably > 5.0 mg/L (Van Wyk et al. 1999). Therefore, generating microbial flocs external from the shrimp production system and then transporting them to the shrimp system makes sense in terms of oxygen demand and operating costs, especially when considering RAS.

Conclusion

Even though numerous studies have demonstrated that shrimp growth and health are improved significantly in ponds that have a high activity of microbial flocs, algae, and other natural biota (Avnimelech 1999; Moss et al. 2000; Moss et al. 2001; Tacon et al. 2002; Cuzon et al. 2004; Izquierdo et al. 2006), pond aquaculture is primarily limited to tropical and subtropical climates. In this study, an alternative method for harnessing the benefits of microbial flocs for shrimp culture in RAS, while potentially reducing limitations associated with climate, environmental concerns, biosecurity, and feeding costs, and aeration, was examined. The results of this study demonstrated that microbial flocs generated in bioreactors, and offered as a supplemental feed, significantly improved shrimp growth and SGRs in shrimp fed a restricted ration. Moreover, treating fish

effluent to produce microbial flocs is a means of recycling nutrients in wastewater providing an additional sustainable measure for shrimp culture, as well as a means for fish producers to mitigate their effluent impacts on the environment.

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Literature Cited

Al-Hafedh, Y., A. Alam, and M. Alam. 2003. Performance of plastic biofilter media with different configurations in a water recirculation system for the culture of Nile tilapia, *Oreochromis niloticus*. Aquacultural Engineering 29:139-154.

Avnimelech, Y. 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176:227-235.

APHA (American Public Health Association). 1998. In: Clesceri, Greenberg, Trussell (Eds.), Standard methods for the examination of water and wastewater, 20th ed. Washington, D.C.

Burford, M. A., P. J. Thompson, R. P. McIntosh, R. H. Bauman, and D. C. Pearson. 2003. Nutrient and microbial dynamics in high-intensity zero-exchange shrimp ponds in Belize. Aquaculture 219:393-411.

Boyd, C. E. 2003. Guidelines for aquaculture effluent management at the farm-level. Aquaculture 226:101-112.

Cuzon, G., A. Lawrence, G. Gaxiola, C. Rosas, and J. Guillaume. 2004. Nutrition of *Litopenaeus vannamei* reared in tanks or ponds. Aquaculture 235:513-551.

FAO. 2000. Yearbook of Fishery Statistics 1998, Vol. 86/2. Aquaculture Production. FAO Statistics Series No. 154 and Fisheries No. 56, FAO, Rome, 182 pg.

Fortin, N. Y. and M. A. Deshusses. 1999. Treatment of methyl tert-butyl ether vapors in biotrickling filters. 1. Reactor startup, steady state performance, and culture characteristics. Environmental Science and Technology 33:2980-2986.

Hargreaves, J. A. 2006. Photosynthetic suspended-growth systems in aquaculture. Aquacultural Engineering 34:344-363.

Hari, B., B. Madhusoodana Kurup, J. T. Varghese, J. W. Schrama, and M. C. J. Verdegem. 2004. Effects of carbohydrate addition on production systems in extensive shrimp culture systems. Aquaculture 241:179-194.

Harvey, D. J. 2005. Electronic outlook report for economic research service. Aquaculture Outlook 2004. LDP-AQS-21. USDA, Washington, D.C.

Higgins, M. J. and J. T. Novak. 1997. Characterization of exocellular protein and its role in bioflocculation. Journal of Environmental Engineering - American Society of Chemical Engineers 123:479-485.

Izquierdo, M., I. Forster, S. Divakaran, L. Conquest, and O. Decamp. 2006. Effect of green and clear water and lipid source on survival, growth and biochemical composition of Pacific white shrimp *Litopenaeus vannamei*. Aquaculture Nutrition 12:192-202.

Jiménez-Montealegre, R., M. Verdegem, J. Zamora, and J. Verreth. 2002. Organic matter sedimentation and resuspension in tilapia, *Oreochromis niloticus*, ponds during a production cycle. Aquacultural Engineering 26:1-12.

Lin, Y -C. and J -C. Chen. 2001. Acute toxicity of ammonia on *Litopenaeus vannamei* Boone juveniles at different salinity levels. Journal of Experimental Marine Biology and Ecology 259:109-119.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-Phenol reagents. Journal of Biological Chemistry 193:265-275.

McLean, E., B. Reid, D. Fegan, D. Kuhn, and S. Craig. 2006. Total replacement of fishmeal with an organically certified yeast-based protein in Pacific White shrimp, *Litopenaeus vannamei*: laboratory and field trials. Ribarstvo 64:47-58.

Menasveta, P. 2002. Improved shrimp growout systems for disease prevention and environmental sustainability in Asia. Reviews in Fisheries Science 10: 391-402.

Metcalf and Eddy. 2003. Wastewater engineering: treatment and reuse. 4th ed. New York: McGraw-Hill.

Moore, D. S. 2003. The basic practice of statistics. 3rd ed. New York : W.H. Freeman and Company.

Moss, S. M., B. R. LeaMaster, and J. N. Sweeney. 2000. Relative abundance and species composition of gram-negative, anaerobic bacteria associated with the gut of juvenile white shrimp, *Litopenaeus vannamei*, reared in oligotrophic well water and eutrophic pond water. Journal of the World Aquaculture Society 31:255-263.

Moss, S. M., S. Divakaran, and B. G. Kim. 2001. Stimulating effects of pond water on digestive enzyme activity in the Pacific white shrimp, *Litopenaeus vannamei* (Boone). Aquaculture Research 32:125-131.

Naylor, R. L., R. J. Goldburg, H. Mooney, M. Beveridge, L. Clay, C. Folke, N. Kautsky, J. Lubchenco, J. Primavera, M. Williams. 1998. Nature's subsidies to shrimp and salmon farming. Science 282:883-884.

Paez-Osuna, F. 2001. The environmental impacts of shrimp aquaculture: Causes, effects, and mitigation alternatives. Environmental Management 28:131-140.

Ricker, W. E. 1975. Computation and interpretation of biological statistics of fish population. Bulletin of the Fisheries Research Board of Canada 191:1-382.

Seidman, E. R. and A. L. Lawrence. 1986. Growth, feeding digestibility and proximate body composition of juvenile *Penaeus vannamei* and *Penaeus monodon* growth at different dissolved oxygen levels. Journal of the World Mariculture Society 16:333-346.

Skjølstrup, J., E. McLean, P. H. Nielson, and J. –O. Frier. 2000. The influence of dietary oxolinic acid on fluidised bed biofilter performance in a recrculation system for rainbow trout, Oncorhynchus mykiss. Aquaculture 183:255-268.

Sowers, A., S. P. Young, J. J. Isely, C. L. Browdy, and J. R. Tomasso. 2004. Nitrite toxicity to *Litopenaeus vannamei* in water containing low concentrations of sea salt or mixed solids. Journal of the World Aquaculture Society 35:445-451.

Tacon, A., J. J. Cody, L. D. Conquest, S. Divakaran, I. P. Forster, and O. E. Decamp. 2002. Effect of culture system on the nutrition and growth performance of Pacific white shrimp, *Litopenaeus vannamei* (Boone) fed different diets. Aquaculture Nutrition 8:121-137.

Timmons, M. B., J. M. Ebeling, F. W. Wheaton, S. T. Summerfelt, and B. J. Vinci. 2002. Recirculating Aquaculture Systems, 2nd Ed. Cayuga Aqua Ventures, Ithaca, New York.

Van Wyk, P., M. Davis-Hodgkins, C. R. Laramore, K. Main, J. Mountain, and J. Scarpa. 1999. Farming marine shrimp in recirculating freshwater production systems: a practical manual. FDACS Contract #4520. Florida Department of Agriculture Consumer Services, Tallahassee, FL.

Wickins, J. F. 1976. The tolerance of warm-water prawns to recirculated water. Aquaculture 9:19–37.

Table 3-1. Water quality results observed during the first and second feeding trial, mean values with 95% confidence intervals (n denotes the number of sampling events).

Feeding trial	AHAB system	Dissolved oxygen [mg/l]	Nitrate-N [mg/L]	Nitrite-N [mg/L]	pH	Salinity [ppt]	Total ammonia-N [mg/L]	Temperature [^{o}C]
		n = 13	n = 9	n = 9	n = 11	n = 16	n = 11	n = 28
1	1	5.21 (4.87-5.55)	15 (5.5-25)	0.020 (0-0.072)	8.45 (8.37-8.53)	1.88 (1.60-2.17)	0.018 (0-0.045)	29.8 (28.5-31.1)
1	2	5.18 (4.76-5.61)	16 (5.9-26)	0.022 (0-0.077)	8.48 (8.35-8.62)	1.96 (1.85-2.06)	0.020 (0-0.040)	29.4 (27.7-31.1)
1	3	5.20 (4.82-5.57)	18 (5.5-31)	0.029 (0-0.11)	8.50 (8.37-8.62)	1.97 (1.87-2.06)	0.022 (0.002-0.041)	29.1 (27.9-30.4)
1	4	5.06 (4.73-5.39)	20 (6.7-33)	0.032 (0-0.14)	8.53 (8.37-8.68)	2.15 (1.91-2.39)	0.021 (0-0.048)	30.0 (28.3-31.6)
1	5	5.16 (4.85-5.47)	12 (5.9-18)	0.017 (0-0.049)	8.45 (8.24-8.65)	1.86 (1.39-2.32)	0.025 (0-0.065)	29.4 (28.0-30.7)
1	6	5.10 (4.71-5.50)	18 (5.9-31)	0.012 (0-0.024)	8.49 (8.34-8.65)	2.11 (1.97-2.26)	0.029 (0-0.070)	29.5 (27.7-31.3)
2	1	6.40 (5.90-6.90)	21 (0-46)	0.032 (0-0.063)	8.60 (8.51-8.69)	2.03 (1.87-2.19)	0.100 (0-0.199)	28.9 (27.7-30.2)
2	2	6.34 (5.83-6.84)	19 (0-41)	0.033 (0-0.069)	8.62 (8.52-8.72)	2.03 (1.90-2.16)	0.112 (0-0.223)	29.2 (28.2-30.1)
2	3	6.42 (5.91-6.93)	18 (0-37)	0.025 (0-0.052)	8.61 (8.52-8.71)	2.06 (1.72-2.40)	0.111 (0-0.195)	29.0 (28.1-30.0)

Table 3-2. Shrimp performance results for the first feeding trial, alphas denote significant differences ($P < 0.05$).

Diet	Final mass [mg]	Survival [%]
Diet one: 100% shrimp feed	385.4[a]	73
Diet two: 50% shrimp feed, 50% microbial flocs	259.1[b]	93
Diet three: 50% shrimp feed, 50% untreated solids	225.7[b,c]	87
50% shrimp feed	180.2[c]	93
Pooled error	39.03	15.06
P > F	<0.0001	0.1027

Figure 3-1. Soluble COD degradation over time with first order fits, error bars denote standard errors for each data point (n = 2 for each data point).

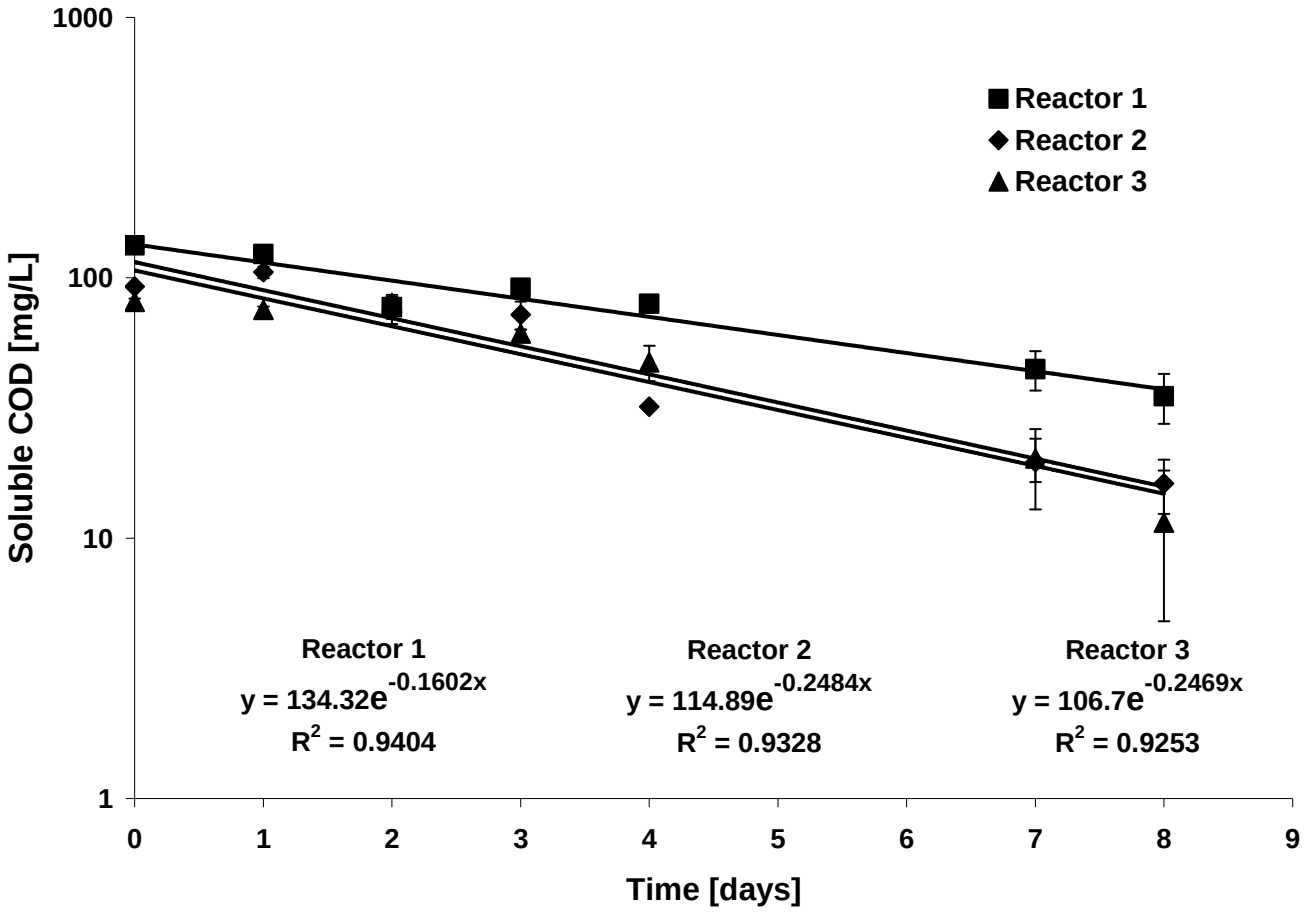


Figure 3-2. Representative particle size distribution for untreated solids (top) and microbial flocs (bottom).

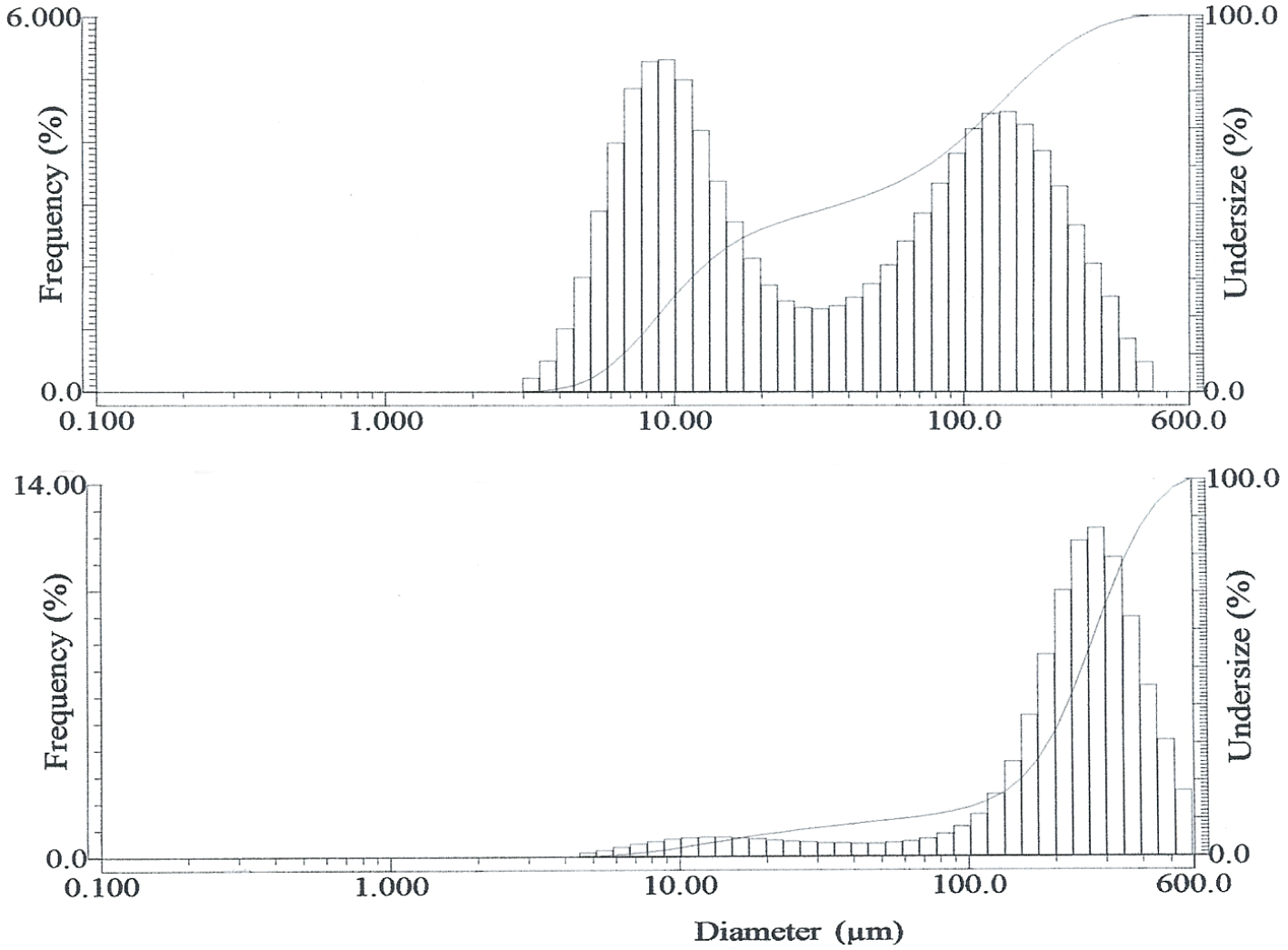


Figure 3-3. Shrimp growth (mean values ± standard errors) observed during the second feeding trial, alphas denote significant differences ($P < 0.05$). Day 21, pooled error = 0.2679, $P > F = 0.0181$. Day 28, pooled error = 0.4215, $P > F = 0.0228$. Day 35, pooled error = 0.5254, $P > F = 0.0074$.

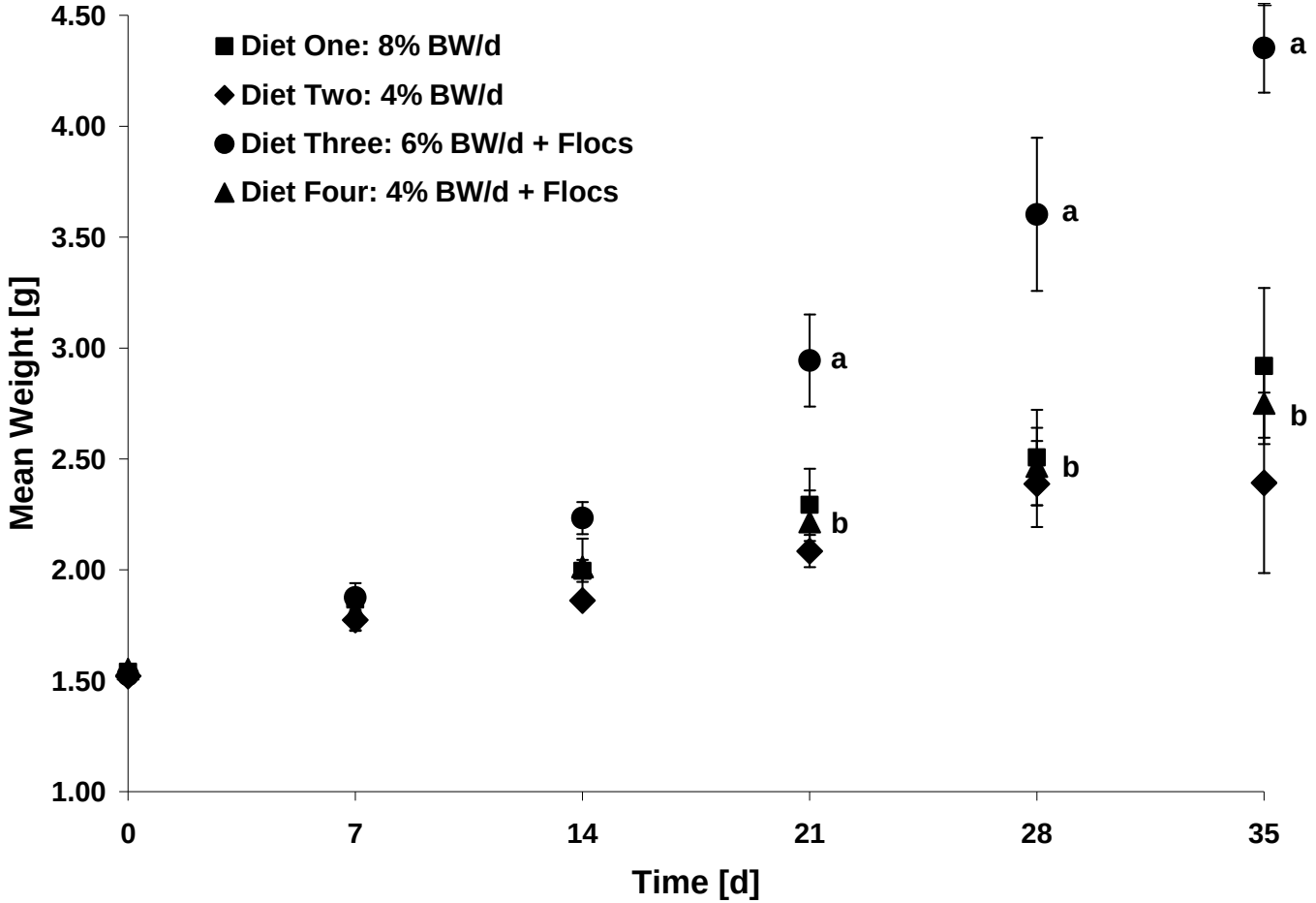


Figure 3-4. Weekly cumulative SGRs (mean values ± standard errors) observed during the second feeding trial, alphas denote significant differences (P < 0.05). Day 21, pooled error = 0.4334, P > F = 0.0072. Day 28, pooled error = 0.5256, P > F = 0.0295. Day 35, pooled error = 0.5157, P > F = 0.0075.

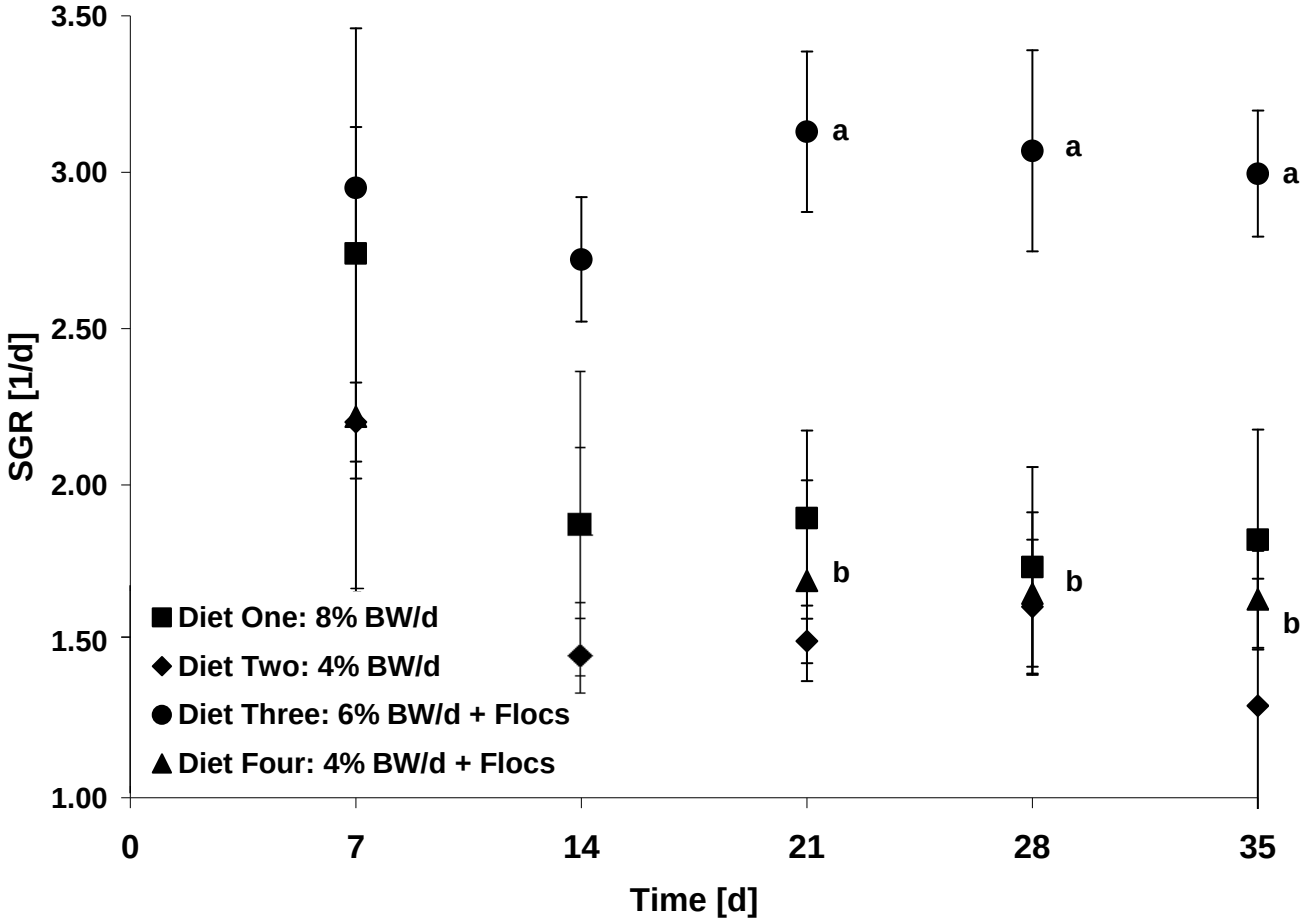
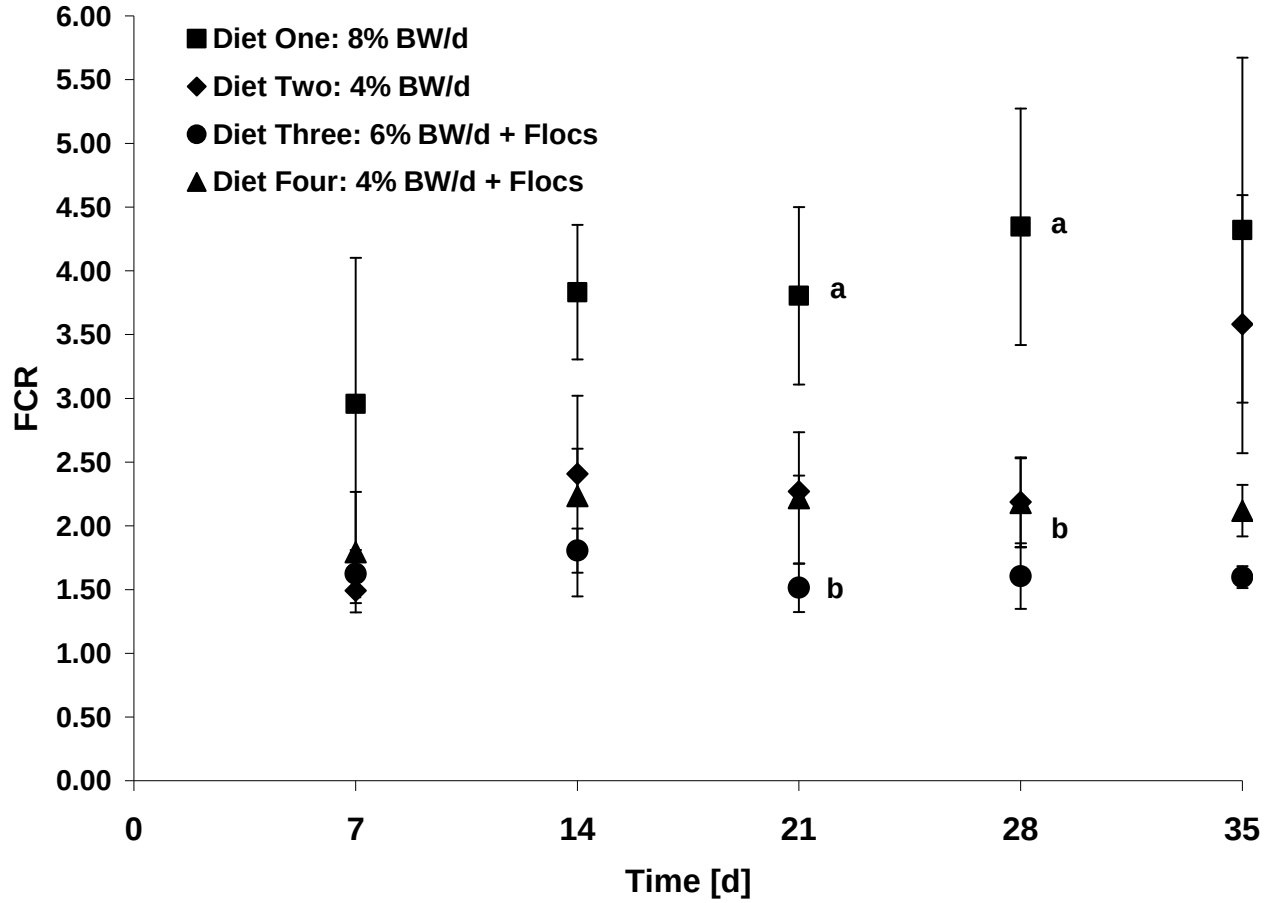


Figure 3-5. Weekly cumulative FCRs (mean values ± standard errors) observed during the second feeding trial, alphas denote significant differences ($P < 0.05$). The raw data failed the Levine's Test for Homogeneity; therefore, a logarithm transform was employed and subsequently passed the test. The following statistical values reflect the transformation; Day 21, pooled error = 0.2816, $P > F = 0.0282$. Day 28, pooled error = 0.3115, $P > F = 0.0284$.



Chapter 4:

Microbial flocs generated in bioreactors as an ingredient for shrimp feed and enhanced shrimp growth

To be submitted to peer reviewed journal, Aquaculture

Abstract

Microbial flocs produced in suspended growth bioreactors could offer the shrimp industry a novel alternative feed ingredient. In this study, microbial flocs were produced in sequencing batch reactors using tilapia effluent and sugar as a growth media. It was determined that 1 kg of microbial floc could be produced per 1.49 kg of sugar. These microbial flocs were tested as an ingredient for shrimp feed over a 35 day feeding trial. Two control diets (absent of microbial flocs) were compared against three dietary treatments (microbial floc inclusion). Control 1 and microbial floc diets (diets 1-3) were formulated to be equivalent for levels of crude protein, total fat, crude fiber, calcium, magnesium, phosphorus, potassium, and sodium. Control 1, as well as a second control, did not contain microbial flocs and differed slightly from each other in soybean oil, krill meal, and mineral/salt levels. For diet 1 (microbial floc 7.8%) and diet 2 (microbial floc 15.6%), soybean protein isolate (on a protein basis) was replaced with microbial flocs at a 7.8 and 15.6% inclusion level (on a dry matter basis). For diet 3, fishmeal was replaced with microbial flocs at 7.8% and fish oil at 0.50% (microbial floc 7.8%+FO). Four juvenile shrimp were stocked per tank and each dietary treatment was tested in 12 replicates over a 35 day feeding trial. No differences were observed between final survival rates (93 to 100%) between any of the dietary treatments. Weight gain (weight gain per week) for control 1, control 2, diet 1, diet 2, and diet 3 were respectively 5.46 ± 0.68 (1.09 ± 0.14), 4.42 ± 0.70 (0.88 ± 0.14), 8.18 ± 0.14 (1.64 ± 0.03), 8.07 ± 0.17 (1.61 ± 0.03), and 8.13 ± 0.20 g (1.63 ± 0.04 g/wk). Tukey's HSD revealed that each of the three microbial floc diets significantly ($P < 0.01$) outperformed each control in terms of weight gain, weight gain per week, and specific growth rates. However, there were no significant differences ($P > 0.05$) between the two controls or between the three diets that included microbial flocs.

Introduction

Traditional Penaeid shrimp culture using extensive techniques has long been practiced in tropical ponds. The global shrimp market has expanded from less than $1 billion to $5.8 billion (US) from 2000 to 2005 (FAO, 2008). To meet this growing demand, the shrimp industry is shifting from small-scale, extensive rearing systems to large-scale, intensive rearing systems. This shift has been mostly successful. However, there are numerous drawbacks and concerns regarding intensive systems, especially those outdoors in an uncontrolled environment. The drawbacks include the possibilities of stressed animals, increased disease, increased oxygen demands, and decreased water quality. Generally, these risks can be reduced, while maintaining a high density of animals, by employing a controlled, indoor environment (e.g. recirculating aquaculture systems). Several water treatment technologies are used in recirculating aquaculture systems to clean water for reuse within the culture system (Timmons et al., 2002) and for reuse of the water in polyculture systems (Kuhn et al., 2007). Often included among these treatment operations are: nitrification (e.g. with fluidized sand filters or plastic media), oxygenation (e.g. with direct aeration or use of Speece cone), disinfection (e.g. with ultraviolet light), and solids removal (e.g. with settling or drum filters) (Skjølstrup et al., 2000; Menasveta, 2002; Timmons et al., 2002).

Even though clear water recirculating systems have numerous benefits over outdoor intensive ponds, implementation of recirculating aquaculture systems has not yet translated into a solution for intensive shrimp culture. This is because numerous studies have demonstrated that shrimp reared in clear water do poorly in terms of growth,

survival, and health. This becomes increasingly evident when shrimp tested in clear water are compared directly to shrimp in systems with a high productivity of natural organisms (e.g. algae, bacteria, and other natural biota) (Tacon et al., 2002; Izquierdo et al., 2006; Moss et al., 2006). McLean et al. (2006) reported that shrimp can grow equally as well on a yeast based diet as a fishmeal diet. It is speculated that these naturally occurring organisms contribute nutritionally and serve as a pre-/probiotic. For these reasons, it was hypothesized that microbial flocs generated in sequencing batch reactors (SBRs) could potentially serve as a valuable alternative ingredient for shrimp feed.

Biological treatment of aquaculture effluent using suspended growth processes has been demonstrated with (Schneider et al., 2006; Schneider et al., 2007) and without (Sharrer et al., 2007) carbon supplementation. In this study, SBRs were used to treat effluent from a tilapia facility that was supplemented with sugar. Microbial flocs produced in SBRs would be different from naturally occurring organisms found in pond systems because they would be produced in the dark external from the shrimp culture. The microbial flocs could be dried and incorporated into a pelleted feed for shrimp. If this alternative feed proved to be successful, it could offer the shrimp industry a new culture option in clear water recirculating aquaculture systems. Thus, the overall goal of this work was to demonstrate the potential of producing a microbial floc in SBRs that would enhance the performance of shrimp feed.

Materials and methods

Experimental design

Microbial flocs generated in SBRs by Virginia Tech researchers at a shrimp pilot facility (Virginia Shrimp Farms, Martinsville, Virginia, US) were used as a test ingredient in shrimp feed, over a 35 day feeding trial. Two control diets (absent of microbial flocs) were compared against three dietary treatments (microbial floc inclusion). Each dietary control/treatment consisted of 12 replicates over six systems in a randomized block design. Each 20 L tank was stocked with four juvenile shrimp. This feeding trial was conducted indoors at the Texas A&M AgriLife Research Mariculture Laboratory (Port Aransas, Texas, US) using indoor recirculating systems with seawater renewal.

Sequencing batch reactors used to produce microbial flocs

Wastewater was diverted from a tilapia farm where recirculating aquaculture systems are used. This effluent was pumped to an aerated, well-mixed equalization tank (11,300 L). Wastewater in the equalization tank was monitored for five days over a one week period for nitrate (95.7 ± 10.9 mg N/L), nitrite (0.56 ± 0.11 mg N/L), pH (7.09 ± 0.02 mg/L), total ammonia nitrogen (TAN, 10.8 ± 2.7 mg/L), and soluble total organic carbon (STOC, 20.8 ± 3.1 mg/L). Effluent was manually drained into two commercially sized, 5,100 L SBRs (Model CA-15d, Cromaglass Corp., Williamsport, Pennsylvania, US). The SBRs were well-mixed using water pumps and aeration. Carbon in the form of granulated white sugar (Kroger Co., Cincinnati, Ohio, US) was added to the wastewater

at a target rate of 80 mg C/L. The SBRs were operated inside a building and had manhole covers to prevent light penetration. Aerobic batch tests were performed in the SBRs with and without carbon supplementation in duplicate at 19 ^{o}C.

Total ammonia nitrogen (TAN) was determined spectrophotometrically using methods approved by the US Environmental Protection Agency for the analysis of wastewater (HACH Co., Loveland, Colorado, US). Soluble chemical oxygen demand (SCOD), STOC, and volatile suspended solids (VSS) were measured in accordance with APHA (2005). The VSS concentration is commonly used as a measurement of microbial mass (Metcalf and Eddy, 2003).

Microbial flocs as an ingredient for shrimp feed

The nutritional composition of the microbial flocs was analyzed by A&L Eastern Laboratories, Inc. (Richmond, Virginia, US) and is reported in Table 1. Samples of the flocs were collected twice (14 days between sampling events) to determine microbial floc nutritional consistency. Microbial flocs were harvested as a dietary ingredient for the shrimp feeding trial during the first sampling event. Settled microbial flocs were harvested from SBRs by siphoning and were air dried in 5 cm layers to solids levels greater than 86%. Microbial flocs were subsequently ground into a fine material, using a stand mixer with grain mill attachment (KitchenAid® Professional 600 Series, Saint Joseph, Michigan, US).

Shrimp

The shrimp used in this study came from Oceanic Institute (OI) and were free of pathogens listed by the US Marine Shrimp Farming Program (2006), including the Taura syndrome virus (TSV), White spot syndrome virus (WSSV), Yellow head virus (YHV), Infectious hypodermal and hematopoietic necrosis virus (IHHNV), and Infectious myonecrosis virus (IMNV). These shrimp were from OI's "Kona" line which is a reference strain of shrimp that originated in Sinaloa, Mexico. This shrimp line has been domesticated for over 15 years and is often used as a positive control in disease-challenge studies because of its consistent performance over time (Hennig et al. 2004).

Experimental systems for shrimp

Six recirculating systems were used to culture shrimp. Each system was comprised of two rows (front and back) with 12 tanks per row (24 tanks per system). Five out of the 12 tanks in a row were dedicated to this study. Each opaque tank had a cover, an air diffuser, and a volume of 20 L with a bottom area of 0.09 m^2. Each system included 100 μm filtration, an aerated sump for nitrification, and ultraviolet irradiation. Each tank had a recirculation rate of 5,400% per day, and seawater renewal rate of 85.7% per day. Seawater was pumped from the Corpus Christi Ship Channel about 1 km from the Gulf of Mexico. This seawater was pumped through a series of pressurized sand filters (50, 25, and 1 μm) and was stored in three, 53,000 L opaque fiberglass reservoirs prior to use.

Water quality in experimental systems used for shrimp

Each experimental system for shrimp was monitored daily for dissolved oxygen (DO), salinity, and temperature using a YSI 85 meter (Yellow Springs, Ohio, US). Nitrate, nitrite, pH, and (TAN) were measured weekly using methods designed for seawater samples (Spotte, 1979). More detail for nitrate, nitrite, and TAN procedures can be found in Stickland and Parsons (1972), Mullin and Riley (1955), and Solorzano (1969), respectively.

Diets and feeding regime for shrimp

Control 1 and microbial floc diets (diets 1-3) were formulated to be equivalent for levels of crude protein, total fat, crude fiber, calcium, magnesium, phosphorus, potassium, and sodium (Table 2). Controls 1 and 2 did not contain microbial flocs and differed slightly from each other in soybean oil, krill meal, and mineral/salt levels. Inclusion of microbial flocs was used in diets 1, 2, and 3. For diet 1 (microbial floc 7.8%) and diet 2 (microbial floc 15.6%), soybean protein isolate was replaced on a protein basis with microbial flocs at a 7.8 and 15.6% inclusion level. For diet 3 (microbial floc 7.8%+FO), fishmeal was replaced on a protein basis with microbial flocs at 7.8% and fish oil at 0.50%. Measured nutrient levels (A&L Eastern Laboratories, Inc., Richmond, Virginia, US) for each formulated diet are reported in Table 3. Shrimp were fed 15 times daily using modified automatic feeders (Lifegard Automatic Fish Feeder, Pentair Aquatics®, El Monte, California, US) at a rate based on an excess food conversion ratio (FCR) of 2, assuming a weight gain of 1 g/wk. Uneaten feed was removed daily by siphoning.

Shrimp performance indicators

Group weights of all shrimp were recorded on a per tank basis at study initiation. Shrimp were stocked at a density of 4 shrimp per tank (57 shrimp/m^2, or 0.20 shrimp/L). Survival rates were recorded daily and any moribund/dead shrimp were removed immediately from the study. At the termination of the experiment, final group weights of shrimp were made on a per tank basis. Survival, weight gain, weight gain per week, and specific growth rates (SGRs) were used to asses dietary effects on shrimp performance.

Analysis of data

Statistical analysis was performed using SAS v9.1.3 for Windows (Cary, North Carolina, US). Differences in water quality were considered significant when $P < 0.05$. Analysis of variance (ANOVA) was used on shrimp performance data to look at effects of diet (5 levels). Additionally, weight gain data was subjected to ANOVA with two blocking factors (system at 6 levels, row at 2 levels) to see if there were any effects of rows or systems. When appropriate, a post-hoc test (Tukey's HSD) was employed to check for differences between means. The 5% significance level was used for all tests. With the exception to initial weight and survival, the following transformation (Equation 4-1) was used to equalize the variance of all shrimp performance data.

$$Transformation = \sqrt{e^{X}} \qquad \text{Equation 4-1}$$

Results

Microbial floc generation in SBRs

Carbon supplementation enhanced removal rates of TAN (Figure 1) and microbial floc generation (Figure 2) in SBR batch tests with the tilapia effluent. Without carbon supplementation, TAN treatment averaged < 1.0 %/h. Carbon supplementation increased the observed mean rate to 26.0 %/h. Generation of microbial flocs without carbon supplementation averaged < 1.0 (mg VSS)/(L·h), while carbon supplementation increased the observed mean rate to 95.1 (mg VSS)/(L·h). This sugar source is 42 % carbon based on soluble COD and STOC correlations. The following data represent calculated kinetic coefficients when sugar was used as a carbon supplement. Uptake of soluble COD as microbial flocs were generated can be observed in Figure 3. The average uptake rate of STOC was 0.14 (g carbon)/(g VSS·h) or 0.32 (g sugar)/(g VSS·h). Meanwhile, the observed mean specific growth rate was 0.22 1/h. The observed yield coefficient was calculated to be 1.6 (g VSS)/(g carbon) or 0.67 (g VSS)/(g sugar).

Diets and Water quality

The composition of diets is provided in Table 3. A one-way ANOVA did not reveal any differences between means for proximate values between diets. More specifically, no differences were observed between diets for crude protein ($P = 0.1244$), calculated carbohydrate ($P = 0.0528$), total fat ($P = 0.5646$), and fiber ($P = 0.8822$).

Table 4 summarizes the water quality parameters measured during the 35 day feeding trial for each of the six experimental systems. No differences were observed

between any water quality parameters, thereby confirming system uniformity. Water quality levels were within safe levels for normal shrimp health, growth, and survival.

Shrimp performance

As presented in Table 5, no differences were observed using an ANOVA between initial weights ($P = 0.9032$) or survival ($P = 0.2079$). Tukey's HSD revealed that that all three microbial floc diets independently and significantly ($P < 0.01$) outperformed either control in terms of weight gain, weight gain per week, and SGRs (Table 5). Microbial floc diets enhanced shrimp growth by an average of 65.1% over the mean growth of control diets. More specifically, shrimp fed microbial flocs on average grew 49.2 and 84.8% faster than the shrimp in controls 1 and 2, respectively. No differences ($P = 0.4904$) in weight gain were detected between front and back rows within the systems. Even though systems had an effect on weight gain ($P = 0.0365$), no pair wise differences were detected using Tukey's HSD.

Discussion

Microbial flocs were produced in SBRs using tilapia effluent and sugar as a carbon source. Carbon supplementation selects for heterotrophic bacteria (Avnimelech, 1999; Ebeling et al., 2006) and other heterotrophic microorganisms (Metcalf, Eddy, 2003). Batch tests in this study provided evidence of heterotrophic domination. Removal of TAN without carbon supplementation was not efficient (Figure 1) and was 26 times slower than batches that were supplemented with carbon. Similar benefits of carbon supplementation were observed for microbial floc production. Without carbon

supplementation, no generation of microbial flocs was observed over a 240 minute period (Figure 2).

Sirianuntapiboon and Prasertsong (2008) used SBRs to treat a waste stream that contained molasses and found that glucose supplementation significantly increased treatability. Molasses is a sugar by-product with a high sugar content (48-50%). This inexpensive carbon source is often used in light-based, heterotrophic shrimp systems (Burford et al., 2003, Samocha et al., 2007). The major components of molasses are polysaccharides which are too complex to be readily biodegraded. Studies (Najafpour and Shan, 2003; Quan et al., 2005) have shown that molasses reduced to sugars such as sucrose, glucose, and fructose significantly enhances its treatment in bioreactors. Sugar (sucrose) was therefore chosen in this study because it is readily assimilated by microbes and easy to use.

The calculated yield coefficient, 0.67 (g VSS)/(g sugar), represents the amount of microbial floc generated per unit sugar consumed by the heterotrophic microorganisms. Alternatively, this could be expressed as 1 kg of microbial flocs produced per 1.5 kg of sugar added to the SBRs. Even though the maximum specific growth rate was not determined in this study, the observed mean specific growth rate was 0.22 1/h, twice as great as the maximum values observed when molasses was used in the treatment of aquaculture wastewater (0.10-0.12 1/h; Schneider et al., 2006). However, specific growth rates seen in this study are similar to the growth rates of yeast (*Candida utilis*) grown on sugar cane stillage (0.20–0.27 1/h; Cabib et al., 1983; Bottaro Castilla et al., 1984) or for more generally described, aerobically produced heterotrophic organisms (0.2-0.5 1/h; Rittmann and McCarty, 2001; Metcalf and Eddy, 2003).

Proximate values were not different from each other between any of the diets (Table 3) and water quality in the shrimp systems did not vary from each other (Table 4). Formulated diets and water quality values were considered optimal for shrimp culture (Van Wyk et al., 1999; Cuzon et al., 2004). This allowed direct comparisons between diets with and without microbial flocs to be made. Survival rates did not vary between dietary treatments; however, shrimp growth was significantly improved ($P < 0.01$) by microbial flocs (Table 5). Even though numerous studies have reported enhanced survival, health, and growth rates of shrimp raised in ponds with high algal activity, microbial flocs, and other natural biota (Avnimelech 1999; Moss et al. 2000; Moss et al. 2001; Tacon et al. 2002; Burford; 2004; Cuzon et al. 2004; Izquierdo et al. 2006; Wasielesky et al., 2006), none of these studies were performed with microbial flocs produced in the dark. The results of this study are consistent with Kuhn et al. (2008) who reported that shrimp growth was enhanced by microbial flocs which were produced in the treatment of tilapia aquacultural wastewater with an enclosed reactor and no carbon additions. In the previous study, microbial flocs was fed directly to the shrimp, as opposed to being dried and pelleted in this current work. The enhancement of shrimp growth was actually more consistent in this study than in the earlier work (Kuhn et al., 2008), as was the treatment of tilapia effluent with carbon supplementation.

It is not known exactly how microbial flocs enhance growth. Izquierdo et al. (2006) suggested that the lipid contributions of microbial flocs are important. Microbial flocs in this study were low in fats (1.13 ± 0.09 %) and contributed very low lipid levels to the microbial floc diets (e.g. < 0.1 % of diet 1 or 3). Therefore, the contribution of lipids was limited and was not likely the reason for enhanced growth. Perhaps, enhanced

growth was due to the microbial flocs having a more favorable amino acid profile or being more digestible. It is also speculated that microbial flocs are probiotics (Kesarcodi-Watson et al., 2008). For example, Bairagi et al. (2002) noted that the aerobic bacteria in fish gastrointestinal tracts produced digestive enzymes that improved feed utilization and digestion. More recently, Bairagi et al. (2004) reported that *Bacillus subtilis* and *Bacillus circulans* in a feed for rohu (*Labeo rohita*) significantly enhanced growth, food conversion ratios, and protein efficiency ratios. These authors attributed enhanced performance to extracellular cellulolytic (digestion of plant tissue) and amylolytic (conversion of starch to sugar via amylase) enzyme production by the bacteria. Numerous digestive enzymes contribute to shrimp digestion, and the three major groups include lipases, proteases, and carbohydrases (e.g. amylases) (Cuzon et al., 2004). Arena (2003) discovered that domestication of *L. vannamei* over generations (wild, 7th, and 25th generations) had significantly decreased amylase activity. He attributed this to a reduction in the alleles for the α-amylase gene. Since the majority of *L. vannamei* are domesticated, a probiotic that could enhance amylolytic enzyme activity is even more crucial. Ultimately, more work is needed to fully realize the contributions of microbial flocs produced in biological reactors and in ponds.

Conclusion

Numerous studies have demonstrated that shrimp are healthiest and grow best in ponds or systems that have high levels of algae, bacteria, and other natural biota. This study differed significantly because microbial flocs were generated externally from

shrimp systems in dark reactors, using a simple sugar and tilapia wastewater. Microbial floc inclusion significantly enhanced shrimp growth ($P < 0.01$) over a 35 day feeding trial. Addition of dried microbial flocs into a feed is also a novel approach. Not only did this allow for a controlled study, but this technique can also be readily adopted by the shrimp industry. This could be especially important for inland industries that would like to implement clear water recirculating aquaculture systems. These clear water recirculating systems generally have benefits over traditional pond or flow-through systems in terms of biosecurity, conservation of water and salts, wastewater treatment, solids management, feed management, and oxygen demand. It is hoped that results from this study can be adopted by the shrimp industry as a means to increase economic and environmental sustainability.

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Literature Cited

APHA. 2005. Standard Methods for the Examination of Water and Wastewater, 21st edition. Edited by Clesceri, Greenberg and Trussell. Washington DC, US.

Arena, L., Cuzon, G., Pascual, C., Gaxiola, G., Soyez, C., Van Wormhoudt, A., Rosas, C., 2003. Physiological and genetic variations in domesticated and wild populations of *Litopenaeus vannamei* fed with different carbohydrate levels. J. Shellfish Res. 22: 269-279.

Avnimelech, Y., 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176, 227-235.

Bairagi, A., Sakar Ghosh, K., Sen, S.K., Ray, A.K., 2002. Enzyme producing bacterial flora isolated from fish digestive tracts. Aquacult. Int. 10, 109–121.

Bairagi, A., Sarkar Ghosh, K., Sen, S.K., Ray, A.K., 2004. Evaluation of the nutritive value of *Leucaena leucocephala* leaf meal, inoculated with fish intestinal bacteria *Bacillus subtilis* and *Bacillus circulans* in formulated diets for rohu, *Labeo rohita* (Hamilton) fingerlings. Aquac. Res. 35, 436-446.

Bottaro Castilla, R., Waehner, R.S., Giulietti, A.M., 1984. Aerobic microbial treatment of sugar cane stillage by *Candida utilis* and *Paecilomyces variotii* in two steps continuous culture. Biotechnol. Lett. 6, 195–198.

Burford, M.A., Thompson, P.J., McIntosh, R.P., Bauman, R.H., Pearson, D.C., 2003. Nutrient and microbial dynamics in high-intensity, zero-exchange shrimp ponds in Belize. Aquaculture 219, 393-411.

Burford, M.A., Thompson, P.J., McIntosh, R.P., Bauman, R.H., Pearson, D.C., 2004. The contribution of flocculated material to shrimp (*Litopenaeus vannamei*) nutrition in a high-intensity, zero-exchange system. Aquaculture 232, 525-537.

Cabib, G., Silva, H.J., Giulietti, A., Ertola, R., 1983. The use of sugar cane stillage for single cell protein production. J. Chem. Technol. Biotechnol. 33B, 21–28.

Cuzon, G., Lawrence, A., Gaxiola, G., Rosas, C., Guillaume, J., 2004. Nutrition of *Litopenaeus vannamei* reared in tanks or in ponds. Aquaculture 235, 513-551.

Ebeling, J.M., Timmons, M.B., Bisogni, J.J., 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia–nitrogen in aquaculture systems. Aquaculture 257, 346-358.

FAO (Food and Aqriculture Organization of the United Nations). 2008. Cultured aquaculture species information programme *Penaeus vannamei* (Boone, 1931). Food and Aqriculture Organization of the United Nations, Online: www.fao.org.

Hennig, O.L., Keller, K., Rasmussen, L., Arce, S.M., Moss, S.M., White-Noble, B., Lightner, D.V., Breland, V., Lotz, J., 2004. Strain of reference shrimp aids researchers, farmers. Global Aquaculture Advocate 7,74.

Izquierdo, M., Forster, I., Divakaran, S., Conquest, L., Decamp, O., 2006. Effect of green and clear water and lipid source on survival, growth and biochemical composition of Pacific white shrimp *Litopenaeus vannamei*. Aquacult. Nutr. 12, 192-202.

Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J., Gibson, L., 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. Aquaculture 274, 1-14.

Kuhn, D.D., Boardman, G.D., Craig, S.R., Flick, G.J., Mclean, E., 2007. Evaluation of tilapia effluent with ion supplementation for marine shrimp production in a recirculating aquaculture system. J. World Aquacult. Soc. 38, 74-84.

Kuhn, D.D., Boardman, G.D., Craig, S.R., Flick, G.J., Mclean, E., 2008. Use of microbial flocs generated from tilapia effluent as a nutritional supplement for shrimp, *Litopenaeus vannamei*, in recirculating aquaculture systems. J. World Aquacult. Soc. 39, 72-82.

McLean, E., Reid, B., Fegan, D., Kuhn, D., Craig, S., 2006. Total replacement of fishmeal with an organically certified yeast-based protein in Pacific White Shrimp (*Litopenaeus vannamei)* diets: laboratory and field trials. Ribarstvo 64: 47-58.

Menasveta, P., 2002. Improved shrimp growout systems for disease prevention and environmental sustainability in Asia. Reviews in Fisheries Science 10, 391-402.

Merrill, A.L., Watt, B.K., 1973. Energy value of foods: basis and derivation. United States Department of Agriculture (USDA), Handbook No. 74.

Metcalf and Eddy, 2003. Wastewater Engineering: Treatment and Reuse, 4th edition. McGraw-Hill, New York, New York, US.

Moss, S.M., LeaMaster, B.R., Sweeney, J. N., 2000. Relative abundance and species composition of gram-negative, anaerobic bacteria associated with the gut of juvenile white shrimp, *Litopenaeus vannamei*, reared in oligotrophic well water and eutrophic pond water. J. World Aquacult. Soc. 31, 255-263.

Moss, S.M., Divakaran, S., Kim, B.G., 2001. Stimulating effects of pond water on digestive enzyme activity in the Pacific white shrimp, *Litopenaeus vannamei* (Boone). Aquac. Res. 32, 125-131.

Moss, S.M., Forster, I.P., Tacon, A.G.J., 2006. Sparing effect of pond water on vitamins in shrimp diets. Aquaculture 258, 388-395.

Mullin, J.D., Riley, J.P., 1955. The spectrophotometric determination of nitrate in natural waters, with particular reference to sea-water. Anal. Chim. Acta 12 (1955), pp. 464–480.

Najafpour, G.D., Shan, C.P., 2003. Enzymatic hydrolysis of molasses. Bioresour. Technol. 86, 91-94.

Quan, Z-X., Jin, Y-S., Yin, C-R., Lee, J.J., Lee, S-T., 2005. Hydrolyzed molasses as an external carbon source in biological nitrogen removal. Bioresour. Technol. 96, 1690-1695.

Rittmann, B.E., McCarty, P.L., 2001. Environmental Biotechnology: Principles and Applications. McGraw-Hill, New York, New York, US.

Samocha, T.M., Patnaik, S., Speed, M., Ali, A.-M, Burger, J.M., Almeida, R.V., Margasanto Harisanto, Z.A., Horowitz A., Brock, D.L., 2007. Use of molasses as carbon source in limited discharge nursery and grow-out systems for Litopenaeus vannamei. Aquacult. Eng. 36, 184-191.

Schneider, O., Sereti, V., Eding, E.H., 2006. Molasses as C source for heterotrophic bacteria production on solid fish waste. Aquaculture 261, 1239-1248.

Schneider, O., Sereti, V., Eding, E.H., Verreth, J.A.J., Klapwijk, B., 2007. Kinetics, design and biomass production of a bacteria reactor treating RAS effluent streams. Aquacult. Eng. 36, 24-35.

Sharrer, M.J., Tal, Y., Ferrier, D., Hankins, J.A., Summerfelt, S.T., 2007. Membrane biological reactor treatment of a saline backwash flow from a recirculating aquaculture system. Aquacult. Eng. 36, 159-176.

Sirianuntapiboon, S., Prasertsong, K., 2008. Treatment of molasses wastewater by acetogenic bacteria BP103 in sequencing batch reactor (SBR) system. Biores. Technol. 99, 1806-1815.

Skjølstrup, J., McLean, E., Nielson, P. H., Frier, J.-O., 2000. The influence of dietary oxolinic acid on fluidized bed biofilter performance in a recirculation system for rainbow trout, Oncorhynchus mykiss. Aquaculture 183, 255-268.

Solorzano, L., 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. 14, 799–801.

Spotte, S., 1979. Fish and Invertebrate Culture: Water Management in Closed Systems, 2nd edition. Wiley, New York, New York, US.

Strickland, J.D.H., Parsons, T.R., 1972. A Practical Handbook of Seawater Analysis, 2nd edition. Bulletin, vol. 167. J. Fish. Res. Board Can., Ottawa. 310 pp..

Tacon, A., Cody, J.J., Conquest, L.D., Divakaran, S., Forster, I.P., Decamp, O.E., 2002. Effect of culture system on the nutrition and growth performance of Pacific white shrimp, Litopenaeus vannamei (Boone) fed different diets. Aquacult. Nutr. 8,121-137.

Timmons, M.B., Ebeling, J.M., Wheaton, F.W., Summerfelt, S.T., Vinci, B.J., 2002. Recirculating Aquaculture Systems, 2nd Ed. Cayuga Aqua Ventures, Ithaca, New York, US.

United States Marine Shrimp Farming Program, 2006. FY05 Progress Report, vol. I & II. U.S. Marine Shrimp Farming Consortium, Oceanic Institute, Honolulu, Hawaii, US.

Van Wyk, P., Davis-Hodgkins, M., Laramore, C.R., Main, K., Mountain, J., Scarpa, J., 1999. Farming Marine Shrimp in Recirculating Freshwater Production Systems: A Practical Manual. FDACS Contract #4520. Florida Department of Agriculture Consumer Services, Tallahassee, Florida, US.

Wasielesky, W., Atwood, H., Stokes, A., Browdy, C.L., 2006. Effect of natural production in a zero exchange suspended microbial floc based super-intensive culture system for white shrimp Litopenaeus vannamei. Aquaculture 258, 396-403.

Table 4-1. Composition of microbial flocs, mean values with standard errors, as determined by laboratory analysis (n=2).

Parameter	Microbial flocs [g/100g dry matter]
Crude protein	49.0 ± 1.5
Ash	13.4 ± 0.6
Total fat	1.13 ± 0.09
Fiber	12.6 ± 0.1
Calcium	1.28 ± 0.07
Phosphorus	1.29 ± 0.08
Potassium	0.75 ± 0.13
Magnesium	0.41 ± 0.05
Sodium	1.27 ± 0.03

Table 4-2. Ingredients of diets used in feeding trial.

Ingredients	Treatment diets (g/100g dry matter)				
	Control 1	Control 2	Diet 1 Microbial Floc 7.8%	Diet 2 Microbial Floc 15.6%	Diet 3 Microbial floc 7.8%+FO
Microbial floc[1]	0	0	7.8	15.7	7.8
Wheat starch[2]	29.6	29.0	27.6	25.3	27.9
Fish meal[3]	15.0	15.0	15.0	15.0	9.4
Squid meal[4]	15.0	15.0	15.0	15.0	15.0
Krill meal[4]	10.0	10.5	10.0	10.0	10.0
Soybean protein isolate[4]	7.9	7.9	4.0	0.0	7.9
Lecithin[5]	4.0[6]	4.2	4.0	4.0	4.0
Dicalcium phosphate[2]	3.0	6.6	2.8	2.8	3.6
Calcium Carbonate[7]	2.0	1.5	2.0	1.8	2.1
Alginate[8]	2.0	2.0	2.0	2.0	2.0
Potassium chloride[4]	2.0	1.9	1.9	1.8	2.0
Cellulose[9]	2.5	2.0	1.6	0.80	1.6
Diatomaceous earth[9]	2.0	1.0	1.6	1.2	1.5
Magnesium oxide[7]	1.5	1.7	1.5	1.5	1.5
Chromic oxide[10]	1.0	0.0	1.0	1.0	1.0
Sodium hexametaphosphate[10]	1.0	1.0	1.0	1.0	1.0
Sodium chloride[2]	0.5	0	0.40	0.30	0.40
Mineral/Vitamin Premix[4]	0.46	0.50	0.32	0.32	0.32
Soybean oil[11]	0.30	0	0.20	0.20	0.20
Cholesterol[2]	0.20	0.20	0.20	0.20	0.20
Vitamin C (stay C)[4]	0.04	0.05	0.04	0.04	0.04
Fish oil[3]	0	0	0	0	0.50

[1] Produced in SBRs as part of this study.

[2] MP Biomedicals, Solon, Ohio, US.

[3] Omega Protein, Houston, Texas, US.

[4] Zeigler Bros., Inc., Gardners, Pennsylvania, US.

[5] Cargill, Hamburg, Germany.

[6] Solae Co., Saint Louis, Missouri, US.

[7] VWR International, West Chester, Pennsylvania, US.

[8] ChemPoint, Bellevue, Washington, US.

[9] Sigma-Aldrich, Saint Louis, Missouri, US.

[10] Fisher Scientific, Pittsburg, Pennsylvania, US.

[11] Federated Group Inc., Arlington Heights, Illinois.

Table 4-3. Composition of diets, mean values with standard errors, as determined by laboratory analysis (n=2).

Parameter	Treatment diets (g/100g dry matter)				
	Control 1	Control 2	Diet 1 Microbial floc 7.8%	Diet 2 Microbial floc 15.6%	Diet 3 Microbial floc 7.8%+FO
Crude protein	38.4 ± 1.6	41.9 ± 0.3	41.8 ± 0.1	41.2 ± 1.3	42.7 ± 0.1
Carbohydrate[1]	28.6 ± 1.6	21.9 ± 1.0	24.7 ± 0.6	22.2 ± 0.7	21.9 ± 2.0
Ash	19.7 ± 0.1	19.7 ± 0.1	19.9 ± 0.1	21.6 ± 0.3	19.7 ± 0.1
Total fat	5.70 ± 0.12	7.09 ± 1.42	5.88 ± 0.65	7.54 ± 0.38	8.19 ± 2.11
Fiber	1.87 ± 0.21	1.43 ± 0.16	1.31 ± 0.94	1.79 ± 0.29	1.60 ± 0.05
Calcium	2.82 ± 0.08	3.42 ± 0.08	2.94 ± 0.09	3.19 ± 0.01	3.16 ± 0.07
Phosphorus	1.98 ± 0.04	2.62 ± 0.06	2.04 ± 0.12	2.34 ± 0.02	2.22 ± 0.09
Potassium	1.34 ± 0.04	1.28 ± 0.02	1.38 ± 0.03	1.32 ± 0.01	1.33 ± 0.01
Magnesium	1.01 ± 0.02	1.16 ± 0.02	1.06 ± 0.01	1.23 ± 0.02	1.02 ± 0.00
Sodium	1.03 ± 0.03	0.88 ± 0.04	1.09 ± 0.02	1.06 ± 0.01	1.06 ± 0.01

[1] Calculated value (Merrill and Watt, 1973): carbohydrate = total - (ash + crude protein + moisture + total fat)

Table 4-4. Water quality results in the systems used to test dietary effects on shrimp performance. Mean values with 95% confidence intervals (n denotes the number of sampling events).

System	Dissolved oxygen [mg/l]	Nitrate-N [mg/L]	Nitrite-N [mg/L]	pH	Salinity [ppt]	Total ammonia-N [mg/L]	Temperature [^{o}C]
	n = 100	n = 5	n = 5	n = 4	n = 100	n = 5	n = 100
1	5.76 (5.32-6.19)	0.78 (0-2.11)	0.08 (0-0.19)	8.39 (8.26-8.52)	30.6 (29.1-32.1)	0.11 (0.01-0.22)	30.0 (29.2-30.9)
2	5.72 (5.34-6.09)	0.91 (0-2.58)	0.09 (0-0.23)	8.31 (8.12-8.50)	30.3 (28.7-32.0)	0.12 (0-0.27)	30.1 (29.7-30.5)
3	5.74 (5.24-6.25)	0.74 (0-2.06)	0.10 (0-0.25)	8.33 (8.18-8.48)	30.5 (28.9-32.1)	0.12 (0-0.26)	29.9 (29.4-30.5)
4	5.85 (5.37-6.32)	0.76 (0-2.13)	0.09 (0-0.19)	8.34 (8.21-8.47)	30.5 (28.9-32.0)	0.12 (0.01-0.24)	30.1 (29.5-30.8)
5	5.62 (5.11-6.11)	0.73 (0-2.02)	0.08 (0-0.15)	8.31 (8.14-8.47)	30.3 (28.8-31.8)	0.13 (0-0.29)	30.0 (29.5-30.6)
6	5.77 (5.35-6.19)	0.80 (0-2.28)	0.07 (0-0.15)	8.31 (8.16-8.47)	30.2 (28.7-31.7)	0.09 (0-0.21)	30.0 (29.6-30.4)

Table 4-5. Mean values (mean transformation value) of initial weights and shrimp performance indicators at the end of the 35 day feeding trial.[1]

Diet	Initial weight [g]	Survival [%]	Weight gain [g]	Weight gain per week [g/wk]	SGR [1/d]
Control 1	0.44	93	5.46[a] (26.1)	1.09[a] (1.77)	7.17[a] (41.7)
Control 2	0.43	93	4.42[a] (19.0)	0.88[a] (1.60)	6.63[a] (35.5)
Diet 1: Microbial floc 7.8%	0.43	100	8.18[b] (61.5)	1.64[b] (2.27)	8.54[b] (71.8)
Diet 2: Microbial floc 15.6%	0.44	98	8.07[b] (58.7)	1.61[b] (2.24)	8.45[b] (69.4)
Diet 3: Microbial floc 7.8%+FO	0.43	100	8.13[b] (61.5)	1.63[b] (2.26)	8.54[b] (72.2)
Pooled error	0.09937	9.897	19.62[2]	0.2648[2]	16.51[2]
P > F	0.9032	0.2079	<0.0001	<0.0001	<0.0001

[1] Superscript letters denote significant differences (P<0.01).

[2] Reflects transformation values.

Figure 4-1. Removal of TAN observed during batch treatment of aquaculture wastewater using microbial flocs in SBRs, with and without carbon supplementation. With carbon supplementation (mean value ± standard error); slope = 0.4341 ± 0.0029, R^2 = 0.9807 ± 0.0095. Without carbon supplementation; slope = 0.0152 ± 0.0060, R^2 = 0.6084 ± 0.1380.

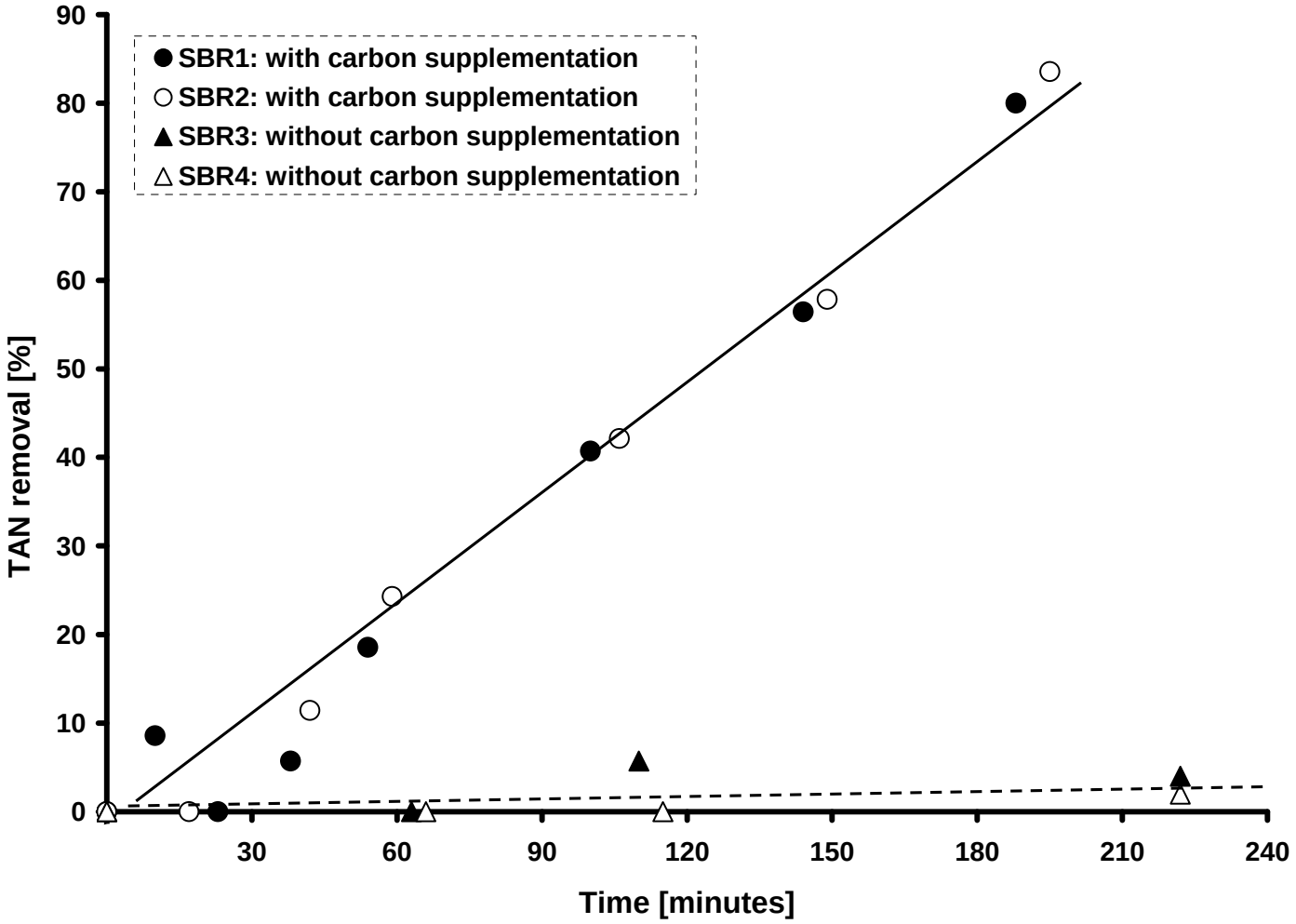


Figure 4-2. Microbial floc generation observed during batch treatment of aquaculture wastewater in SBRs, with and without carbon supplementation. With carbon supplementation (mean value ± standard error); slope = 1.5851 ± 0.0452, R^2 = 0.9660 ± 0.0115. Without carbon supplementation; slope = 0.0089 ± 0.0178, R^2 = 0.0723 ± 0.0680.

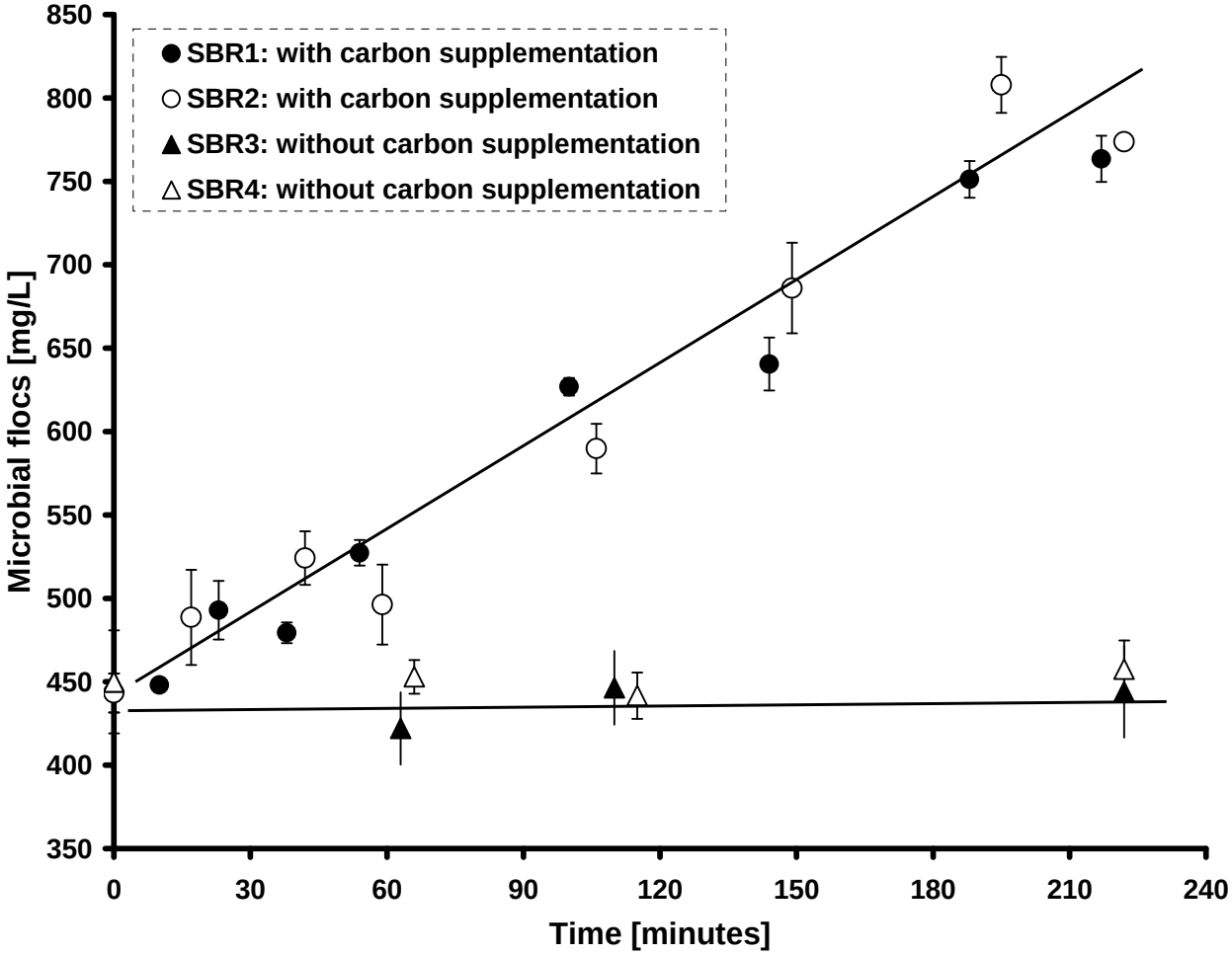
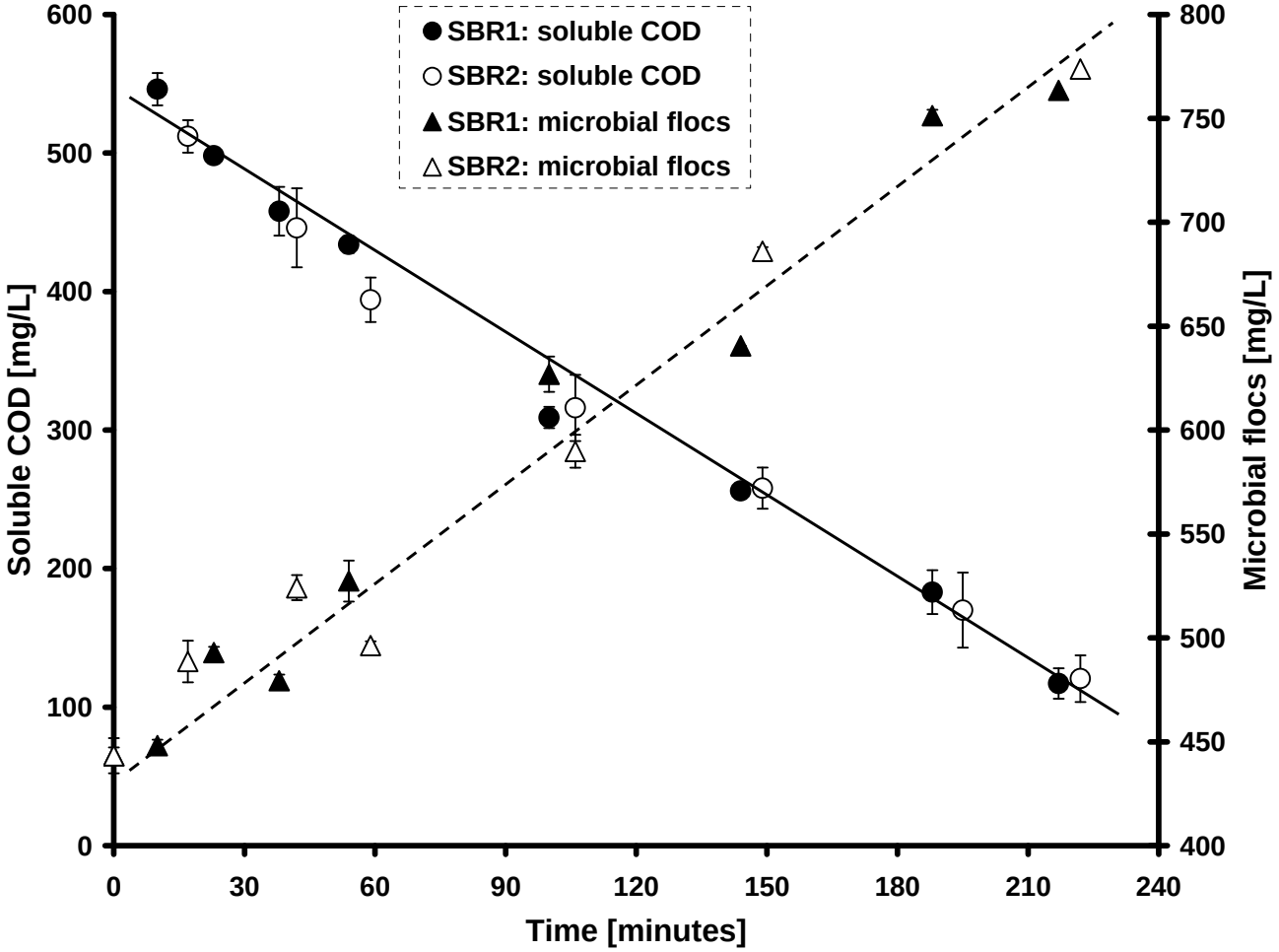


Figure 4-3. Carbon supplementation effects on generation of microbial flocs with simultaneous removal of soluble COD (mean values with standard error bars). Soluble COD (mean value ± standard error); slope = -1.9009 ± 0.0792, R^2 = 0.9896 ± 0.0016. Microbial flocs; slope = 1.5851 ± 0.0452, R^2 = 0.9660 ± 0.0115.



Chapter 5:

Production and Nutritional Value of Microbial Flocs Generated Through Biological Treatment of Tilapia Wastewaters

Report of unpublished data

Introduction

Overfishing of natural fisheries is a global issue that is becoming more urgent as the human population continues to increase. According to the Food and Agriculture Organization of the United Nations (FAO, 2002), approximately 47% of the natural fisheries are fully exploited and an additional 18% are overexploited. This high demand for seafood protein is not going away; and one out of five people in this world depend on this source of protein. To meet the growing demand for seafood, aquaculture is on the rise and is reportedly the fastest growing sector of agriculture worldwide. Traditional aquaculture practices use pond and flow-through systems which are often responsible for discharging pollutants (e.g. nutrients and solids) into the environment. Furthermore, aquacultural feeds often contain high levels of fish or seafood protein, so the demand on wild fisheries is not completely eased. Even though traditional aquaculture has these drawbacks, there is a significant movement towards more sustainable practices, especially in developed countries (Avnimelech 1999; Hargreaves 2006). For example, implementing recirculating aquaculture systems (RAS) maximizes the reuse of culture water which consequently decreases water demand and minimizes the levels of pollutants being discharged to the environment (Skjølstrup et al. 2000; Menasveta 2002; Timmons et al. 2002). Alternative proteins (e.g., yeast-based proteins) are also replacing the fish and seafood proteins in aquaculture diets (McLean et al., 2006). Accordingly, the research described in this paper focused on maximizing the reuse of freshwater fish effluent to culture marine shrimp. More specifically, by using suspended-growth biological reactors to treat a tilapia effluent waste stream and to generate microbial flocs that could be used as an alternative feed to support shrimp culture.

In order to reuse the freshwater tilapia effluent for marine shrimp culture, understanding which ions are critical for the survival and normal growth of the shrimp is essential. It is also very important to understand how to convert an effluent's organic matter into a consistent and controlled food for the shrimp. Results from Kuhn et al. (2007) revealed that the marine shrimp (*Litopenaeus vannamei*) can be raised in freshwater effluent when supplemented with synthetic sea salt (0.6 g/L), CaO (50 mg/L Ca^{2+}) and $MgSO_4$ (30 mg/L Mg^{2+}). Kuhn et al. (2008) looked into using nutrients in effluents from a commercial tilapia farm as supplemental feed to *L. vannamei* directly and in the form of microbial flocs generated from biological treatment of the effluents. The results from this study are encouraging. For example, microbial flocs generated in bioreactors, and offered as a supplemental feed, significantly ($P < 0.05$) improved shrimp growth and growth rates (SGRs) in shrimp fed a restricted ration of commercial shrimp feed. Since this previous research demonstrated the potential benefits of implementing suspended growth biological treatment to aid in the polyculture of shrimp, it is important to understand how to best treat the effluent while producing biomass that can be utilized by the shrimp as a supplemental feed. Therefore, this study investigated the treatability of this effluent stream using sequencing batch reactors (SBRs). Treatment with and without carbon supplementation was evaluated and compared. Biological kinetic data and nutritional properties of SBR produced biomass are also reported.

Materials and Methods

Effluent handling and storage

Tilapia effluent was collected from a local commercial RAS tilapia facility (Blue Ridge Aquaculture Inc., Martinsville, VA, USA). The effluent was collected as settling basins at the farm were drained as part of normal operations. Variability of constituents in this effluent was minimal because the settling basins were only flushed once 500 pounds of feed was fed to the tilapia. Approximately 250 gallons of this effluent was hauled offsite once a week and was stored in the laboratory in a 300 gallon storage tank. During one of the trials (discussed below), this effluent was stored at -20 C in 19 L buckets. Untreated solids, collected directly from tilapia effluent after a 45 min settling period were characterized and compared against biomass generated in SBRs.

Bioreactor operation (Trials one to three)

Three trials were undertaken. These are summarized in Table 1. Trial one consisted of twelve 1 L Beakers in a 29 C water batch (this temperature will be the future operational temperature at Virginia Shrimp Farms, Martinsville, VA). These beakers were operated like a SBR with a hydraulic residence time (HRT) of 24 hours with no carbon supplementation. Effluent was stored in numerous 19 L buckets in a -20 C freezer. Every 24 hours a bucket was removed and thawed out so a fresh source of effluent could be manually exchanged for treated effluent at the appropriate rates to evaluate biological solids residence times (SRTs) of 3, 6, 10, and 15 days in triplicate. The SBRs were

operated manually using a 23 h well-mixed aeration, 45 min settle, and 15 min decant/idle/fill period. This trial lasted for 50 d.

Trials two and three (Table 1) were conducted in three SBRs illustrated in Figure 1 and were maintained at 28 C. Dissolved oxygen (DO) levels were greater than 5 mg/L during the aeration cycle. These 5 L SBRs were operated in triplicate with a 4 h well-mixed aeration, 1 h settling, 45 min draw, and 15 min idle/fill period. Water was pumped every 24 h from the 250 gallon storage tank into a well-mixed 76 L equalization (EQ) tank. Biomass was wasted at a rate that provided an SRT of 10 d. Trial two was conducted for 45 d with no carbon supplementation. Trial three dosed 500 mg/L (210 mg of carbon/L) of sugar (Granulated white sugar, Kroger Co., Cincinnati, OH, USA) directly into the SBRs 5 min after each aeration cycle began using peristaltic dosing pumps (Reefdoser RD4 Quadro, Aqua Medic©, Bissendorf, Denmark). Trial three was conducted for 30 to 35 d until the reactors were dominated by fungus and operation became difficult with this laboratory SBR setup.

Bioreactor operation (Trial four)

Every 24 h the 76 L EQ tank was cleaned using pressurized well water. This EQ tank was well mixed without aeration using a submersible Rio® 200 pump (TAAM Inc., Camarillo, CA, USA) and was maintained at 29 C. Sugar was added directly to the EQ tank (500 mg/L sugar, 210 mg of carbon/L) to promote denitrification and a heterotrophic population of biomass. The resulting food to microorganism ratio (F:M) over the stabilized period from day 30 to 50 was 0.15 ± 0.01.

Three 5 L SBRs were operated with a 4 h well-mixed aeration, 1 h settling, 45 min draw, and 15 min idle/fill period (Figure 1). The target SRT was 10 d. The temperature in the SBRs were maintained at 28.7 ± 0.2 C (mean ± standard error) using a water bath and DO levels were always greater than 5 mg/L. Effluent was collected in 19 L buckets and volumetric measurements of treated water were determined every 24 h for each reactor to ensure proper operation. Two independent batch trials were performed on random stabilized SBRs on day 50 to determine kinetic coefficients from concentrations of biomass (MLVSS), soluble total organic carbon (sTOC), and soluble chemical oxygen demand (sCOD) versus time (n = 17). Initial levels of MLVSS and sugar spike concentrations to initiate kinetic batch experiments were similar to levels used during the 50 day trial. More specifically, initial F:M for the two kinetic trials were respectively 0.14 and 0.17.

Laboratory analysis

After samples were filtered through a 1.5 µm filter, the filtrate was analyzed for nitrite-N, nitrate-N, orthophosphate (OP), and total ammonia-N (TAN) in accordance with HACH spectrophometric methods 8507, 8039, 8048, and 8038, respectively. Sludge volume index (SVI), sCOD, sTOC, total solids (TS), total suspended solids (TSS) and volatile suspended solids (VSS) were determined using method 2710D, 5310B, 5220D, 2540B, 2540D, and 2540E referenced in *Standard Methods* (APHA, 1998). Crude protein levels were determined in accordance with AOAC (1994). Temperature and DO were determined with an YSI 85 probe (Yellow Springs Inc., Yellow Springs, OH, USA). A

HI 9024 pH meter (HANNA Instruments, Woonsocket, RI, USA) was used to determine pH.

Results

Trials one to three

Results for trials one to three are summarized in Table 1. For trial one, treatment of sCOD and TAN ranged from 58 to 72% and 79 to 83%, respectively and both increased with increasing SRT. Volatile suspended solids ranged from 100 to 200 mg/L and increased with increasing SRT. Trial two resulted in highly variable treatment, ranging from 18 to 80% for sCOD while MLVSS concentrations remained less than 200 mg/L. Trial three generated levels of MLVSS greater than 1,000 mg/L. Treatment of sCOD and TAN were both greater than 80%. However, fungus (Figure 6) became dominate starting between days 30 and 35. Even though fungus was present during trial 3, it was not present during trials one and two.

Trial four

A strong linear correlation (R^2 of 0.9930) was observed between sCOD and sTOC (Figure 2). This function yielded a slope of 2.26 (mg sCOD)/(mg sTOC) and was determined over a range of sTOC (11-230 mg/L) and sCOD (12-510 mg/L) which was reflective of the range observed during this 50 day study. Similarly, ratios of COD to TOC were 2.33 ± 0.063 when treatment of sTOC, or sCOD, was less than 85% (Figure

3). However, for treatment levels greater than 85%, this ratio was significantly ($P < 0.05$) reduced to 1.36 ± 0.099.

During the stabilized period from day 30 to 50 (Figure 4), the overall mean concentration of MLVSS in the three SBRs was 1,383 ± 151 mg/L. No differences ($P > 0.05$) were observed for the mean MLVSS concentrations between the different days. During this stabilized period, treatment of sTOC was always greater than 89% with an average treatment of 93.0 ± 0.8%. Furthermore, the mean effluent concentration of sTOC was 14.7 ± 1.7 mg/L. Figure 5 illustrates the treatment effects of various constituents between the storage tank, equalization tank, and treatment from the SBRs. Overall, the percent change of TAN, NO2, pH, NO3, and OP were respectively –91, 0, +9, -60, and –23 %.

Kinetic coefficients are reported in Table 2. No significant differences ($P > 0.05$) were reported between the anoxic/oxic and oxic yield coefficients values for either substrate. Correlations were strong for all determined normalized rate values, R^2 values were never less than 0.92. For both substrates, correlation and standard error values were respectively higher and lower for zero-order rates compared to first order-rates.

Biomass characterization for miocrobial flocs and untreated solids are compared in Table 3. Protein levels, determined as crude protein or Lowry protein, were significantly higher ($P < 0.01$) in microbial flocs compared to untreated solids. More specifically, crude protein and Lowry protein values of microbial flocs were respectively 95% and 69% greater than untreated solids. The organic fraction of microbial flocs was significantly greater ($P < 0.01$) compared to untreated solids. Some fungus growth was observed in the SBRs (Figure 6).

Discussion

As expected, the results summarized in Table 1 demonstrate that operational inputs significantly influenced various parameters, ranging from treatment efficiencies to biomass production to fungus development. Trial one treatment levels were encouraging, but were likely limited by the low biomass concentration in the SBRs. Furthermore, the HRT of 24 h was perhaps too long and could have also contributed to these low efficiency levels. The biomass concentration could have theoretically been four times greater if the HRT was decreased to the levels (HRT of 6 h) used in trials two to four. Hence, trial two was conducted to test this theory. This trial yielded similar results in terms of MLVSS concentrations. However, during this trial, these insufficient biomass levels and highly variable treatment efficiencies could have been due to: (1) the tilapia wastewater not being fresh (it was used over the course of 7 days until a new batch was transported 130 km from Blue Ridge Aquaculture), and/or (2) a low biodegradable fraction of sCOD that would need carbon supplementation (Metcalf and Eddy, 2003), especially when attempting to biologically treat an aquacultural waste using heterotrophic microorganisms (Avnimelech, 1999; Ebeling et al., 2006). For this reason, trial three was conducted with carbon supplementation. This trial resulted in better treatment of sCOD and TAN than seen in trials one and two. Biomass concentrations greater than 1,000 mg/L were also achieved. Even though this trial yielded desirable levels of treatment and biomass, beginning on day 30, fungus (Figure 6) populations began to proliferate and eventually became excessive and interfered with the decant cycle. This type of filamentous organism is not uncommon in aerobic systems when a readily degradable

substance, such as a simple sugar, is being treated (Eckenfelder, 2000; Elmaslar et al., 2004). Trials one through three were informative, but were not completely successful. However, trial four was successful. Therefore, this article focuses more on the results obtained during trial four.

Since there was a strong correlation between sCOD and sTOC (Figure 2), one can be accurately estimated from measuring the other. Typically, the higher the COD:TOC, the more available the carbon is for oxidation via heterotrophic microorganisms (Metcalf and Eddy, 2003; Kleerebezem and Van Loosdrecht, 2006). Plotting sCOD:sTOC versus percent treatment of sCOD (Figure 3) demonstrated the importance of this ratio because this ratio was significantly reduced ($P < 0.05$) when the treatment was greater than 85%.

It is often assumed that a bioreactor is stable when the reactor has been operated for a period of time which is three times its average SRT. Therefore, during trial four, it was assumed that the three SBRs were stable after 30 days. This was verified by measuring the MLVSS concentrations and treatment performance from days 30 to 50 (Figure 4). As expected there were no significant differences between MLVSS concentrations during this time period and treatment of sTOC was consistently greater than 90%. Effluent concentrations of sCOD were calculated to be 20.6 ± 2.2 mg/L.

Total ammonia nitrogen is typically reduced in SBRs by assimilation via heterotrophic microorganisms and via oxidation by autotrophic microorganisms (Metcalf and Eddy; Ebeling et al., 2006). Nitrite remained low, less than 0.11 mg/L, in all stages. The pH increased after treatment by the SBRs. As expected, denitrification was only accomplished in the anoxic portion of the treatment train because nitrate becomes the electron acceptor for microbial metabolism in the absence of oxygen (Metcalf and Eddy,

2003; Boopathy et al., 2005). Nitrate was reduced by 65% during the anaerobic stage and increased by 5% during the aerobic phase. This increase in nitrate is likely due to oxidation of reduced nitrogen by autotrophic microorganisms.

Kinetic coefficients of biomass production and substrate removal presented in Table 2 are important because they help the operator understand how to best manage the systems and additions of supplemental carbon. Yield coefficients represent the amount of biomass produced per unit of substrate consumed. Typically, an operator would like low yield coefficients because they have to dispose of this sludge, which can be time consuming and expensive. However, in this case, a high yield coefficient is beneficial because the biomass can be used as a supplemental feed for shrimp culture and reduce the amount of commercial feed required (Kuhn et al., 2008). Interestingly, the anoxic/oxic yield coefficients in this study were not significantly different ($P > 0.05$) from the oxic yield coefficients. Typically, anaerobic yield coefficients are significantly lower than aerobic yield coefficients (Metcalf and Eddy, 2003).

Biomass growth rates (μ) of 0.27 ± 0.028 1/h observed in this study were higher than those observed for treating aquaculture wastewater using molasses (0.10-0.12 1/h; Schneider et al., 2006). This is because the granulated sugar (i.e., sucrose) used in this study is readily biodegradable, and molasses is a more complex polysaccharides and not as biodegradable (Najafpour and Shan, 2003; Quan et al., 2005). Fungi were observed in the MLVSS (Figure 6). Even though these organisms were observed in low numbers during trial four, they did not adversely affect treatment performance or reactor operation. Although uptake rates for both substrates related well to zero-order and first-order rate equations (Table 2), zero-order rates represented the data sets more accurately.

Biomass generated in the SBRs had significantly higher ($P < 0.01$) nutritional values than untreated solids. Since microbes assimilate organic material and nutrients during biological treatment (Metcalf and Eddy, 2003), this result was expected. Furthermore, these microbial flocs are a combination of microorganisms and exocellular biopolymers. The biopolymers are a conglomerate of multivalent cations, polysaccharides, and proteins (Higgins and Novak, 1997). Sludge volume indices were not indicative of bulking because they were less than 150 ml/g biomass (Eckenfelder, 2000).

Conclusion

The poorer treatment of tilapia wastewater noted without carbon supplementation may have been due to the age of the effluent used at the Virginia Tech laboratories. Therefore, a follow-up study needs to be conducted onsite with a fresh source of tilapia effluent. However, trial four was successful in terms of treatment and biomass production. Since tilapia production at BRA involves autotrophic systems which accumulate nitrate that can be a limiting factor for water reuse, the denitrification observed during trial four becomes even more important. Ultimately, it is hoped that the results from this study can be adopted by the aquaculture industry as an alternative method for treating effluent while producing a supplemental feed for shrimp culture. This approach could result in cost savings through conserving water and reducing feed.

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Literature Cited

AOAC. 1994. *Official Methods of Analysis*, 15th ed. Association of Official Analytical Chemistry. Arlington, Virginia.

APHA. 1998. *Standard Methods for the Examination of Water and Wastewater*, 20th ed. Edited by Clesceri, Greenberg and Trussell. Washington, D.C.

Avnimelech, Y, 1999. Carbon/nitrogen ratio as a control element in aquaculture systems. Aquaculture 176: 227-235.

Boopathy, R., Q. Fontenot, M.B, Kilgen, 2005. Biological treatment of sludge from a recirculating aquaculture system using a sequencing batch reactor. Journal of the World Aquaculture Society 36: 542-545.

Ebeling, J.M., M.B. Timmons, J.J. Bisogni, 2006. Engineering analysis of the stoichiometry of photoautotrophic, autotrophic, and heterotrophic removal of ammonia-nitrogen in aquaculture systems. Aquaculture 257: 346-358.

Eckenfelder, W.W., 2000. *Industrial water pollution control*. 3rd ed. Edited by Tchobanoglous. New York: McGraw Hill.

Elmaslar, E., N. Tufekci, S. Ovez, 2004. Aerobic treatability of fruit juice industry effluents in sequencing batch and activated sludge reactors. Fresenius Environmental Bulletin 13: 985-988.

FAO. 2002. *The State of World Fisheries and Aquaculture 2002*. Food and Agriculture Organization of the United Nations, Rome, Italy.

Hargreaves, J.A. 2006. Photosynthetic suspended-growth systems in aquaculture. Aquacultural Engineering 34: 344-363.

Higgins, M. J. and J. T. Novak. 1997. Characterization of exocellular protein and its role in bioflocculation. Journal of Environmental Engineering - American Society of Chemical Engineers 123: 479-485.

Kleerebezem, R., M.C.M. Van Loosdrecht, 2006. Waste characterization for implementation in ADM1. Water Science and Technology 54: 167-174.

Kuhn, D.D., G.D. Boardman, S.R. Craig, G.J. Flick Jr., E. McLean, 2007. Evaluation of tilapia effluent with ion supplementation for marine shrimp production in a RAS. Journal of the World Aquaculture Society 38: 74-84.

Kuhn, D.D., Boardman, G.D., Craig, S.R., Flick, G.J., Mclean E, 2008. Use of microbial flocs generated from tilapia effluent as a nutritional supplement for shrimp, *Litopenaeus vannamei*, in recirculating aquaculture systems. J. World Aquacult. Soc. 39, 72-82.

McLean, E., B. Reid, D. Fegan, D. Kuhn and S. Craig, 2006. Total replacement of fishmeal with an organically certified yeast-based protein in Pacific White Shrimp (*Litopenaeus vannamei)* diets: laboratory and field trials. Ribarstvo 64: 47-58.

Menasveta, P. 2002. Improved shrimp growout systems for disease prevention and environmental sustainability in Asia. Reviews in Fisheries Science 10: 391-402.

Metcalf and Eddy. 2003. *Wastewater engineering: treatment and reuse*. 4th ed. Edited by Tchobanoglous, Burton, and Stensel. New York: McGraw Hill.

Najafpour, G.D., C.P. Shan, 2003. Enzymatic hydrolysis of molasses. Bioresource Technology 86: 91-94.

Quan, Z-X., Y-S. Jin, C-R. Yin, J.J. Lee, and S-T. Lee, 2005. Hydrolyzed molasses as an external carbon source in biological nitrogen removal. Bioresource Technology 96: 1690-1695.

Schneider, O., V. Sereti, E.H. Eding, 2006. Molasses as C source for heterotrophic bacteria production on solid fish waste. Aquaculture 261: 1239-1248.

Skjølstrup, J., E. McLean, P. H. Nielson, and J. –O. Frier, 2000. The influence of dietary oxolinic acid on fluidised bed biofilter performance in a recrculation system for rainbow trout, *Oncorhynchus mykiss*. Aquaculture 183: 255-268.

Timmons, M.B., J.M. Ebeling, F.W. Wheaton, S.T. Summerfelt, and B.J. Vinci. 2002. *Recirculating Aquaculture Systems*. 2nd ed. New York: Cayuga Aqua Ventures.

Figure 5-1. Diagram of SBRs used for trials two through three: a) anoxic EQ tank, b) submersible pump on float switch, c) aerobic SBR, d) float switch, e) solenoid valve, f) air flow meter, g) air stone, h) peristaltic pump, 1) tilapia effluent, 2) compressed air, 3) treated effluent.

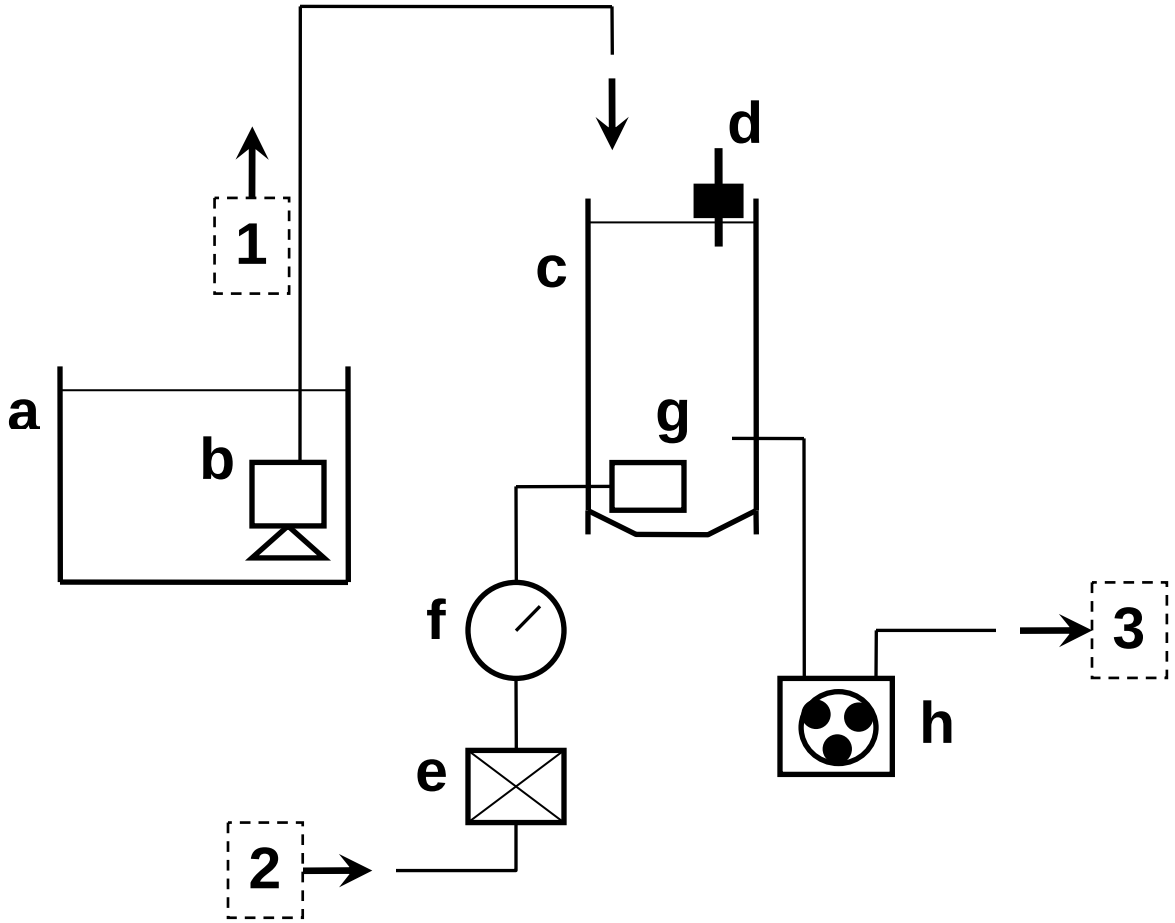


Figure 5-2. Correlation relationship between sCOD and sTOC.

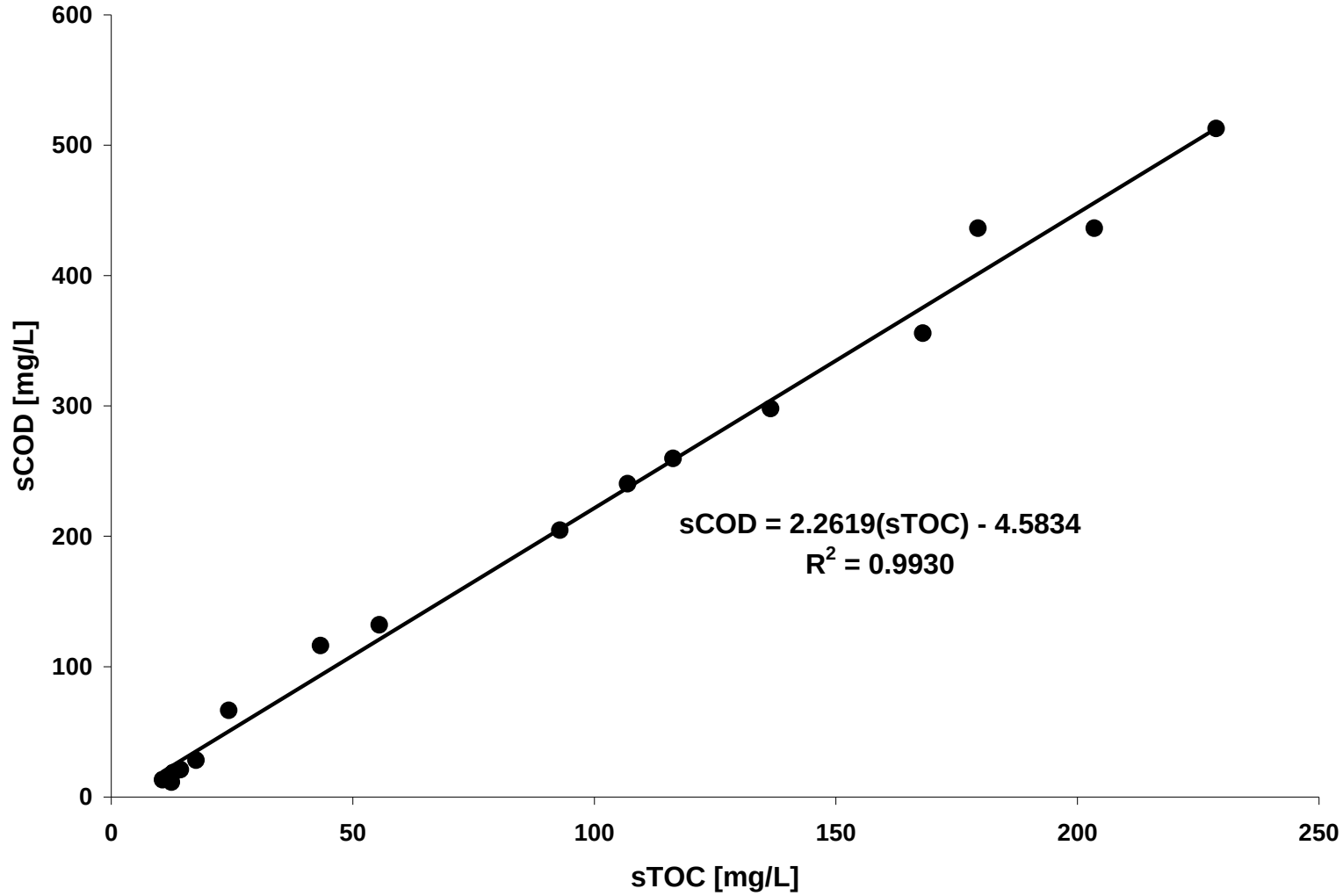


Figure 5-3. Oxidation state versus % treatment.

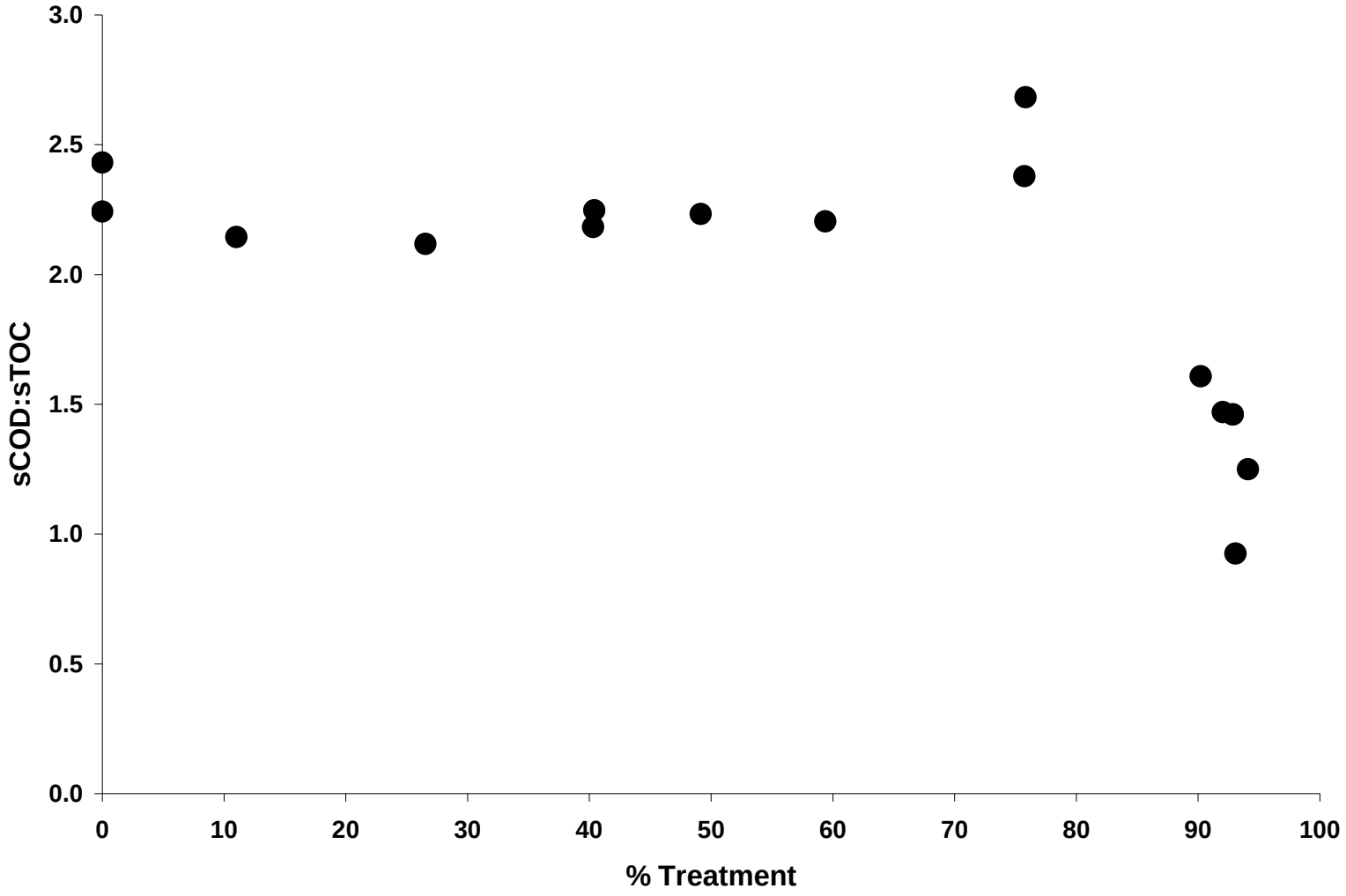


Figure 5-4. Biomass concentration and % soluble TOC treated (mean values ± standard errors) for the three stabilized SBRs.

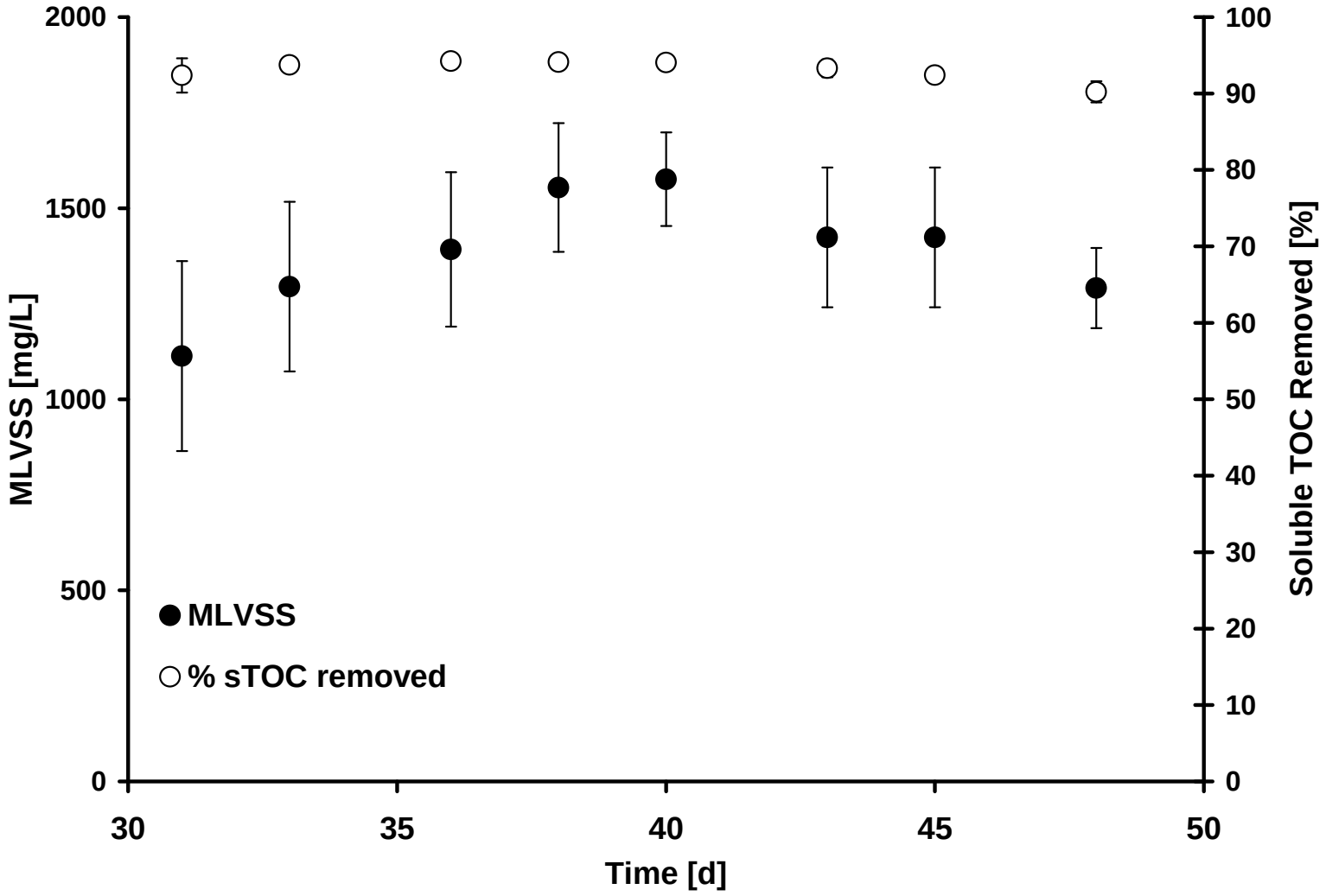


Figure 5-5. Constituent levels determined in storage tank, equalization tank, and effluent after SBR treatment.

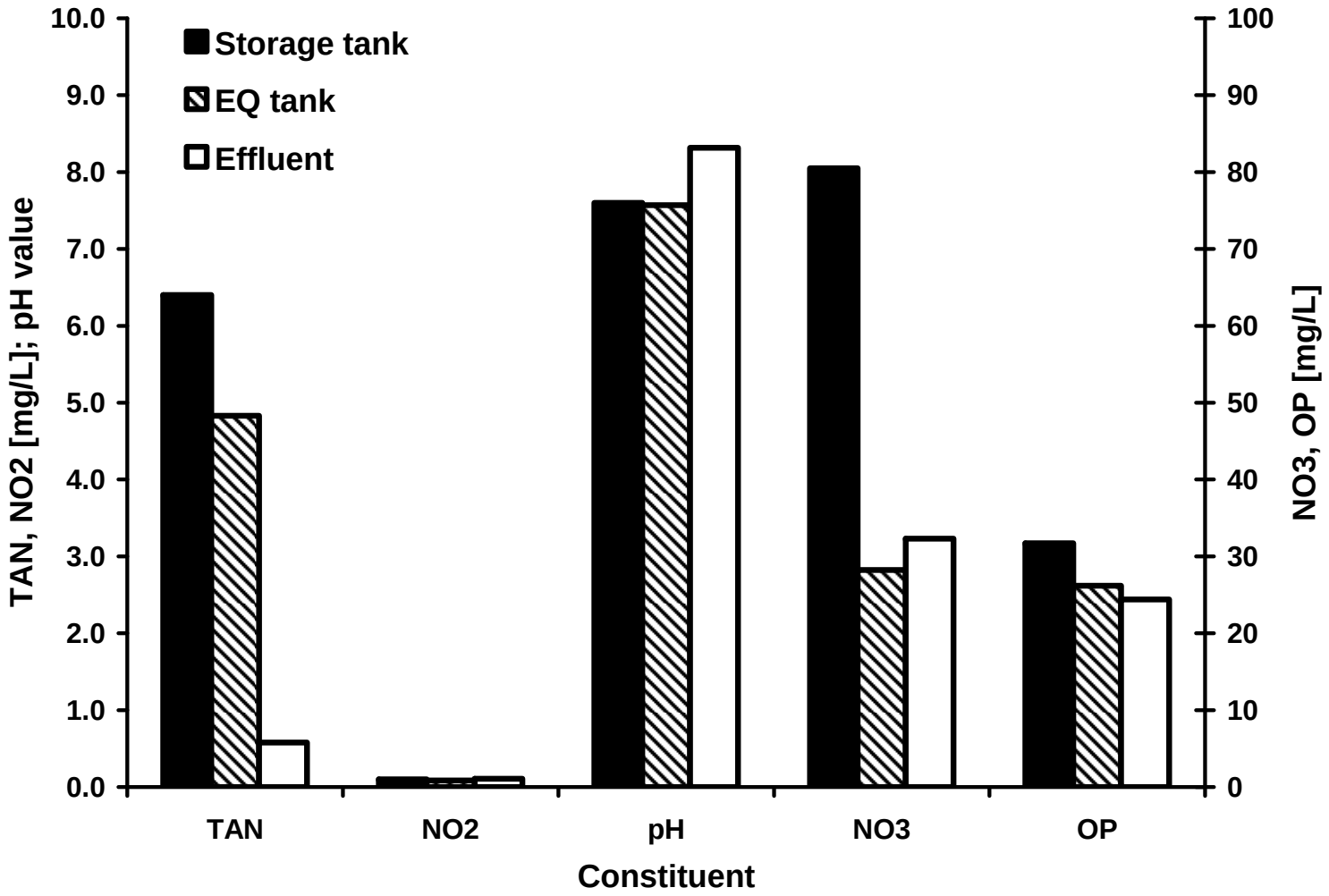


Figure 5-6. Macro-photograph of fungus.

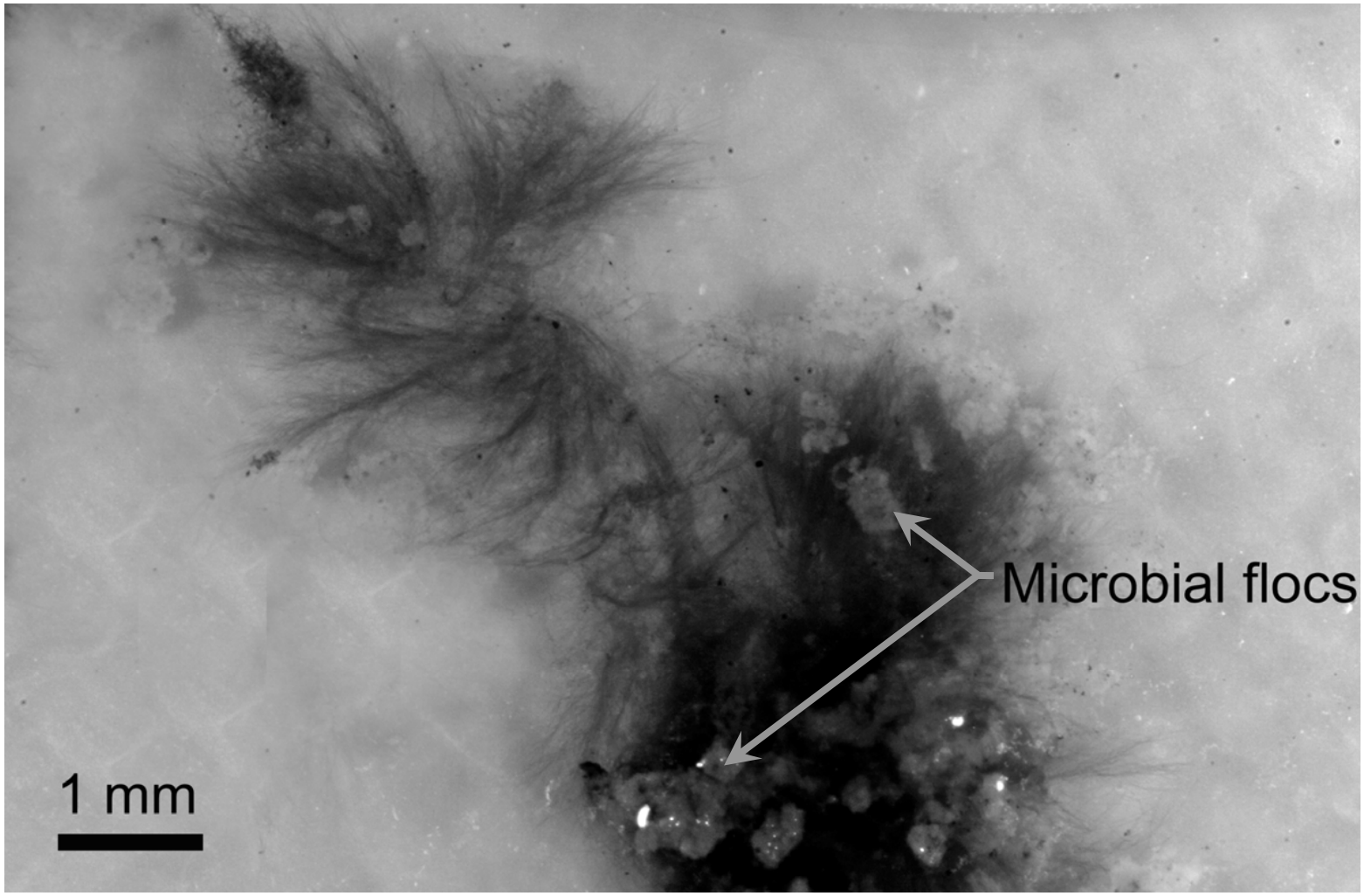


Table 5-1. Comparison of various treatment and operation schemes performed at the laboratory scale.

Trial	Operation/input	Treatment	Biomass production	Fungus production	Comments
One: Aerobic (Beaker SBR)	HRT = 24 hours SRT = 3,6,10,15 days CS = no	Moderate 58-72% sCOD 79-83% TAN	Insufficient <200 mg/L	None	Fresh wastewater from freezer every 24 hours
Two: Aerobic (SBR)	HRT = 6 hours SRT = 10 days CS = no	Highly variable (e.g., 18 to 80% sCOD treatment)	Insufficient <200 mg/L	None	Up to 7 day old wastewater
Three: Aerobic (SBR)	HRT = 6 hours SRT = 10 days CS = yes	Sufficient > 80% sCOD > 80% TAN	Sufficient >1,000 mg/L	Excessive	Up to 7 day old wastewater
Four: anaerobic/aerobic (EQ tank/SBR)	HRT = 6 hours SRT = 10 days CS = yes	Sufficient > 80% sCOD > 80% TAN	Sufficient >1,000 mg/L	Limited	Up to 7 day old wastewater

Note: CS = carbon supplementation as sugar

Table 5-2. Normalized kinetic coefficients based on two separate kinetic trials except for Yanoxic/oxic (determined from 8 data points from day 30 to 50). Mean values with standard error (mean R^2 from two trials).

Kinetic Coefficients	Substrate	
	sTOC	sCOD
Yanoxic/oxic [g biomass/g substrate]	1.54 ± 0.11	0.68 ± 0.05
Yoxic [g biomass/g substrate]	1.60 ± 0.07	0.69 ± 0.02
μ [1/h]	0.27 ± 0.028 (0.9225)	
Zero-order rate [g substrate/(g biomass*h)]	0.17 ± 0.01 (0.9964)	0.39 ± 0.03 (0.9759)
First-order rate [(1/hr)/gVSS]	1.59 ± 0.39 (0.9650)	1.72 ± 0.64 (0.9656)

Table 5-3. Characteristic of SBR biomass versus untreated solids.

	Biomass	Untreated Solids
SVI [ml/g]	129 ± 10.7	-
Crude protein [%]	54.4 ± 0.3	27.9 ± 1.5
Lowry protein [mg/g TSS]	40.2 ± 1.5	23.8 ± 2.5
Organic fraction [%]	89.1 ± 0.3	84.4 ± 0.1

Note: Lowry protein results from Kuhn et al. (2008).

APPENDEX A: Data Associated with Figures and Tables in Chapter 2

Table A1: Water quality results observed during preliminary trial in control treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	23	0	0.22	0.06652	0.06379	0.12758	-0.06106	0.1941
Alkalinity	6	364	431	397.67	25.74	51.48	346.19	449.15
Color	16	5	64	23.81	16.59	33.18	-9.37	56.99
Dissolved oxygen	19	5.9	6.3	6.1221	0.09402	0.18804	5.93406	6.31014
Nitrite	12	0.006	0.033	0.016083	0.0085754	0.0171508	-0.0010678	0.0332338
Nitrate	12	5.2	9.7	7.892	1.608	3.216	4.676	11.108
Orthophosphate	7	0.06	1.02	0.6486	0.3327	0.6654	-0.0168	1.314
pH	20	8.33	8.98	8.6685	0.1601	0.3202	8.3483	8.9887
Salinity	26	17.6	19.8	18.808	0.655	1.31	17.498	20.118
Temperature	24	26.1	28.6	27.429	0.836	1.672	25.757	29.101
Total solids	4	19962	23625	21243	1720.47	3440.94	17802.06	24683.94
Total suspended solids	4	52	120	75.05	30.67	61.34	13.71	136.39
Turbidity	14	0.23	1.13	0.4664	0.2993	0.5986	-0.1322	1.065

Table A2: Water quality results observed during preliminary trial in freshwater treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	22	0	0.2	0.07136	0.04921	0.09842	-0.02706	0.16978
Alkalinity	6	261	297	283.67	13.41	26.82	256.85	310.49
Color	13	5	40	20.15	13.96	27.92	-7.77	48.07
Dissolved oxygen	19	6.73	7.09	6.95	0.1145	0.229	6.721	7.179
Total hardness	6	437	519	484.1667	30.1026	60.2052	423.9615	544.3719
Nitrite	13	0.002	0.014	0.0066154	0.0032797	0.0065594	5.6E-05	0.0131748
Nitrate	13	2.5	17.2	4.815	3.859	7.718	-2.903	12.533
Orthophosphate	7	0.28	0.96	0.4829	0.2309	0.4618	0.0211	0.9447
pH	20	8.53	9.09	8.817	0.1447	0.2894	8.5276	9.1064
Salinity	26	1	1.2	1.05	0.06481	0.12962	0.92038	1.17962
Temperature	23	26.6	27.7	27.165	0.339	0.678	26.487	27.843
Total solids	4	1308	1808	1484	233.17	466.34	1017.66	1950.34
Total suspended solids	4	0	8	4.25	3.3	6.6	-2.35	10.85
Turbidity	13	0.19	0.72	0.3362	0.1375	0.275	0.0612	0.6112

Table A3: Water quality results observed during preliminary trial in effluent 1 treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	23	0.06	0.41	0.1548	0.07458	0.14916	0.00564	0.30396
Alkalinity	6	186	253	228.83	28.94	57.88	170.95	286.71
Color	16	19	85	44.44	16.23	32.46	11.98	76.9
Dissolved oxygen	20	6.75	7.14	6.935	0.1348	0.2696	6.6654	7.2046
Total hardness	6	323	417	375.1667	34.5856	69.1712	305.9955	444.3379
Nitrite	13	0.005	0.049	0.017	0.011811	0.023622	-0.006622	0.040622
Nitrate	13	15.2	30.3	23.377	4.525	9.05	14.327	32.427
Orthophosphate	7	0.75	1.16	0.8614	0.1531	0.3062	0.5552	1.1676
pH	20	8.36	8.99	8.667	0.1708	0.3416	8.3254	9.0086
Salinity	26	1.2	1.6	1.492	0.116	0.232	1.26	1.724
Temperature	24	26.3	27.3	26.792	0.2	0.4	26.392	27.192
Total solids	4	1583	2000	1809.25	203.84	407.68	1401.57	2216.93
Total suspended solids	4	0	20	9.07	8.24	16.48	-7.41	25.55
Turbidity	13	0.74	3.35	1.6762	0.8984	1.7968	-0.1206	3.473

Table A4: Water quality results observed during preliminary trial in effluent 2 treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	23	0	0.75	0.1813	0.1473	0.2946	-0.1133	0.4759
Alkalinity	6	192	256	227	27.6	55.2	171.8	282.2
Color	15	0	101	42.6	25.16	50.32	-7.72	92.92
Dissolved oxygen	20	6.63	7.12	6.92	0.143	0.286	6.634	7.206
Total hardness	6	304	380	347.3333	29.1868	58.3736	288.9597	405.7069
Nitrite	13	0.007	0.038	0.018538	0.0087903	0.0175806	0.0009574	0.0361186
Nitrate	13	15.4	33.1	26.523	4.778	9.556	16.967	36.079
Orthophosphate	7	0.63	0.85	0.7957	0.08121	0.16242	0.63328	0.95812
pH	20	8.44	9.03	8.689	0.1599	0.3198	8.3692	9.0088
Salinity	26	1	1.2	1.096	0.05987	0.11974	0.97626	1.21574
Temperature	24	26.7	29	27.092	0.456	0.912	26.18	28.004
Total solids	4	1240	1640	1431.5	188.63	377.26	1054.24	1808.76
Total suspended solids	4	4	13	9.33	4.08	8.16	1.17	17.49
Turbidity	14	0.69	3.42	1.7979	0.9159	1.8318	-0.0339	3.6297

Table A5: Water quality results observed during trial A in control treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	18	0.19	1.82	0.5611	0.5417	1.0834	-0.5223	1.64
Alkalinity	6	415	491	446.5	30.24	60.48	386.02	506.98
Color	8	5	81	33.75	23.53	47.06	-13.31	80.81
Dissolved oxygen	20	5.03	6.23	5.8658	0.37	0.74	5.1258	6.6058
Nitrite	14	0.021	0.36	0.13757	0.13254	0.26508	-0.12751	0.40265
Nitrate	13	2.5	5.3	3.86	1.09	2.18	1.68	6.04
Orthophosphate	6	1.12	4.48	2.8867	1.3502	2.7004	0.1863	5.5871
pH	16	8.16	8.58	8.4213	0.1121	0.2242	8.1971	8.6455
Salinity	22	17.6	19.9	18.514	0.775	1.55	16.964	20.064
Temperature	19	23.3	26.6	25.6	0.773	1.546	24.054	27.146
Total solids	4	19985	21150	20368.75	528.65	1057.3	19311.45	21426.05
Total suspended solids	4	25	95	58.25	28.79	57.58	0.67	115.83
Turbidity	8	0.51	1.61	0.815	0.4336	0.8672	-0.0522	1.6822

Table A6: Water quality results observed during trial A in freshwater treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	18	0.11	2.1	0.5767	0.6363	1.2726	-0.6959	1.8493
Alkalinity	4	317	372	339	23.93	47.86	291.14	386.86
Color	8	4	44	24.63	15.27	30.54	-5.91	55.17
Dissolved oxygen	20	5.37	6.33	6.0435	0.2548	0.5096	5.5339	6.5531
Total hardness	6	521	620	566.4	36.29	72.58	493.82	638.98
Nitrite	13	0.011	0.061	0.025538	0.013055	0.02611	-0.000572	0.051648
Nitrate	13	4.3	8	5.69	1.324	2.648	3.042	8.338
Orthophosphate	6	0.9	2.56	1.87	0.6085	1.217	0.653	3.087
pH	16	8.26	8.74	8.4775	0.1467	0.2934	8.1841	8.7709
Salinity	22	1	1.2	1.059	0.07341	0.14682	0.91218	1.20582
Temperature	20	24	27.2	25.91	0.777	1.554	24.356	27.464
Total solids	4	1205	1685	1437.5	219.26	438.52	998.98	1876.02
Total suspended solids	4	5	15	8.75	4.79	9.58	-0.83	18.33
Turbidity	8	0.35	0.82	0.54	0.1829	0.3658	0.1742	0.9058

Table A7: Water quality results observed during trial A in effluent 1 treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	18	0.14	1.51	0.3556	0.3065	0.613	-0.2574	0.9686
Alkalinity	6	301	358	320.33	20.99	41.98	278.35	362.31
Color	8	42	73	59.75	12.48	24.96	34.79	84.71
Dissolved oxygen	20	5.84	6.95	6.614	0.3138	0.6276	5.9864	7.2416
Total hardness	6	348	399	377.33	20.09	40.18	337.15	417.51
Nitrite	14	0.02	0.276	0.066143	0.075423	0.150846	-0.084703	0.216989
Nitrate	13	44.2	88.7	62.89	12.298	24.596	38.294	87.486
Orthophosphate	6	6.44	7.92	7.2783	0.6534	1.3068	5.9715	8.5851
pH	16	8.28	8.78	8.5819	0.1334	0.2668	8.3151	8.8487
Salinity	22	2	2.2	2.109	0.04264	0.08528	2.02372	2.19428
Temperature	20	24.5	26.6	25.82	0.426	0.852	24.968	26.672
Total solids	4	2240	2515	2393.25	121.12	242.24	2151.01	2635.49
Total suspended solids	4	10	38	19	12.83	25.66	-6.66	44.66
Turbidity	8	0.68	1.99	1.01	0.4337	0.8674	0.1426	1.8774

Table A8: Water quality results observed during trial A in effluent 2 treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	17	0.16	0.57	0.3106	0.1302	0.2604	0.0502	0.571
Alkalinity	6	295	408	335	43.59	87.18	247.82	422.18
Color	8	35	128	63.75	28.42	56.84	6.91	120.59
Dissolved oxygen	20	6.15	7.01	6.687	0.2302	0.4604	6.2266	7.1474
Total hardness	6	281	318	300.17	13.96	27.92	272.25	328.09
Nitrite	13	0.012	0.039	0.027154	0.0095118	0.0190236	0.0081304	0.0461776
Nitrate	13	48.6	84.7	66.63	10.234	20.468	46.162	87.098
Orthophosphate	6	6.16	8.2	6.8783	0.7921	1.5842	5.2941	8.4625
pH	16	8.38	8.83	8.6006	0.1393	0.2786	8.322	8.8792
Salinity	22	1.4	1.8	1.586	0.08888	0.17776	1.40824	1.76376
Temperature	20	24.4	26.6	25.715	0.484	0.968	24.747	26.683
Total solids	4	1980	2330	2146.25	145.68	291.36	1854.89	2437.61
Total suspended solids	4	19	30	25	5.35	10.7	14.3	35.7
Turbidity	8	0.63	1.18	0.8513	0.174	0.348	0.5033	1.1993

Table A9: Water quality results observed during trial B in control treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	20	0.03	0.62	0.221	0.1344	0.2688	-0.0478	0.4898
Alkalinity	8	380	560	460.38	61.12	122.24	338.14	582.62
Color	10	13	54	35.7	13.17	26.34	9.36	62.04
Dissolved oxygen	21	5.05	6.72	5.6443	0.3146	0.6292	5.0151	6.2735
Nitrite	12	0.004	0.14	0.057	0.036895	0.07379	-0.01679	0.13079
Nitrate	8	4.8	20.5	8.1	5.109	10.218	-2.118	18.318
Orthophosphate	8	1.01	5.3	2.78	1.541	3.082	-0.302	5.862
pH	15	8.2	8.62	8.414	0.1243	0.2486	8.1654	8.6626
Salinity	25	16.3	20.1	18.244	1.09	2.18	16.064	20.424
Temperature	29	26.9	29.6	27.969	0.612	1.224	26.745	29.193
Total solids	4	20990	22000	21273.75	485.07	970.14	20303.61	22243.89
Total suspended solids	4	36	75	54	18.17	36.34	17.66	90.34
Turbidity	8	0.57	0.99	0.7275	0.1338	0.2676	0.4599	0.9951

Table A10: Water quality results observed during trial B in freshwater treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	20	0	0.15	0.084	0.04044	0.08088	0.00312	0.16488
Alkalinity	8	322	484	368	55.43	110.86	257.14	478.86
Chloride	11	335	413	395.09	22.94	45.88	349.21	440.97
Color	10	2	48	17.6	14.05	28.1	-10.5	45.7
Dissolved oxygen	21	5.82	6.76	6.0029	0.1841	0.3682	5.6347	6.3711
Calcium hardness	10	77.6	119	96.16	13.116	26.232	69.928	122.392
Total hardness	8	596	714	651.13	46.65	93.3	557.83	744.43
Nitrite	12	0.002	0.038	0.0125	0.011493	0.022986	-0.010486	0.035486
Nitrate	8	9.2	41.5	17.888	9.989	19.978	-2.09	37.866
Orthophosphate	8	0.92	3.2	1.86	0.849	1.698	0.162	3.558
pH	15	8.2	8.68	8.44	0.1013	0.2026	8.2374	8.6426
Salinity	24	1	1.1	1.029	0.04643	0.09286	0.93614	1.12186
Temperature	29	27.8	28.9	28.366	0.297	0.594	27.772	28.96
Total solids	4	1195	1465	1336.25	120.03	240.06	1096.19	1576.31
Total suspended solids	4	5	13	8.25	3.95	7.9	0.35	16.15
Turbidity	8	0.3	1	0.45	0.2282	0.4564	-0.0064	0.9064

Table A11: Water quality results observed during trial B in effluent 1 treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	20	0.09	0.32	0.2175	0.05937	0.11874	0.09876	0.33624
Alkalinity	8	458	536	507	26.58	53.16	453.84	560.16
Chloride	11	498	657	548.55	51.34	102.68	445.87	651.23
Color	10	14	93	46.4	23.75	47.5	-1.1	93.9
Dissolved oxygen	21	5.74	6.67	5.9933	0.1902	0.3804	5.6129	6.3737
Calcium hardness	10	73.3	122	97.83	14.34	28.68	69.15	126.51
Total hardness	8	544	668	616	46.27	92.54	523.46	708.54
Nitrite	12	0.004	0.142	0.045583	0.038738	0.077476	-0.031893	0.123059
Nitrate	8	57	158	99.813	35.218	70.436	29.377	170.249
Orthophosphate	8	3.8	5.44	4.3038	0.5547	1.1094	3.1944	5.4132
pH	15	8.39	8.8	8.6313	0.1088	0.2176	8.4137	8.8489
Salinity	24	2	2.2	2.104	0.03586	0.07172	2.03228	2.17572
Temperature	29	27.6	29.5	28.69	0.565	1.13	27.56	29.82
Total solids	4	2300	2445	2347.5	66.65	133.3	2214.2	2480.8
Total suspended solids	4	10	45	23	15.47	30.94	-7.94	53.94
Turbidity	8	0.35	1.92	0.8038	0.4958	0.9916	-0.1878	1.7954

Table A12: Water quality results observed during trial B in effluent 2 treatment [mg/L, C, NTU].

	Descriptive Statistics							
	n	Minimum	Maximum	Mean	Standard Deviation	2x Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	20	0.09	0.31	0.198	0.06606	0.13212	0.06588	0.33012
Alkalinity	8	435	536	476	34.92	69.84	406.16	545.84
Chloride	12	288	338	307.83	14.18	28.36	279.47	336.19
Color	10	13	69	50.1	16.93	33.86	16.24	83.96
Dissolved oxygen	21	5.78	6.74	6.1914	0.1686	0.3372	5.8542	6.5286
Calcium hardness	11	72	106	89.2	12.432	24.864	64.336	114.064
Total hardness	8	460	534	493.38	26.31	52.62	440.76	546
Nitrite	12	0.004	0.102	0.033333	0.030404	0.060808	-0.027475	0.094141
Nitrate	8	57.3	121	94.288	23.044	46.088	48.2	140.376
Orthophosphate	8	3.5	5.02	4.22	0.4582	0.9164	3.3036	5.1364
pH	15	8.37	8.82	8.64	0.1291	0.2582	8.3818	8.8982
Salinity	25	1.4	1.6	1.52	0.07071	0.14142	1.37858	1.66142
Temperature	29	27.1	29.6	28.169	0.552	1.104	27.065	29.273
Total solids	4	1725	1940	1850	91.2	182.4	1667.6	2032.4
Total suspended solids	4	5	22	12.5	7.14	14.28	-1.78	26.78
Turbidity	8	0.8	1.95	1.1725	0.4823	0.9646	0.2079	2.1371

Table A13: Water quality results observed during trial C in control treatment [mg/L, C, NTU].

	Descriptive Statistics						
	n	Minimum	Maximum	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	19	0.1	0.23	0.168	0.035	0.098	0.237
Alkalinity	8	422	556	484.000	36.956	410.089	557.911
Color	7	0	57	24.143	23.061	-21.979	70.265
Dissolved oxygen	18	5	6.29	5.527	0.371	4.786	6.269
Nitrite	12	0.004	0.049	0.012	0.012	-0.013	0.037
Nitrate	10	2.5	15.5	8.500	4.562	-0.623	17.623
Orthophosphate	9	1.93	3.1	2.730	0.362	2.006	3.454
pH	12	8.39	8.67	8.572	0.093	8.386	8.757
Salinity	18	16.6	20.4	18.039	1.158	15.724	20.354
Temperature	21	26.9	30.7	29.300	1.010	27.279	31.321
Turbidity	5	0.44	0.76	0.606	0.119	0.367	0.845

Table A14: Water quality results observed during trial C in freshwater treatment [mg/L, C, NTU].

	Descriptive Statistics						
	n	Minimum	Maximum	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	19	0	0.16	0.0794737	0.0432658	-0.007	0.166
Alkalinity	8	246	282	266.25	12.623674	241.003	291.497
Chloride	7	355	550	451.42857	75.469325	300.490	602.367
Color	7	0	19	7.4285714	8.1005585	-8.773	23.630
Dissolved oxygen	18	5.58	6.89	6.225	0.402569	5.420	7.030
Calcium hardness	7	72	126	95	19.174636	56.651	133.349
Total hardness	7	400	480	450	26.708301	396.583	503.417
Nitrite	12	0	0.005	0.0026667	0.0017753	-0.001	0.006
Nitrate	10	0	7.9	4.45	2.573044	-0.696	9.596
Orthophosphate	9	0.29	0.72	0.4422222	0.125676	0.191	0.694
pH	12	8.19	8.86	8.5525	0.1948951	8.163	8.942
Salinity	18	0.9	1.3	1.0333333	0.1188177	0.796	1.271
Temperature	21	27	29.6	28.328571	0.7342635	26.860	29.797
Turbidity	5	0.19	0.52	0.32	0.1278671	0.064	0.576

Table A15: Water quality results observed during trial C in effluent 1 treatment [mg/L, C, NTU].

	Descriptive Statistics						
	n	Minimum	Maximum	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	19	0	0.33	0.1663158	0.1080746	-0.050	0.382
Alkalinity	8	324	410	370.25	32.556544	305.137	435.363
Chloride	7	365	662	553.14286	94.612241	363.918	742.367
Color	7	2	73	24.285714	23.535181	-22.785	71.356
Dissolved oxygen	18	5.42	6.6	5.9955556	0.3446263	5.306	6.685
Calcium hardness	7	82	130	103.71429	17.913815	67.887	139.542
Total hardness	7	416	564	487.71429	58.096799	371.521	603.908
Nitrite	12	0	0.013	0.00675	0.0041806	-0.002	0.015
Nitrate	10	16.7	170	60.99	47.899929	-34.810	156.790
Orthophosphate	9	1.75	9.1	4.7333333	2.6858425	-0.638	10.105
pH	12	8.42	8.86	8.6333333	0.1576725	8.318	8.949
Salinity	18	1.1	2.1	1.5941176	0.3071453	0.980	2.208
Temperature	21	26.8	31.1	29.042857	1.0614007	26.920	31.166
Turbidity	5	0.42	1.27	0.76	0.329621	0.101	1.419

Table A16: Water quality results observed during trial C in effluent 2 treatment [mg/L, C, NTU].

	Descriptive Statistics						
	n	Minimum	Maximum	Mean	Standard Deviation	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Total ammonia-N	19	0.08	0.28	0.1836842	0.0613017	0.061	0.306
Alkalinity	8	348	456	402.375	41.300078	319.775	484.975
Chloride	7	765	940	844.28571	65.137583	714.011	974.561
Color	7	7	72	38.714286	28.946585	-19.179	96.607
Dissolved oxygen	18	5.28	6.71	6.0538889	0.3921605	5.270	6.838
Calcium hardness	7	105	143	121	14.764823	91.470	150.530
Total hardness	7	572	710	622.28571	46.78217	528.721	715.850
Nitrite	12	0.003	0.019	0.0098333	0.0052714	-0.001	0.020
Nitrate	10	26.3	172	74.03	39.42543	-4.821	152.881
Orthophosphate	9	3.26	8.2	5.0122222	1.5134297	1.985	8.039
pH	12	8.43	8.86	8.6516667	0.1484363	8.355	8.949
Salinity	18	2.2	2.4	2.2555556	0.0783823	2.099	2.412
Temperature	21	27.2	30.2	28.72381	0.9104421	26.903	30.545
Turbidity	5	0.54	1.32	0.814	0.2985465	0.217	1.411

Table A17: Observed mean survival rates during preliminary trial.

	Observed Survival Rate [%]			
Day	Control	Freshwater	Effluent 1	Effluent 2
0	100	100	100	100
1	100	100	95	86
2	100	98	95	86
3	95	98	95	86
4	96	98	95	86
5	96	100	95	79
6	96	100	95	79
7	96	100	95	79
8	.	.	.	.
9	.	.	.	.
10	96	100	95	79
11	96	93	90	76
12	91	95	90	76
13	89	95	90	79
14	89	89	90	79
15	.	.	.	.
16	.	.	.	.
17	.	.	.	.
18	89	86	85	66
19	87	86	85	66
20	89	86	85	59
21	.	.	.	.
22	75	86	85	59
23	70	86	85	59
24	70	86	80	59
25	70	84	80	55
26	70	84	80	48
27	.	.	.	.
28	61	84	80	41
29	.	.	.	.
30	.	.	.	.
31	61	84	80	41
32	.	.	.	.
33	66	80	80	48
34	68	77	80	45
35	63	80	80	45
36	.	.	.	.
37	.	.	.	.
38	63	75	80	45
39	59	73	80	45
40	63	75	80	48
41				
42	61	75	75	48

Table A18: Observed mean survival rates during trial A.

	Observed Survival Rate [%]			
Day	Control	Freshwater	Effluent 1	Effluent 2
0	100	100	100	100
1	96	97	96	93
2	96	96	93	92
3	96	94	71	70
4	96	96	70	73
5	97	100	68	64
6	92	97	68	51
7	96	94	60	51
8				
9				
10	92	98	62	47
11				
12	81	96	59	43
13			59	43
14	92	96	60	39
15	92	97	57	32
16				
17	89	90	51	27
18	88	87	56	32
19	87	89	49	28
20	87	88	49	29
21				
22	81	88	40	17
23	79	89	41	13
24	79	89	41	13
25	78	91	41	9
26				
27	77	89	43	9
28	76	86	51	8
29	73	80	38	4
30	78	81	37	4
31	72	73	36	4
32				
33	72	80	32	3
34	69	77	33	2
35	70	73	33	2
36				
37	72	73	33	0
38	68	72	33	0
39				
40	68	71	33	0
41				
42	67	73	33	0

Table A19: Observed mean survival rates during trial B.

	Observed Survival Rate [%]			
Day	Control	Freshwater	Effluent 1	Effluent 2
0	100	100	100	100
1	100	100	100	100
2	100	98	99	97
3	99	97	98	71
4	99	89	97	73
5	98	92	93	61
6	98	86	93	58
7	94	86	89	54
8	92	86	89	52
9	91	87	89	52
10	89	86	84	48
11	92	84	84	50
12	90	84	86	48
13	84	84	80	47
14	83	83	84	40
15	.	.	.	.
16	.	.	.	.
17	76	72	73	43
18	72	78	72	41
19	69	79	66	43
20	66	77	66	38
21	.	87	61	39
22	.	72	.	.
23	58	70	48	37
24	57	70	58	33
25	56	69	58	34
26	53	61	66	32
27	51	60	56	31
28	.	.	.	.
29	.	.	.	.
30	51	62	49	33
31	51	49	50	32
32	.	.	.	.
33	51	49	48	33
34	51	50	47	33
35	.	47	.	.
36	.	.	.	.
37	50	47	46	29
38	.	.	.	.
39	.	.	.	.
40	50	47	46	28
41	.	.	.	.
42	50	46	46	27

Table A20: Observed mean survival rates during trial C.

	Observed Survival Rate [%]			
Day	Control	Freshwater	Effluent 1	Effluent 2
0	100	100	100	100
2	100	94	93	96
4	98	84	91	94
7	96	82	83	94
21	86	60	75	91
42	69	50	61	74

Table A21: Observed ion concentrations in controls.

Control	Barium (mg/l)	Calcium (mg/l)	Iron (mg/l)	Potassium (mg/l)	Magnesium (mg/l)	Manganese (mg/l)	Sodium (mg/l)	Lead (mg/l)	Chloride (mg/l)	Sulfate (mg/l)
trial prelim	0.2	296	<0.01	294	669	<0.01	6740	<0.01	9909	1789
trial a	<0.05	219	<0.01	176	527	<0.01	5973	<0.01	-	-
trial a	<0.05	160	<0.01	204	608	<0.01	5729	<0.01	8515	1937
trial b	<0.05	325	0.11	205	471	<0.01	6071	<0.01	10243	1372
trial b	<0.05	319	0.13	202	504	<0.01	5972	<0.01	12108	1702
trial b	<0.05	248	0.13	174	467	<0.01	5124	<0.01	12398	1698
trial c	<0.05	220.4	<0.01	235.8	498	<0.01	7137	<0.01	14366	2123
trial c	<0.05	203.9	<0.01	212.8	511	<0.01	7593	<0.01	14012	2232
trial c	<0.05	199.5	<0.01	207.4	505	<0.01	7505	<0.01	14828	2378

Table A22: Observed ion concentrations freshwater treatments.

Freshwater	Barium (mg/l)	Calcium (mg/l)	Iron (mg/l)	Potassium (mg/l)	Magnesium (mg/l)	Manganese (mg/l)	Sodium (mg/l)	Lead (mg/l)	Chloride (mg/l)	Sulfate (mg/l)
trial prelim	0.09	93	<0.01	14	74	<0.01	253	<0.01	379	138
trial a	<0.05	121	<0.01	13	70	<0.01	280	<0.01	878	275
trial a	<0.05	58	<0.01	14	78	<0.01	256	<0.01	830	286
trial b	<0.05	57	0.08	11	49	<0.01	227	<0.01	488	120
trial b	<0.05	98	0.09	16	57	<0.01	242	<0.01	494	127
trial b	<0.05	137	0.09	19	60	<0.01	257	<0.01	569	151
trial c	<0.05	62.6	<0.01	11.3	44	<0.01	224	<0.01	492	133
trial c	<0.05	63.0	<0.01	15.2	52	<0.01	227	<0.01	498	144
trial c	<0.05	64.7	<0.01	16.8	67	<0.01	229	<0.01	509	148

Table A23: Observed ion concentrations in effluent 1 treatments.

Effluent 1	Barium (mg/l)	Calcium (mg/l)	Iron (mg/l)	Potassium (mg/l)	Magnesium (mg/l)	Manganese (mg/l)	Sodium (mg/l)	Lead (mg/l)	Chloride (mg/l)	Sulfate (mg/l)
trial prelim	0.056	78	<0.01	56	57	<0.01	466	<0.01	508	194
trial a	<0.05	41	<0.01	73	56	<0.01	677	<0.01	1372	350
trial a	<0.05	14	<0.01	83	63	<0.01	438	<0.01	1466	388
trial b	<0.05	66	0.09	73	53	<0.01	606	<0.01	704	270
trial b	<0.05	74	0.10	75	58	<0.01	579	<0.01	711	285
trial b	<0.05	98	0.10	80	64	<0.01	597	<0.01	694	283
trial c	<0.05	89.8	<0.01	98.9	53	<0.01	599	<0.01	723	344
trial c	<0.05	87.2	<0.01	96.8	53	<0.01	525	<0.01	714	385
trial c	<0.05	97.1	<0.01	98.7	57	<0.01	602	<0.01	718	388

Table A24: Observed ion concentrations in effluent 2 treatments.

Effluent 2	Barium (mg/l)	Calcium (mg/l)	Iron (mg/l)	Potassium (mg/l)	Magnesium (mg/l)	Manganese (mg/l)	Sodium (mg/l)	Lead (mg/l)	Chloride (mg/l)	Sulfate (mg/l)
trial prelim	0.052	76	<0.01	49	48	<0.01	285	0.08	315	124
trial a	<0.05	32	<0.01	84	43	<0.01	515	<0.01	681	274
trial a	<0.05	11	<0.01	67	20	<0.01	473	<0.01	655	207
trial b	<0.05	67	0.09	65	48	<0.01	433	<0.01	353	215
trial b	<0.05	59	0.09	74	54	<0.01	450	<0.01	440	263
trial b	<0.05	62	n/a	69	59	<0.01	419	<0.01	374	225
trial c	<0.05	90.8	<0.01	88.5	64	<0.01	689	<0.01	1017	364
trial c	<0.05	95.5	<0.01	82.3	62	<0.01	618	<0.01	991	342
trial c	<0.05	100.9	<0.01	86.2	69	<0.01	619	<0.01	1004	373

Figure A1: Survival rates with error bars as a function of time during preliminary trial. Alpha-numeric values denote significant differences between survival rates.

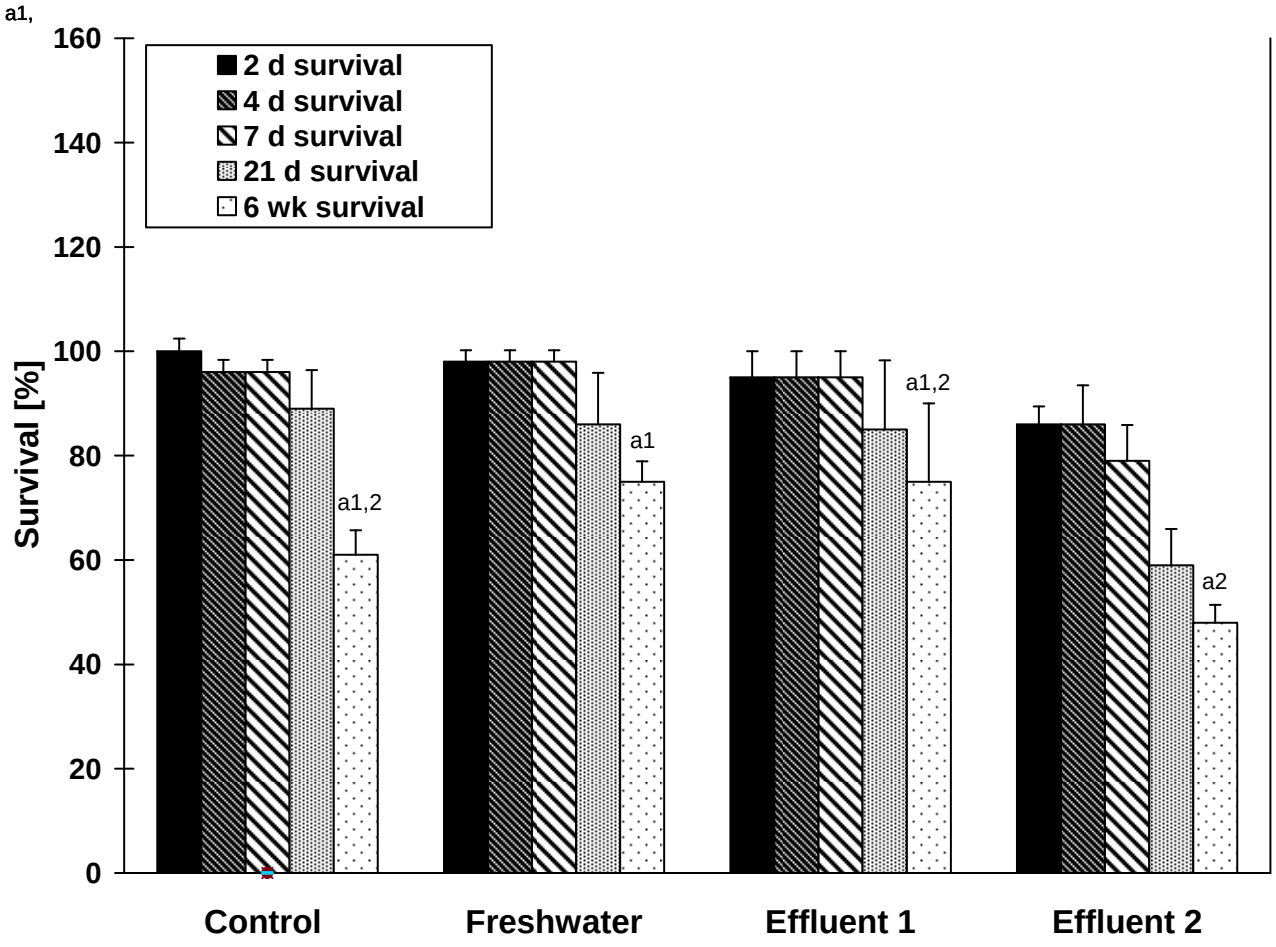


Figure A2: Survival rates with error bars as a function of time during trial A. Alpha-numeric values denote significant differences between survival rates.

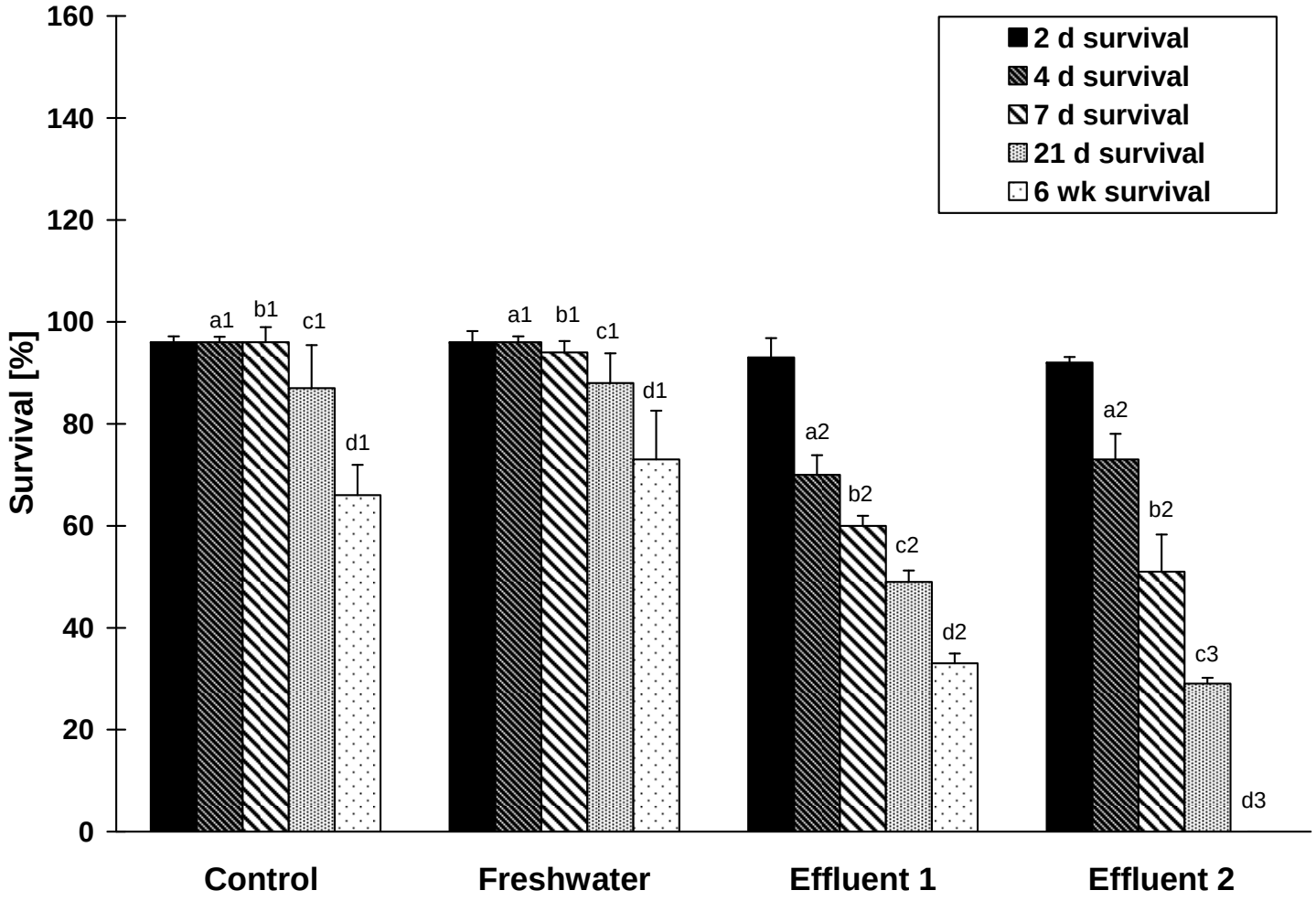


Figure A3: Survival rates with error bars as a function of time during trial B. Alpha-numeric values denote significant differences between survival rates.

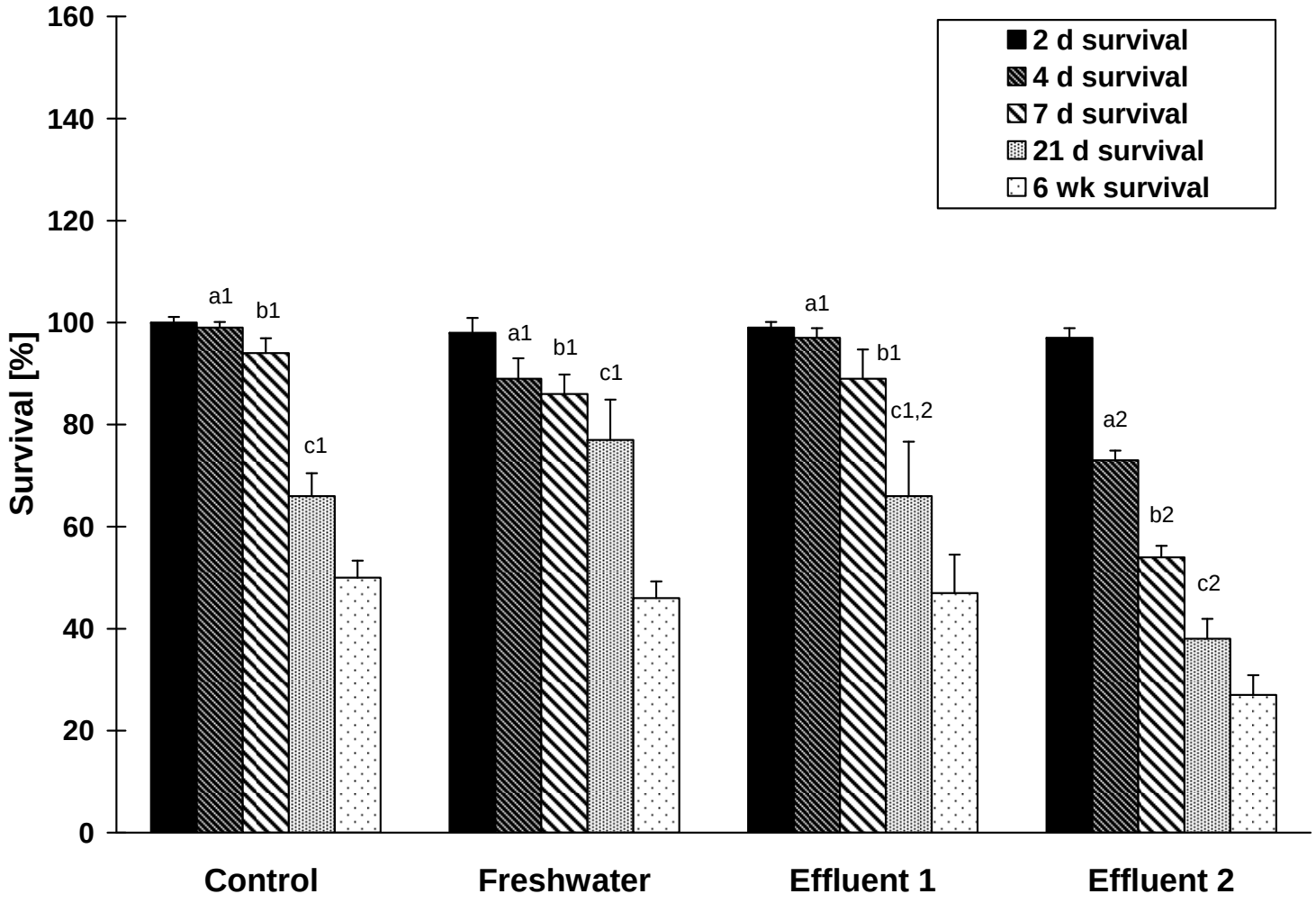
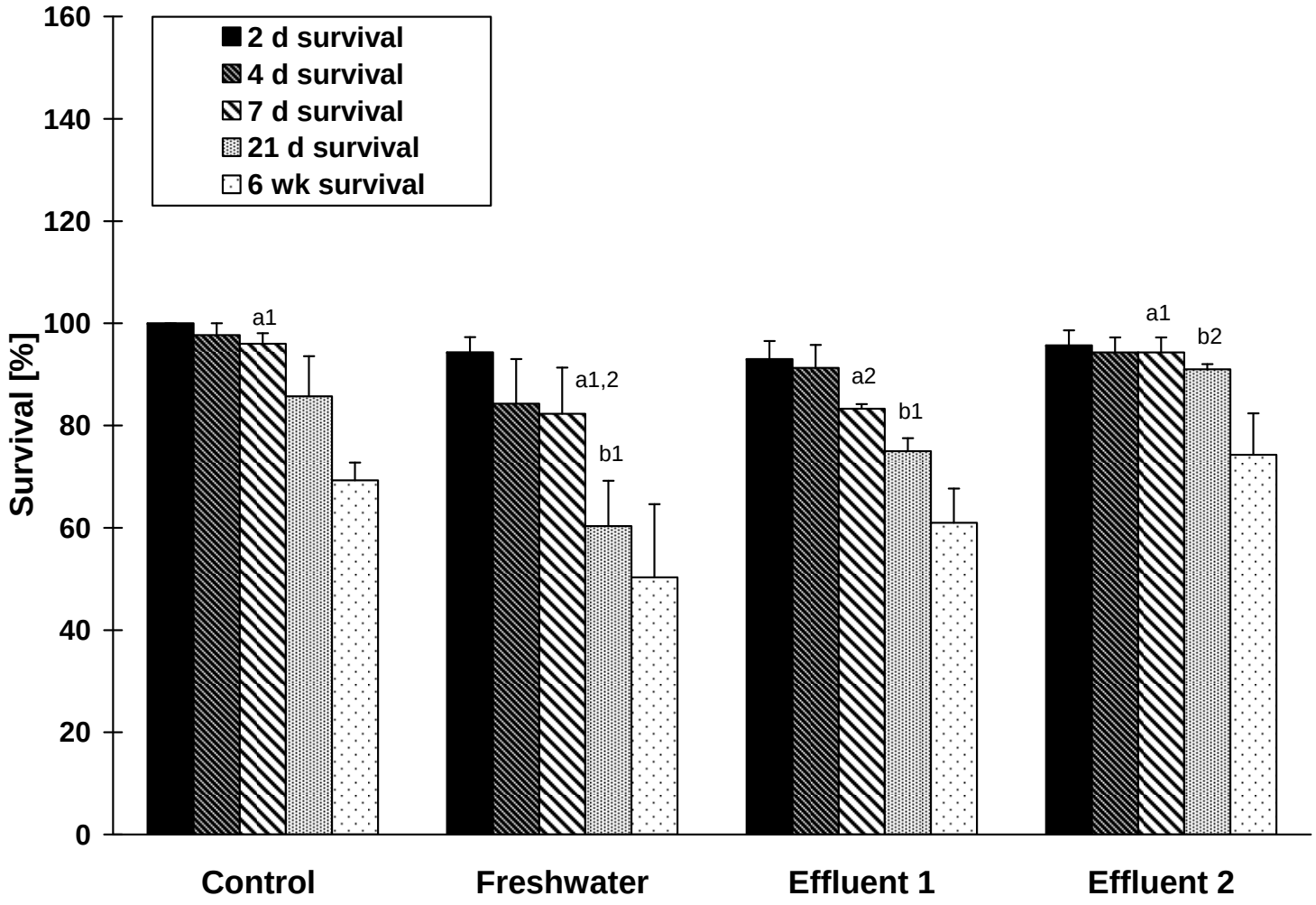


Figure A4: Survival rates with error bars as a function of time during trial C. Alpha-numeric values denote significant differences between survival rates.



APPENDEX B: Data Associated with Figures and Tables in Chapter 3

Table B1. Total ammonia-N [mg/L] data collected with basic statistics during first trial.

	System 1	System 2	System 3	System 4	System 5	System 6
	0.03	0.03	0.02	0.01	0.06	0.02
	0.02	0.04	0.02	0.03	0.02	0.03
	0	0.01	0.03	0.02	0.01	0.03
	0.01	0.01	0.02	0.03	0.02	0.07
	0.01	0.01	0.02	0.01	0.01	0.01
	0	0.02	0	0	0	0
	0.04	0.03	0.04	0.05	0.06	0.05
	0.02	0.01	0.02	0.03	0.03	0.02
	0.01	0.02	0.02	0.01	0.01	0.02
	0.03	0.02	0.02	0.02	0.03	0.02
	0.03	0.02	0.03	0.02	0.03	0.05
Mean	0.018	0.020	0.022	0.021	0.025	0.029
Std. Deviation	0.013	0.010	0.010	0.014	0.020	0.020
Low 95% Confidence Interval	-0.008	0.000	0.002	-0.007	-0.014	-0.011
High 95% Confidence Interval	0.045	0.040	0.041	0.048	0.065	0.070

Table B2. Nitrate-N [mg/L] data collected with basic statistics during first trial.

	System 1	System 2	System 3	System 4	System 5	System 6
	6.3	6.5	4.5	5.2	5.1	5.8
	10.7	11.8	13.3	14.9	9.8	13.9
	11.6	14.5	15.5	17.2	11.2	16.1
	13.9	15.9	18.9	20.2	13.5	18.6
	17.1	18.2	23.2	23	13.5	21.9
	17.2	21	20.9	24.6	14.3	23.4
	19.3	21.9	22.7	22.6	13.8	24.7
	18.8	21.3	20.3	23.6	12.7	15.3
	21	13.8	25.1	25.9	15.2	24.3
Mean	15.100	16.100	18.267	19.689	12.122	18.222
Std. Deviation	4.809	5.095	6.364	6.488	3.097	6.179
Low 95% Confidence Interval	5.481	5.911	5.539	6.713	5.929	5.865
High 95% Confidence Interval	24.719	26.289	30.995	32.665	18.316	30.580

Table B3. Dissolved Oxygen [mg/L] data collected with basic statistics during first trial.

	System 1	System 2	System 3	System 4	System 5	System 6
	4.78	4.75	4.86	4.76	4.87	4.7
	5.04	5.02	5.04	4.96	4.99	4.96
	5.11	4.97	5.01	4.94	5.02	4.93
	5.19	5.11	5.13	5.03	5.11	5.03
	5.36	5.39	5.41	5.31	5.33	5.36
	5.24	5.12	5.14	4.96	5.13	5.05
	5.23	5.11	5.13	4.99	5.14	5.1
	5.21	5.13	5.11	4.95	5.15	5.07
	5.17	5.18	5.21	4.97	5.08	5.04
	5.23	5.26	5.29	5.15	5.32	5.13
	5.45	5.56	5.55	5.3	5.39	5.48
	5.31	5.35	5.38	5.25	5.31	5.24
	5.38	5.4	5.29	5.17	5.26	5.25
Mean	5.208	5.181	5.196	5.057	5.162	5.103
Std. Deviation	0.170	0.213	0.186	0.165	0.154	0.199
Low 95% Confidence Interval	4.868	4.755	4.825	4.727	4.855	4.705
High 95% Confidence Interval	5.548	5.606	5.568	5.387	5.469	5.501

Table B4. Temperature [C] data collected with basic statistics during first trial.

	System 1	System 2	System 3	System 4	System 5	System 6
	31	30.5	29.9	30.4	30.2	31.1
	29.9	29.6	29.3	29.8	29.9	30
	30.2	29.5	29.4	29.6	29.7	30.4
	30.2	30	29.9	29.8	29.2	30
	29.8	29.9	29.5	29.9	29.8	30.2
	29.5	29.3	29.2	29.7	29.4	29.9
	29.5	28.3	28.4	28.5	28	28.3
	29.5	28.2	28.7	28.7	28.3	28.4
	28	27.9	28	28.4	28.1	27.4
	30.2	29.4	29.5	30	29.3	30.1
	30.1	30.5	29.7	30.2	30.7	30.1
	29.9	28.6	28.5	29.1	29.3	28.6
	30.3	29.9	29.9	30.9	29.3	29.7
	30.2	30.2	29.5	30.9	29.4	30.7
	30.1	30.2	29.6	30.9	30	30.1
	29.9	29.7	29.4	30.4	29.7	29.4
	29.9	30	29.5	30.7	29.8	30
	29.9	29.9	29.4	30.6	29.8	29.9
	29.5	29.4	29.5	30	29	29.6
	29.5	28.5	28.9	29.4	28.7	28.5
	29.7	29.6	29.2	30.3	29.7	29.7
	30.7	30.8	29	31	30.3	30.1
	29.8	29	28.5	30.4	29.7	29.5
	29.8	28.7	28.3	29	28.7	28.5
	27.7	27.1	27.2	28.5	28.1	27.3
	29.7	29.6	28.9	30	29.7	29.7
	29.5	29.8	29.8	30.9	29.7	29.6
	29.7	29.1	29.2	30.8	29	29
Mean	29.775	29.4	29.13571	29.95714	29.375	29.49286
Std. Deviation	0.655	0.859	0.640	0.806	0.685	0.921
Low 95% Confidence Interval	28.466	27.683	27.857	28.345	28.005	27.650
High 95% Confidence Interval	31.084	31.117	30.415	31.570	30.745	31.336

Table B5. Nitrite [mg/L] data collected with basic statistics during first trial.

	System 1	System 2	System 3	System 4	System 5	System 6
	0.088	0.093	0.139	0.017	0.013	0.012
	0.011	0.02	0.009	0.013	0.008	0.008
	0.007	0.009	0.025	0.023	0.012	0.015
	0.009	0.018	0.014	0.006	0.01	0.01
	0.021	0.021	0.018	0.011	0.008	0.011
	0.01	0.017	0.016	0.169	0.01	0.02
	0.011	0.012	0.014	0.016	0.056	0.021
	0.016	0.011	0.015	0.026	0.028	0.013
	0.011	0	0.01	0.003	0.006	0.001
Mean	0.020	0.022	0.029	0.032	0.017	0.012
Std. Deviation	0.026	0.027	0.042	0.052	0.016	0.006
Low 95% Confidence Interval	-0.031	-0.032	-0.054	-0.073	-0.015	0.000
High 95% Confidence Interval	0.072	0.077	0.112	0.136	0.049	0.024

Table B6. pH data collected with basic statistics during first trial.

	System 1	System 2	System 3	System 4	System 5	System 6
	8.47	8.39	8.48	8.45	8.44	8.46
	8.46	8.59	8.62	8.65	8.64	8.63
	8.51	8.55	8.57	8.59	8.58	8.58
	8.45	8.47	8.46	8.49	8.45	8.47
	8.47	8.46	8.44	8.45	8.41	8.42
	8.4	8.48	8.47	8.52	8.41	8.45
	8.43	8.48	8.5	8.55	8.41	8.48
	8.47	8.53	8.53	8.58	8.43	8.51
	8.43	8.5	8.49	8.58	8.42	8.55
	8.37	8.37	8.41	8.4	8.26	8.37
Mean	8.446	8.482	8.497	8.526	8.445	8.492
Std. Deviation	0.040	0.067	0.062	0.078	0.103	0.077
Low 95% Confidence Interval	8.366	8.348	8.373	8.370	8.239	8.337
High 95% Confidence Interval	8.526	8.616	8.621	8.682	8.651	8.647

Table B7. Salinity [ppt] data collected with basic statistics during first trial.

	System 1	System 2	System 3	System 4	System 5	System 6
	1.8	2	2	2.3	1.6	2.2
	1.7	2	2	2.3	1.6	2.2
	1.7	2	2	2.3	1.6	2.2
	1.7	2	2	2.3	1.5	2.2
	1.7	2	2	2.3	1.5	2.2
	1.7	2	2	2.2	2.1	2.1
	1.8	2	2	2.2	2.3	2.1
	2	2	2	2	2	2.1
	2	2	2	2.1	1.9	2.1
	2	1.9	2	2.1	1.9	2.1
	2	1.9	2	2.1	1.9	2.1
	2	1.9	1.9	2.1	1.9	2.1
	2	1.9	1.9	2.1	1.9	2.1
	2	1.9	1.9	2	1.9	2
	2	1.9	1.9	2	2	2
	2	1.9	1.9	2	2.1	2
Mean	1.881	1.956	1.969	2.150	1.856	2.113
Std. Deviation	0.142	0.051	0.048	0.121	0.234	0.072
Low 95% Confidence Interval	1.596	1.854	1.873	1.908	1.389	1.969
High 95% Confidence Interval	2.166	2.059	2.064	2.392	2.324	2.256

Table B8. Shrimp performance per tank at end of feeding trial one for diet one.

Diet one: 100% Shrimp Feed					
Final Count	Total Mass [g]	Individual Mass [g]	**SGR**		
3	1.145	0.38166667	**6.687447**	**% Growth =**	**1363.921**
4	1.9908	0.4977	**7.351071**	**SGR =**	**6.709258**
4	1.3911	0.347775	**6.454967**		
5	1.6555	0.3311	**6.332129**		
4	1.2905	0.322625	**6.267304**		
2	0.8584	0.4292	**6.980885**		

Table B9. Shrimp performance per tank at end of feeding trial one for diet two.

Diet two: 50/50 feed/floc					
Final Count	Total Mass [g]	Individual Mass [g]	SGR		
5	1.2798	0.25596	**5.688631**	**% Growth =**	**885.1711**
5	1.3975	0.2795	**5.908583**	**SGR =**	**5.719113**
5	1.2608	0.25216	**5.651237**		
5	1.3699	0.27398	**5.858715**		
4	1.0519	0.262975	**5.756225**		
4	0.9201	0.230025	**5.421548**		

Table B10. Shrimp performance per tank at end of feeding trial one for diet three.

Diet three: 50/50 feed/untreated solids					
Final Count	Total Mass [g]	Individual Mass [g]	SGR		
4	0.7808	0.1952	**5.011139**	**% Growth =**	**757.913**
5	1.0828	0.21656	**5.270747**	**SGR =**	**5.373331**
5	1.0365	0.2073	**5.161495**		
5	1.0328	0.20656	**5.152555**		
3	0.755	0.25166667	**5.646341**		
4	1.106	0.2765	**5.881605**		

Table B11. Shrimp performance per tank at end of feeding trial one for diet four.

Diet four: 50% feed					
Final Count	Total Mass [g]	Individual Mass [g]	SGR		
5	0.8792	0.17584	**4.750014**	**% Growth =**	**585.0539**
5	1.05	0.21	**5.193846**	**SGR =**	**4.810818**
5	0.809	0.1618	**4.54198**		
4	0.7056	0.1764	**4.757963**		
4	0.7311	0.182775	**4.846717**		
4	0.6968	0.1742	**4.726588**		

TableB12. Soluble COD measurements with basic statistics for reactors 1, 2, and 3, during second feeding trial.

	Reactor 1			**Reactor 2**			**Reactor 3**		
Days	sCOD	Average	Standard Deviation	sCOD	Average	Standard Deviation	sCOD	Average	Standard Deviation
0	134.5	133.0	2.1	93.2	92.4	1.2	82.5	80.8	2.5
0	131.6			91.5			79.1		
1	121.0	123.3	3.3	108.7	104.9	5.3	76.9	75.4	2.1
1	125.7			101.2			73.9		
2	82.5	77.1	7.7	69.0	74.9	8.4	84.2	80.0	6.0
2	71.7			80.9			75.8		
3	90.4	91.4	1.5	65.8	72.1	9.0	62.6	61.2	2.0
3	92.4			78.5			59.8		
4	77.0	79.5	3.6	31.6	32.0	0.5	52.6	47.4	7.4
4	82.0			32.4			42.2		
7	50.0	44.6	7.6	14.9	19.6	6.7	17.6	20.3	3.8
7	39.2			24.3			23.0		
8	40.5	35.1	7.6	13.5	16.2	3.8	6.8	11.5	6.7
8	29.7			18.9			16.2		

Table B13. Lowry protein in reactor over course of second feeding trial.

	TSS [mg/L]	LOWRY [ug/mL]	LOWRY [mg/gTSS]	AVG LOWRY [mg/gTSS]	STD ERROR LOWRY
Start	2030	83.25368725	41.0116686	60.144	9.584
	490	34.652551	70.71949184		
	1430	98.2432	68.70153846		
1 wk	2810	138.2860473	49.21211646	50.747	1.535
	1010	spill	spill		
	1210	63.26020525	52.28116136		
2 wk	3590	160.638775	44.74617688	55.437	9.956
	2650	122.522459	46.23489019		
	910	68.550064	75.32974066		
3 wk	1170	60.09689525	51.36486774	62.638	5.808
	790	52.02298525	65.85188006		
	610	43.1258	70.69803279		
4 wk	1230	63.419216	51.56033821	58.052	6.298
	1230	63.89673125	51.94856199		
	550	38.85532525	70.64604591		
5 wk	510	40.02662525	78.48357892	59.629	9.462
	1290	62.94242525	48.79257771		
	850	43.86893125	51.61050735		

Table B14. Water quality statistics for system 1 during second feeding trial.

DO [mg/L]		*Nitrate-N [mg/L]*	
Mean	6.400	Mean	21.383
Standard Error	0.080	Standard Error	5.027
Median	6.420	Median	24.550
Range	0.730	Range	31.800
Minimum	6.010	Minimum	1.000
Maximum	6.740	Maximum	32.800

Salinity [ppt]		*TAN [mg/L]*	
Mean	2.030	Mean	0.100
Standard Error	0.026	Standard Error	0.016
Median	2.050	Median	0.100
Range	0.200	Range	0.130
Minimum	1.900	Minimum	0.040
Maximum	2.100	Maximum	0.170

Nitrite-N [mg/L]		*pH*	
Mean	0.032	Mean	8.597
Standard Error	0.005	Standard Error	0.015
Median	0.034	Median	8.600
Range	0.046	Range	0.160
Minimum	0.010	Minimum	8.510
Maximum	0.056	Maximum	8.670

Temp [C]	
Mean	28.938
Standard Error	0.134
Median	28.900
Range	2.200
Minimum	28.000
Maximum	30.200

Table B15. Water quality statistics for system 2 during second feeding trial.

DO [mg/L]		*Nitrate-N [mg/L]*	
Mean	6.337	Mean	19.367
Standard Error	0.080	Standard Error	4.386
Median	6.355	Median	20.850
Range	0.730	Range	31.200
Minimum	5.980	Minimum	1.100
Maximum	6.710	Maximum	32.300

Salinity [ppt]		*TAN [mg/L]*	
Mean	2.030	Mean	0.112
Standard Error	0.021	Standard Error	0.018
Median	2.000	Median	0.115
Range	0.200	Range	0.170
Minimum	1.900	Minimum	0.030
Maximum	2.100	Maximum	0.200

Nitrite-N [mg/L]		*pH*	
Mean	0.033	Mean	8.620
Standard Error	0.006	Standard Error	0.016
Median	0.033	Median	8.630
Range	0.052	Range	0.160
Minimum	0.010	Minimum	8.530
Maximum	0.062	Maximum	8.690

Temp [C]	
Mean	29.157
Standard Error	0.102
Median	29.200
Range	1.800
Minimum	28.100
Maximum	29.900

Table B16. Water quality statistics for system 3 during second feeding trial.

DO [mg/L]		*Nitrate-N [mg/L]*	
Mean	6.422	Mean	17.583
Standard Error	0.080	Standard Error	4.055
Median	6.365	Median	19.000
Range	0.700	Range	25.400
Minimum	6.140	Minimum	1.600
Maximum	6.840	Maximum	27.000

Salinity [ppt]		*TAN [mg/L]*	
Mean	2.060	Mean	0.111
Standard Error	0.054	Standard Error	0.013
Median	2.100	Median	0.115
Range	0.500	Range	0.130
Minimum	1.800	Minimum	0.050
Maximum	2.300	Maximum	0.180

Nitrite-N [mg/L]		*pH*	
Mean	0.025	Mean	8.613
Standard Error	0.004	Standard Error	0.016
Median	0.025	Median	8.620
Range	0.040	Range	0.170
Minimum	0.010	Minimum	8.520
Maximum	0.050	Maximum	8.690

Temp [C]	
Mean	29.019
Standard Error	0.103
Median	29.100
Range	2.000
Minimum	27.900
Maximum	29.900

Table B17. Raw mean weights [g] of shrimp per tank basis during second feeding trial.

	start	wk1	wk2	wk3	wk4	final
Diet 1: 8% BW/d	1.57	1.72	1.91	2.61	2.42	3.39
Diet 1: 8% BW/d	1.53	1.96	2.08	2.19	2.19	2.23
Diet 1: 8% BW/d	1.52	1.92	2.01	2.08	2.92	3.14
Diet 2: 4% BW/d	1.59	1.86	1.90	2.22	2.76	3.21
Diet 2: 4% BW/d	1.49	1.71	1.80	1.98	2.10	2.00
Diet 2: 4% BW/d	1.49	1.76	1.88	2.05	2.31	1.98
Diet 3: 6% BW/d + Flocs	1.49	1.81	2.29	2.58	2.91	4.24
Diet 3: 6% BW/d + Flocs	1.59	1.93	2.32	3.31	4.00	4.75
Diet 3: 6% BW/d + Flocs	1.50	1.90	2.09	2.94	3.90	4.08
Diet 4: 4% BW/d + Flocs	1.55	1.99	2.20	2.41	2.77	2.96
Diet 4: 4% BW/d + Flocs	1.56	1.72	1.77	1.94	2.16	2.45
Diet 4: 4% BW/d + Flocs	1.56	1.74	2.08	2.30	2.47	2.85

Table B18. Mean weights [g] of shrimp per diet basis, averaging weights from tanks (n=3) during second feeding trial.

	start	**wk1**	**wk2**	**wk3**	**wk4**	**wk5**
Diet 1: 8% BW/d	1.54	1.87	2.00	2.29	2.51	2.92
Diet 2: 4% BW/d	1.52	1.77	1.86	2.08	2.39	2.39
Diet 3: 6% BW/d + Flocs	1.53	1.88	2.23	2.94	3.60	4.35
Diet 4: 4% BW/d + Flocs	1.55	1.81	2.01	2.22	2.47	2.75

Table B19. Standard error of mean weights [g] of shrimp per diet basis (n=3) during second feeding trial.

	start	**wk1**	**wk2**	**wk3**	**wk4**	**wk5**
Diet 1: 8% BW/d	0.0164	0.0740	0.0494	0.1631	0.2151	0.3517
Diet 2: 4% BW/d	0.0333	0.0465	0.0303	0.0723	0.1937	0.4064
Diet 3: 6% BW/d + Flocs	0.0310	0.0366	0.0724	0.2083	0.3458	0.2015
Diet 4: 4% BW/d + Flocs	0.0025	0.0885	0.1278	0.1428	0.1747	0.1549

Table B20. Mean SGRs [1/d] of shrimp per diet basis, averaging weights from tanks (n=3) during second feeding trial.

	wk1	wk2	wk3	wk4	wk5
Diet 1: 8% BW/d	2.74	1.85	1.89	1.74	1.83
Diet 2: 4% BW/d	2.20	1.44	1.50	1.61	1.29
Diet 3: 6% BW/d + Flocs	2.95	2.72	3.13	3.07	3.00
Diet 4: 4% BW/d + Flocs	2.22	1.86	1.69	1.65	1.63

Table B21. Standard error of SGRs [1/d] of shrimp per diet basis (n=3) during second feeding trial.

	wk1	wk2	wk3	wk4	wk5
Diet 1: 8% BW/d	0.720	0.242	0.280	0.319	0.352
Diet 2: 4% BW/d	0.126	0.116	0.071	0.214	0.406
Diet 3: 6% BW/d + Flocs	0.195	0.199	0.257	0.322	0.202
Diet 4: 4% BW/d + Flocs	0.705	0.474	0.321	0.260	0.155

Table B22. FCR shrimp data per tank basis during second feeding trial.

	0-1 wk	0-2 wk	0-3 wk	0-4 wk	0-5 wk
Diet 1: 8% BW/d	5.248	4.875	2.458	4.537	2.776
Diet 1: 8% BW/d	1.775	3.167	4.167	5.851	7.016
Diet 1: 8% BW/d	1.845	3.456	4.789	2.651	3.168
Diet 2: 4% BW/d	1.421	2.696	2.072	1.597	1.572
Diet 2: 4% BW/d	1.683	2.495	2.496	2.815	4.386
Diet 2: 4% BW/d	1.369	2.033	2.242	2.147	4.788
Diet 3: 6% BW/d + Flocs	1.746	1.527	1.885	2.118	1.487
Diet 3: 6% BW/d + Flocs	1.724	1.764	1.255	1.402	1.536
Diet 3: 6% BW/d + Flocs	1.402	2.125	1.403	1.297	1.768
Diet 4: 4% BW/d + Flocs	0.861	1.345	1.641	1.646	1.907
Diet 4: 4% BW/d + Flocs	2.392	3.803	3.248	2.837	2.521
Diet 4: 4% BW/d + Flocs	2.126	1.550	1.766	2.056	1.928

Table B23. Mean FCRs of shrimp per diet basis, averaging from tanks (n=3) during second feeding trial.

Day	Diet 1: 8% BW/d	Diet 2: 4% BW/d	Diet 3: 6% BW/d + Flocs	Diet 4: 4% BW/d + Flocs
7	2.956	1.491	1.624	1.793
14	3.833	2.408	1.805	2.232
21	3.805	2.270	1.514	2.218
28	4.346	2.186	1.606	2.180
35	4.320	3.582	1.597	2.119

Table B24. Standard errors of FCRs of shrimp per diet basis, averaging from tanks (n=3) during second feeding trial.

Day	Diet 1: 8% BW/d	Diet 2: 4% BW/d	Diet 3: 6% BW/d + Flocs	Diet 4: 4% BW/d + Flocs
7	1.146	0.097	0.111	0.472
14	0.528	0.196	0.174	0.787
21	0.697	0.123	0.190	0.516
28	0.929	0.352	0.258	0.349
35	1.353	1.012	0.086	0.201

Table B25. Delta mass of accumulated feed [g] fed to shrimp per tank basis during second feeding trial. Accounted for 87.91% dry matter.

	0-1 wk	0-2 wk	0-3 wk	0-4 wk	0-5 wk
Diet 1: 8% BW/d	0.774135	1.620885	2.558708	3.845242	5.036598
Diet 1: 8% BW/d	0.754444	1.718113	2.739627	3.817755	4.893422
Diet 1: 8% BW/d	0.747059	1.693498	2.681782	3.704527	5.13957
Diet 2: 4% BW/d	0.39076	0.849211	1.317507	1.864776	2.542914
Diet 2: 4% BW/d	0.366145	0.785827	1.229509	1.716882	2.233178
Diet 2: 4% BW/d	0.366145	0.798135	1.260893	1.765497	2.334098
Diet 3: 6% BW/d + Flocs	0.550141	1.216586	2.060874	3.014698	4.090364
Diet 3: 6% BW/d + Flocs	0.58614	1.297815	2.155641	3.37592	4.850962
Diet 3: 6% BW/d + Flocs	0.553833	1.253509	2.025183	3.111926	4.551892
Diet 4: 4% BW/d + Flocs	0.380914	0.870749	1.411043	2.003645	2.684244
Diet 4: 4% BW/d + Flocs	0.38276	0.804904	1.239765	1.716472	2.248152
Diet 4: 4% BW/d + Flocs	0.38276	0.809827	1.321199	1.88816	2.496966

APPENDEX C: Data Associated with Figures and Tables in Chapter 4

Table C1. Proximate analysis of microbial flocs produced in pilot scale SBRs (% on dry basis), n=2.

	Sample 1	Sample 2	Average	Standard Deviation	Standard Error	Lower 95% Confidence Interval	Upper 95% Confidence Interval
Crude protein	50.49	47.56	49.03	2.07	1.46	44.88	53.17
Carbohydrate[a]	35.49	37.33	36.41	1.30	0.92	33.81	39.01
Ash	12.81	14.07	13.44	0.89	0.63	11.66	15.22
Total fat	1.21	1.04	1.13	0.12	0.09	0.88	1.37
Fiber	12.62	12.64	12.63	0.01	0.01	12.60	12.66
Calcium	1.22	1.35	1.29	0.09	0.06	1.10	1.47
Phosphorus	1.21	1.37	1.29	0.11	0.08	1.06	1.52
Potassium	0.62	0.87	0.75	0.18	0.13	0.39	1.10
Magnesium	0.36	0.46	0.41	0.07	0.05	0.27	0.55
Sodium	1.24	1.29	1.27	0.04	0.03	1.19	1.34

[a] Calculated value: carbohydrate = total - (ash + crude protein + moisture + total fat)

Table C2. Control 1 proximate and mineral values.

	Sample 1	Sample 2	Average	Standard Deviation	Standard error	Lower 95% Confidence Interval	Higher 95% Confidence Interval
Crude protein	39.94	36.75	38.35	2.26	1.59	33.83	42.86
Calculated Carbohydrate	27.01	29.84	28.43	2.00	1.42	24.42	32.43
Ash	19.59	19.72	19.66	0.09	0.06	19.47	19.84
Total fat	5.58	5.81	5.70	0.16	0.11	5.37	6.02
Calcium	2.74	2.89	2.82	0.11	0.07	2.60	3.03
Phosphorus	1.94	2.02	1.98	0.06	0.04	1.87	2.09
Potassium	1.30	1.38	1.34	0.06	0.04	1.23	1.45
Magnesium	0.99	1.03	1.01	0.03	0.02	0.95	1.07
Sodium	1.00	1.05	1.03	0.04	0.03	0.95	1.10
Fiber	2.08	1.66	1.87	0.30	0.21	1.28	2.46
Moisture	7.88	7.88	7.88	n/a	n/a	n/a	n/a

Table C3. Control 2 proximate and mineral values.

	Sample 1	Sample 2	Average	Standard Deviation	Standard error	Lower 95% Confidence Interval	Higher 95% Confidence Interval
Crude protein	41.50	42.19	41.85	0.49	0.34	40.87	42.82
Calculated Carbohydrate	20.88	22.91	21.90	1.44	1.01	19.02	24.77
Ash	19.69	19.80	19.75	0.08	0.05	19.59	19.90
Total fat	8.50	5.67	7.09	2.00	1.42	3.08	11.09
Calcium	3.50	3.34	3.42	0.11	0.08	3.19	3.65
Phosphorus	2.68	2.56	2.62	0.08	0.06	2.45	2.79
Potassium	1.29	1.26	1.28	0.02	0.02	1.23	1.32
Magnesium	1.18	1.14	1.16	0.03	0.02	1.10	1.22
Sodium	0.91	0.84	0.88	0.05	0.04	0.78	0.97
Fiber	1.27	1.58	1.43	0.22	0.16	0.99	1.86
Moisture	9.43	9.43	9.43	n/a	n/a	n/a	n/a

Table C4. Diet 1 (7.8% floc) proximate and mineral values.

	Sample 1	Sample 2	Average	Standard Deviation	Standard error	Lower 95% Confidence Interval	Higher 95% Confidence Interval
Crude protein	41.81	41.69	41.75	0.085	0.060	41.58	41.92
Calculated Carbohydrate	24.04	25.26	24.65	0.863	0.610	22.92	26.38
Ash	19.79	19.98	19.885	0.134	0.095	19.62	20.15
Total fat	6.52	5.23	5.875	0.912	0.645	4.05	7.70
Calcium	2.85	3.03	2.94	0.127	0.090	2.69	3.19
Phosphorus	1.92	2.16	2.04	0.170	0.120	1.70	2.38
Potassium	1.41	1.35	1.38	0.042	0.030	1.30	1.46
Magnesium	1.06	1.05	1.055	0.007	0.005	1.04	1.07
Sodium	1.11	1.07	1.09	0.028	0.020	1.03	1.15
Fiber	2.24	0.37	1.305	1.322	0.935	-1.34	3.95
Moisture	7.84	7.84		n/a	n/a	n/a	n/a

Table C5. Diet 2 (15.6% floc) proximate values.

	Sample 1	Sample 2	Average	Standard Deviation	Standard error	Lower 95% Confidence Interval	Higher 95% Confidence Interval
Crude protein	39.88	42.56	41.22	1.90	1.34	37.43	45.01
Calculated Carbohydrate	22.88	21.48	22.18	0.99	0.7	20.20	24.16
Ash	21.85	21.32	21.59	0.37	0.265	20.84	22.33
Total fat	7.91	7.16	7.54	0.53	0.375	6.47	8.60
Calcium	3.18	3.2	3.19	0.01	0.01	3.16	3.22
Phosphorus	2.32	2.35	2.34	0.02	0.015	2.29	2.38
Potassium	1.31	1.32	1.32	0.01	0.005	1.30	1.33
Magnesium	1.24	1.21	1.23	0.02	0.015	1.18	1.27
Sodium	1.05	1.07	1.06	0.01	0.01	1.03	1.09
Fiber	2.08	1.5	1.79	0.41	0.29	0.97	2.61
Moisture	7.48	7.48	7.84	n/a	n/a	n/a	n/a

Table C6. Diet 3 (7.8% floc + fish oil) proximate values.

	Sample 1	Sample 2	Average	Standard Deviation	Standard error	Lower 95% Confidence Interval	Higher 95% Confidence Interval
Crude protein	42.63	42.69	42.66	0.042	0.03	42.58	42.74
Calculated Carbohydrate	23.9	19.88	21.89	2.843	2.01	16.20	27.58
Ash	19.83	19.57	19.70	0.184	0.13	19.33	20.07
Total fat	6.08	10.3	8.19	2.984	2.11	2.22	14.16
Calcium	3.09	3.22	3.16	0.092	0.065	2.97	3.34
Phosphorus	2.13	2.3	2.22	0.120	0.085	1.97	2.46
Potassium	1.33	1.32	1.33	0.007	0.005	1.31	1.34
Magnesium	1.02	1.02	1.02	0.000	0	1.02	1.02
Sodium	1.05	1.06	1.06	0.007	0.005	1.04	1.07
Fiber	1.64	1.55	1.60	0.064	0.045	1.47	1.72
Moisture	7.56	7.56	7.56	n/a	n/a	n/a	n/a

Table C7. One-way ANOVA for crude protein.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control 1	2	76.69	38.345	5.08805
control 2	2	83.69	41.845	0.23805
diet 1	2	83.5	41.75	0.0072
diet 2	2	82.44	41.22	3.5912
diet 3	2	85.32	42.66	0.0018

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	21.99014	4	5.497535	3.079403	0.124442	5.192168
Within Groups	8.9263	5	1.78526			
Total	30.91644	9				

Table C8. One-way ANOVA for carbohydrate.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control 1	2	56.85	28.425	4.00445
control 2	2	43.79	21.895	2.06045
diet 1	2	49.3	24.65	0.7442
diet 2	2	44.36	22.18	0.98
diet 3	2	43.78	21.89	8.0802

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	64.02866	4	16.00717	5.043438	0.052805	5.192168
Within Groups	15.8693	5	3.17386			
Total	79.89796	9				

Table C9. One-way ANOVA for total fat.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control 1	2	11.39	5.695	0.02645
control 2	2	14.17	7.085	4.00445
diet 1	2	11.75	5.875	0.83205
diet 2	2	15.07	7.535	0.28125
diet 3	2	16.38	8.19	8.9042

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	9.20264	4	2.30066	0.818833	0.56455	5.192168
Within Groups	14.0484	5	2.80968			
Total	23.25104	9				

Table C10. One-way ANOVA for crude fiber.

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
control 1	2	3.74	1.87	0.0882
control 2	2	2.85	1.425	0.04805
diet 1	2	2.61	1.305	1.74845
diet 2	2	3.58	1.79	0.1682
diet 3	2	3.19	1.595	0.00405

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.45326	4	0.113315	0.275444	0.882219	5.192168
Within Groups	2.05695	5	0.41139			
Total	2.51021	9				

Table C11. Water quality statistical results for system 5.

Statistic	System 5: Water quality results						
	Temperature [C]	Salinity [ppt]	DO [mg/L]	Ammonia-N [mg/L]	Nitrite-N [mg/L]	Nitrate--N [mg/L]	pH
Mean	30.03	30.57	5.76	0.11	0.08	0.78	8.39
Standard Deviation	0.43	0.76	0.22	0.05	0.05	0.66	0.07
n	100.00	100.00	100.00	5.00	5.00	5.00	4.00
Minimum	27.90	29.10	5.00	0.06	0.03	0.09	8.33
Maximum	30.70	31.90	6.70	0.20	0.16	1.65	8.47
Lower 95% Confidence Level	29.17	29.05	5.32	0.01	0.00	0.00	8.26
Upper 95% Confidence Level	30.90	32.10	6.19	0.22	0.19	2.11	8.52

Table C12. Water quality statistical results for system 6.

Statistic	System 6: Water quality results						
	Temperature [C]	Salinity [ppt]	DO [mg/L]	Ammonia-N [mg/L]	Nitrite-N [mg/L]	Nitrate--N [mg/L]	pH
Mean	30.10	30.34	5.72	0.12	0.09	0.91	8.31
Standard Deviation	0.21	0.81	0.19	0.07	0.07	0.84	0.09
n	100.00	100.00	100.00	5.00	5.00	5.00	4.00
Minimum	29.60	29.10	5.10	0.04	0.03	0.09	8.21
Maximum	30.60	32.00	6.10	0.22	0.18	2.08	8.42
Lower 95% Confidence Level	29.68	28.73	5.34	0.00	0.00	0.00	8.12
Upper 95% Confidence Level	30.52	31.96	6.09	0.27	0.23	2.58	8.50

Table C13. Water quality statistical results for system 7.

Statistic	System 7: Water quality results						
	Temperature [C]	Salinity [ppt]	DO [mg/L]	Ammonia-N [mg/L]	Nitrite-N [mg/L]	Nitrate--N [mg/L]	pH
Mean	29.92	30.49	5.74	0.12	0.10	0.74	8.33
Standard Deviation	0.28	0.80	0.25	0.07	0.07	0.66	0.07
n	97.00	97.00	97.00	5.00	5.00	5.00	4.00
Minimum	29.40	29.20	5.30	0.05	0.03	0.09	8.26
Maximum	30.70	31.80	6.80	0.20	0.22	1.66	8.41
Lower 95% Confidence Level	29.37	28.90	5.24	0.00	0.00	0.00	8.18
Upper 95% Confidence Level	30.48	32.08	6.25	0.26	0.25	2.06	8.48

Table C14. Water quality statistical results for system 8.

Statistic	System 8: Water quality results						
	Temperature [C]	Salinity [ppt]	DO [mg/L]	Ammonia-N [mg/L]	Nitrite-N [mg/L]	Nitrate--N [mg/L]	pH
Mean	30.12	30.47	5.85	0.12	0.09	0.76	8.34
Standard Deviation	0.32	0.78	0.24	0.06	0.05	0.69	0.07
n	100.00	100.00	100.00	5.00	5.00	5.00	4.00
Minimum	29.30	29.20	5.30	0.06	0.03	0.09	8.27
Maximum	30.80	31.80	6.70	0.18	0.16	1.75	8.41
Lower 95% Confidence Level	29.49	28.92	5.37	0.01	-0.02	-0.62	8.21
Upper 95% Confidence Level	30.76	32.03	6.32	0.24	0.19	2.13	8.47

Table C15. Water quality statistical results for system 9.

Statistic	System 9: Water quality results						
	Temperature [C]	Salinity [ppt]	DO [mg/L]	Ammonia-N [mg/L]	Nitrite-N [mg/L]	Nitrate--N [mg/L]	pH
Mean	30.04	30.27	5.62	0.13	0.08	0.73	8.31
Standard Deviation	0.26	0.75	0.25	0.08	0.04	0.65	0.08
n	100.00	100.00	100.00	5.00	5.00	5.00	4.00
Minimum	29.40	29.00	4.90	0.06	0.04	0.07	8.22
Maximum	30.70	31.40	6.30	0.22	0.13	1.60	8.40
Lower 95% Confidence Level	29.52	28.76	5.11	0.00	0.00	0.00	8.14
Upper 95% Confidence Level	30.56	31.78	6.12	0.29	0.15	2.02	8.47

Table C16. Water quality statistical results for system 10.

Statistic	System 10: Water quality results						
	Temperature [C]	Salinity [ppt]	DO [mg/L]	Ammonia-N [mg/L]	Nitrite-N [mg/L]	Nitrate--N [mg/L]	pH
Mean	29.96	30.23	5.77	0.09	0.07	0.80	8.31
Standard Deviation	0.21	0.75	0.21	0.06	0.04	0.74	0.08
n	100.00	100.00	100.00	5.00	5.00	5.00	4.00
Minimum	29.30	29.00	5.20	0.03	0.03	0.08	8.23
Maximum	30.40	31.30	6.30	0.19	0.12	1.87	8.40
Lower 95% Confidence Level	29.54	28.74	5.35	0.00	0.00	0.00	8.16
Upper 95% Confidence Level	30.37	31.73	6.19	0.21	0.15	2.28	8.47

Table C17. Shrimp stocking information: including raw shrimp data at commencement and termination. Weights reported in grams.

Diet	Location			Raw Data				
				Initial: 12/10/07			Final: 1/14/08	
	System	Row	Tank	Number: Survival*	Number: Weight	Total Weight	Number	Total Weight
Control 1	5	front	1	4	4	1.56	4	29.62
Control 1	5	back	1	4	4	1.93	4	35.17
Control 1	6	front	4	4	4	1.96	4	33.88
Control 1	6	back	4	4	4	1.45	2	9.12
Control 1	7	front	7	4	4	1.96	4	32.95
Control 1	7	back	7	3	4	1.74	3	9.14
Control 1	8	front	10	4	4	1.86	4	11.72
Control 1	8	back	10	3	4	2.01	3	14.11
Control 1	9	front	13	3	4	1.66	2	6.38
Control 1	9	back	13	2	4	1.57	2	7.74
Control 1	10	front	16	4	4	1.75	4	32.59
Control 1	10	back	16	4	4	1.86	4	30.19
Control 2	5	front	5	4	4	1.67	4	33.74
Control 2	5	back	5	4	4	1.64	3	7.95
Control 2	6	front	8	4	4	1.62	4	12.34
Control 2	6	back	8	4	4	1.79	4	37.35
Control 2	7	front	11	4	4	1.43	4	29.42
Control 2	7	back	11	3	4	1.85	2	10.68
Control 2	8	front	14	3	4	1.70	3	7.92
Control 2	8	back	14	4	4	1.70	4	9.94
Control 2	9	front	17	3	4	1.97	3	8.30
Control 2	9	back	17	4	4	1.45	3	16.07
Control 2	10	front	2	3	4	1.80	3	10.54
Control 2	10	back	2	4	4	1.96	4	20.93
Diet 1	5	front	2	4	4	1.60	4	33.33
Diet 1	5	back	2	4	4	1.76	4	36.60
Diet 1	6	front	5	4	4	1.69	4	35.26
Diet 1	6	back	5	4	4	1.80	4	37.10
Diet 1	7	front	8	4	4	1.75	4	34.22
Diet 1	7	back	8	4	4	1.95	4	33.68
Diet 1	8	front	11	4	4	1.75	4	30.81
Diet 1	8	back	11	4	4	1.95	4	35.23
Diet 1	9	front	14	4	4	1.55	4	33.02
Diet 1	9	back	14	4	4	1.61	4	32.44
Diet 1	10	front	17	4	4	1.74	4	34.40
Diet 1	10	back	17	4	4	1.72	4	37.60
Diet 2	5	front	3	4	4	1.99	4	33.37
Diet 2	5	back	3	4	4	1.82	4	33.00
Diet 2	6	front	6	4	4	1.62	4	33.52
Diet 2	6	back	6	4	4	1.90	4	35.82
Diet 2	7	front	9	4	4	1.74	4	37.72
Diet 2	7	back	9	4	4	1.85	4	35.32
Diet 2	8	front	12	4	4	1.87	4	36.65
Diet 2	8	back	12	4	4	1.42	4	32.69
Diet 2	9	front	15	4	4	1.66	4	32.00
Diet 2	9	back	15	4	4	1.73	4	36.31
Diet 2	10	front	18	4	4	1.70	4	32.68
Diet 2	10	back	18	4	4	1.89	3	22.01
Diet 3	5	front	4	4	4	1.44	4	32.88
Diet 3	5	back	4	4	4	1.66	4	34.74
Diet 3	6	front	7	4	4	1.76	4	32.04
Diet 3	6	back	7	4	4	1.87	4	36.46
Diet 3	7	front	10	4	4	1.94	4	37.88
Diet 3	7	back	10	4	4	1.67	4	38.51
Diet 3	8	front	13	4	4	1.46	4	32.81
Diet 3	8	back	13	4	4	2.00	4	32.34
Diet 3	9	front	16	3	4	1.47	3	22.99
Diet 3	9	back	16	3	4	1.73	3	23.38
Diet 3	10	front	1	4	4	1.74	4	33.23
Diet 3	10	back	1	4	4	2.04	4	38.12

Table C18. Initial weight of shrimp: data with basic statistics organized by diet.

Initial Weights [g]							
			Statistics				
Diet	Mean Initial Weight per Tank	Natural Log Trans-formation	Average	Standard Error	Standard Deviation	Minimum	Maximum
Control 1	0.39	-0.94	0.44	0.013	0.046	0.36	0.50
	0.48	-0.73					
	0.49	-0.71					
	0.36	-1.01					
	0.49	-0.71					
	0.44	-0.83					
	0.47	-0.77					
	0.50	-0.69					
	0.42	-0.88					
	0.39	-0.94					
	0.44	-0.83					
	0.47	-0.77					
Control 2	0.42	-0.87	0.43	0.012	0.043	0.36	0.49
	0.41	-0.89					
	0.41	-0.90					
	0.45	-0.80					
	0.36	-1.03					
	0.46	-0.77					
	0.43	-0.86					
	0.43	-0.86					
	0.49	-0.71					
	0.36	-1.01					
	0.45	-0.80					
	0.49	-0.71					
Diet 1	0.40	-0.92	0.43	0.009	0.031	0.39	0.49
	0.44	-0.82					
	0.42	-0.86					
	0.45	-0.80					
	0.44	-0.83					
	0.49	-0.72					
	0.44	-0.83					
	0.49	-0.72					
	0.39	-0.95					
	0.40	-0.91					
	0.44	-0.83					
	0.43	-0.84					
Diet 2	0.50	-0.70	0.44	0.011	0.039	0.36	0.50
	0.46	-0.79					
	0.41	-0.90					
	0.48	-0.74					
	0.44	-0.83					
	0.46	-0.77					
	0.47	-0.76					
	0.36	-1.04					
	0.42	-0.88					
	0.43	-0.84					
	0.43	-0.86					
	0.47	-0.75					
Diet 3	0.36	-1.02	0.43	0.015	0.051	0.36	0.51
	0.42	-0.88					
	0.44	-0.82					
	0.47	-0.76					
	0.49	-0.72					
	0.42	-0.87					
	0.37	-1.01					
	0.50	-0.69					
	0.37	-1.00					
	0.43	-0.84					
	0.44	-0.83					
	0.51	-0.67					

Table C19. Survival rate of shrimp at the end of the experiment: data with basic statistics organized by diet.

Survival [%]						
		Statistics				
Diet	Mean Survival per Tank	Average	Standard Error	Standard Deviation	Minimum	Maximum
Control 1	100	93.1	4.8	16.6	50.00	100.00
	100					
	100					
	50					
	100					
	100					
	100					
	100					
	67					
	100					
	100					
	100					
Control 2	100	93.1	3.7	12.7	66.67	100.00
	75					
	100					
	100					
	100					
	67					
	100					
	100					
	100					
	75					
	100					
	100					
Diet 1	100	100.0	0.0	0.0	100.00	100.00
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
Diet 2	100	97.9	2.1	7.2	75.00	100.00
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	75					
Diet 3	100	100.0	0.0	0.0	100.00	100.00
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					
	100					

Table C20. Final weight of shrimp at the end of the experiment: data with basic statistics organized by diet.

Final Weight [g]							
			Statistics				
Diet	Mean Final Weight per Tank	Natural Log Trans-formation	Average	Standard Error	Standard Deviation	Minimum	Maximum
Control 1	7.4	2	5.91	0.69	2.38	2.93	8.79
	8.8	2					
	8.5	2					
	4.6	2					
	8.2	2					
	3.0	1					
	2.9	1					
	4.7	2					
	3.2	1					
	3.9	1					
	8.1	2					
	7.5	2					
Control 2	8.4	2	4.85	0.70	2.42	2.49	9.34
	2.7	1					
	3.1	1					
	9.3	2					
	7.4	2					
	5.3	2					
	2.6	1					
	2.5	1					
	2.8	1					
	5.4	2					
	3.5	1					
	5.2	2					
Diet 1	8.3	2	8.62	0.14	0.50	7.70	9.40
	9.2	2					
	8.8	2					
	9.3	2					
	8.6	2					
	8.4	2					
	7.7	2					
	8.8	2					
	8.3	2					
	8.1	2					
	8.6	2					
	9.4	2					
Diet 2	8.3	2	8.51	0.17	0.59	7.34	9.43
	8.3	2					
	8.4	2					
	9.0	2					
	9.4	2					
	8.8	2					
	9.2	2					
	8.2	2					
	8.0	2					
	9.1	2					
	8.2	2					
	7.3	2					
Diet 3	8.2	2	8.56	0.20	0.70	7.66	9.63
	8.7	2					
	8.0	2					
	9.1	2					
	9.5	2					
	9.6	2					
	8.2	2					
	8.1	2					
	7.7	2					
	7.8	2					
	8.3	2					
	9.5	2					

Table C21. Weight gain of shrimp at the end of the experiment: data with basic statistics organized by diet.

Weight Gain [g]							
			Statistics				
Diet	Mean Weight Gain per Tank	Natural Log Trans-formation	Average	Standard Error	Standard Deviation	Minimum	Maximum
Control 1	7.0	2	5.46	0.68	2.36	2.47	8.31
	8.3	2					
	8.0	2					
	4.2	1					
	7.7	2					
	2.6	1					
	2.5	1					
	4.2	1					
	2.8	1					
	3.5	1					
	7.7	2					
	7.1	2					
Control 2	8.0	2	4.42	0.70	2.43	2.06	8.89
	2.2	1					
	2.7	1					
	8.9	2					
	7.0	2					
	4.9	2					
	2.2	1					
	2.1	1					
	2.3	1					
	5.0	2					
	3.1	1					
	4.7	2					
Diet 1	7.9	2	8.18	0.14	0.49	7.27	8.97
	8.7	2					
	8.4	2					
	8.8	2					
	8.1	2					
	7.9	2					
	7.3	2					
	8.3	2					
	7.9	2					
	7.7	2					
	8.2	2					
	9.0	2					
Diet 2	7.8	2	8.07	0.17	0.59	6.86	9.00
	7.8	2					
	8.0	2					
	8.5	2					
	9.0	2					
	8.4	2					
	8.7	2					
	7.8	2					
	7.6	2					
	8.6	2					
	7.7	2					
	6.9	2					
Diet 3	7.9	2	8.13	0.20	0.68	7.30	9.21
	8.3	2					
	7.6	2					
	8.6	2					
	9.0	2					
	9.2	2					
	7.8	2					
	7.6	2					
	7.3	2					
	7.4	2					
	7.9	2					
	9.0	2					

Table C22. Weight gain per week of shrimp at the end of the experiment: data with basic statistics organized by diet.

Weight Gain per Week [g/wk]							
			Statistics				
Diet	Mean Weight Gain per Week per Tank	Natural Log Trans-formation	Average	Standard Error	Standard Deviation	Minimum	Maximum
Control 1	1.40	0.34	1.09	0.14	0.47	0.49	1.66
	1.66	0.51					
	1.60	0.47					
	0.84	-0.17					
	1.55	0.44					
	0.52	-0.65					
	0.49	-0.71					
	0.84	-0.17					
	0.56	-0.59					
	0.70	-0.36					
	1.54	0.43					
	1.42	0.35					
Control 2	1.60	0.47	0.88	0.14	0.49	0.41	1.78
	0.45	-0.80					
	0.54	-0.62					
	1.78	0.58					
	1.40	0.34					
	0.98	-0.02					
	0.44	-0.81					
	0.41	-0.89					
	0.45	-0.79					
	1.00	0.00					
	0.61	-0.49					
	0.95	-0.05					
Diet 1	1.59	0.46	1.64	0.03	0.10	1.45	1.79
	1.74	0.56					
	1.68	0.52					
	1.77	0.57					
	1.62	0.48					
	1.59	0.46					
	1.45	0.37					
	1.66	0.51					
	1.57	0.45					
	1.54	0.43					
	1.63	0.49					
	1.79	0.58					
Diet 2	1.57	0.45	1.61	0.03	0.12	1.37	1.80
	1.56	0.44					
	1.60	0.47					
	1.70	0.53					
	1.80	0.59					
	1.67	0.51					
	1.74	0.55					
	1.56	0.45					
	1.52	0.42					
	1.73	0.55					
	1.55	0.44					
	1.37	0.32					
Diet 3	1.57	0.45	1.63	0.04	0.14	1.46	1.84
	1.65	0.50					
	1.51	0.41					
	1.73	0.55					
	1.80	0.59					
	1.84	0.61					
	1.57	0.45					
	1.52	0.42					
	1.46	0.38					
	1.47	0.39					
	1.57	0.45					
	1.80	0.59					

Table C23. Specific growth rates of shrimp at the end of the experiment: data with basic statistics organized by diet.

Specific Growth Rates [1/d]						
		Statistics				
Diet	Mean Specific Growth Rates per Tank	Average	Standard Error	Standard Deviation	Minimum	Maximum
Control 1	8.4	7.17	0.34	1.19	5.26	8.41
	8.3					
	8.1					
	7.2					
	8.1					
	5.6					
	5.3					
	6.4					
	5.8					
	6.5					
	8.4					
	8.0					
Control 2	8.6	6.63	0.42	1.47	4.93	8.68
	5.3					
	5.8					
	8.7					
	8.6					
	7.0					
	5.2					
	5.0					
	4.9					
	7.7					
	5.9					
	6.8					
Diet 1	8.7	8.54	0.06	0.22	8.14	8.81
	8.7					
	8.7					
	8.6					
	8.5					
	8.1					
	8.2					
	8.3					
	8.7					
	8.6					
	8.5					
	8.8					
Diet 2	8.1	8.46	0.09	0.31	7.84	8.96
	8.3					
	8.7					
	8.4					
	8.8					
	8.4					
	8.5					
	9.0					
	8.5					
	8.7					
	8.4					
	7.8					
Diet 3	8.9	8.54	0.09	0.31	7.95	8.97
	8.7					
	8.3					
	8.5					
	8.5					
	9.0					
	8.9					
	8.0					
	8.7					
	8.3					
	8.4					
	8.4					

Table C24. Final weights of shrimp organized by system.

		Final Weights [g]						
				Statistics				
System	Diet	Mean Final Weight per Tank	Natural Log Trans-formation	Average	Standard Error	Standard Deviation	Minimum	Maximum
5	Control 1	7.41	2.00	7.61	2.20	1.96	2.65	9.15
	Control 1	8.79	2.17					
	Control 2	8.44	2.13					
	Control 2	2.65	0.97					
	Diet 1	8.33	2.12					
	Diet 1	9.15	2.21					
	Diet 2	8.34	2.12					
	Diet 2	8.25	2.11					
	Diet 3	8.22	2.11					
	Diet 3	8.69	2.16					
6	Control 1	8.47	2.14	7.44	2.15	2.40	3.05	9.34
	Control 1	4.56	1.52					
	Control 2	3.09	1.13					
	Control 2	9.34	2.23					
	Diet 1	8.82	2.18					
	Diet 1	9.28	2.23					
	Diet 2	8.38	2.13					
	Diet 2	8.96	2.19					
	Diet 3	8.01	2.08					
	Diet 3	9.12	2.21					
7	Control 1	8.24	2.11	7.16	2.07	2.49	2.93	9.63
	Control 1	3.05	1.11					
	Control 2	7.36	2.00					
	Control 2	5.34	1.68					
	Diet 1	8.56	2.15					
	Diet 1	8.42	2.13					
	Diet 2	9.43	2.24					
	Diet 2	8.83	2.18					
	Diet 3	9.47	2.25					
	Diet 3	9.63	2.26					
8	Control 1	2.93	1.08	5.83	1.68	2.72	2.49	9.16
	Control 1	4.70	1.55					
	Control 2	2.64	0.97					
	Control 2	2.49	0.91					
	Diet 1	7.70	2.04					
	Diet 1	8.81	2.18					
	Diet 2	9.16	2.22					
	Diet 2	8.17	2.10					
	Diet 3	8.20	2.10					
	Diet 3	8.09	2.09					
9	Control 1	3.19	1.16	6.65	1.92	2.22	2.77	9.08
	Control 1	3.87	1.35					
	Control 2	2.77	1.02					
	Control 2	5.36	1.68					
	Diet 1	8.26	2.11					
	Diet 1	8.11	2.09					
	Diet 2	8.00	2.08					
	Diet 2	9.08	2.21					
	Diet 3	7.66	2.04					
	Diet 3	7.79	2.05					
10	Control 1	8.15	2.10	7.58	2.19	1.87	3.51	9.53
	Control 1	7.55	2.02					
	Control 2	3.51	1.26					
	Control 2	5.23	1.65					
	Diet 1	8.60	2.15					
	Diet 1	9.40	2.24					
	Diet 2	8.17	2.10					
	Diet 2	7.34	1.99					
	Diet 3	8.31	2.12					
	Diet 3	9.53	2.25					

Table C25. Final survival rates of shrimp organized by system.

Survival [%]							
			Statistics				
System	Diet	Mean Survival Rate per Tank	Average	Standard Error	Standard Deviation	Minimum	Maximum
5	Control 1	100.00	94	27	16	50	100
	Control 1	100.00					
	Control 2	100.00					
	Control 2	75.00					
	Diet 1	100.00					
	Diet 1	100.00					
	Diet 2	100.00					
	Diet 2	100.00					
	Diet 3	100.00					
	Diet 3	100.00					
6	Control 1	100.00	96	28	14	50	100
	Control 1	50.00					
	Control 2	100.00					
	Control 2	100.00					
	Diet 1	100.00					
	Diet 1	100.00					
	Diet 2	100.00					
	Diet 2	100.00					
	Diet 3	100.00					
	Diet 3	100.00					
7	Control 1	100.00	97	28	10	67	100
	Control 1	100.00					
	Control 2	100.00					
	Control 2	66.67					
	Diet 1	100.00					
	Diet 1	100.00					
	Diet 2	100.00					
	Diet 2	100.00					
	Diet 3	100.00					
	Diet 3	100.00					
8	Control 1	100.00	97	28	10	67	100
	Control 1	100.00					
	Control 2	100.00					
	Control 2	100.00					
	Diet 1	100.00					
	Diet 1	100.00					
	Diet 2	100.00					
	Diet 2	100.00					
	Diet 3	100.00					
	Diet 3	100.00					
9	Control 1	66.67	95	27	11	67	100
	Control 1	100.00					
	Control 2	100.00					
	Control 2	75.00					
	Diet 1	100.00					
	Diet 1	100.00					
	Diet 2	100.00					
	Diet 2	100.00					
	Diet 3	100.00					
	Diet 3	100.00					
10	Control 1	100.00	98	28	8	75	100
	Control 1	100.00					
	Control 2	100.00					
	Control 2	100.00					
	Diet 1	100.00					
	Diet 1	100.00					
	Diet 2	100.00					
	Diet 2	75.00					
	Diet 3	100.00					
	Diet 3	100.00					

Table C26. Final weights of shrimp organized by row.

Final Weights [g]									
					Statistics				
Row	System	Diet	Mean Final Weight	Natural Log Trans-formation	Average	Standard Error	Standard Deviation	Minimum	Maximum
front	5	Control 1	7.41	2.00	7.76	2.24	1.53	3.09	8.82
front	5	Control 2	8.44	2.13					
front	5	Diet 1	8.33	2.12					
front	5	Diet 2	8.34	2.12					
front	5	Diet 3	8.22	2.11					
front	6	Control 1	8.47	2.14					
front	6	Control 2	3.09	1.13					
front	6	Diet 1	8.82	2.18					
front	6	Diet 2	8.38	2.13					
front	6	Diet 3	8.01	2.08					
front	7	Control 1	8.24	2.11	6.64	1.92	2.85	2.64	9.47
front	7	Control 2	7.36	2.00					
front	7	Diet 1	8.56	2.15					
front	7	Diet 2	9.43	2.24					
front	7	Diet 3	9.47	2.25					
front	8	Control 1	2.93	1.08					
front	8	Control 2	2.64	0.97					
front	8	Diet 1	7.70	2.04					
front	8	Diet 2	9.16	2.22					
front	8	Diet 3	8.20	2.10					
front	9	Control 1	3.19	1.16	6.50	1.88	2.59	2.65	8.79
front	9	Control 2	2.77	1.02					
front	9	Diet 1	8.26	2.11					
front	9	Diet 2	8.00	2.08					
front	9	Diet 3	7.66	2.04					
front	10	Control 1	8.15	2.10					
front	10	Control 2	3.51	1.26					
front	10	Diet 1	8.60	2.15					
front	10	Diet 2	8.17	2.10					
front	10	Diet 3	8.31	2.12					
back	5	Control 1	8.79	2.17	7.26	2.10	2.59	2.65	9.34
back	5	Control 2	2.65	0.97					
back	5	Diet 1	9.15	2.21					
back	5	Diet 2	8.25	2.11					
back	5	Diet 3	8.69	2.16					
back	6	Control 1	4.56	1.52					
back	6	Control 2	9.34	2.23					
back	6	Diet 1	9.28	2.23					
back	6	Diet 2	8.96	2.19					
back	6	Diet 3	9.12	2.21					
back	7	Control 1	3.05	1.11	6.40	1.85	2.53	2.49	9.63
back	7	Control 2	5.34	1.68					
back	7	Diet 1	8.42	2.13					
back	7	Diet 2	8.83	2.18					
back	7	Diet 3	9.63	2.26					
back	8	Control 1	4.70	1.55					
back	8	Control 2	2.49	0.91					
back	8	Diet 1	8.81	2.18					
back	8	Diet 2	8.17	2.10					
back	8	Diet 3	8.09	2.09					
back	9	Control 1	3.87	1.35	7.33	2.11	1.92	3.87	9.53
back	9	Control 2	5.36	1.68					
back	9	Diet 1	8.11	2.09					
back	9	Diet 2	9.08	2.21					
back	9	Diet 3	7.79	2.05					
back	10	Control 1	7.55	2.02					
back	10	Control 2	5.23	1.65					
back	10	Diet 1	9.40	2.24					
back	10	Diet 2	7.34	1.99					
back	10	Diet 3	9.53	2.25					

Table C27. Final survival rates of shrimp organized by system.

Survival [%]								
				Statistics				
Row	System	Diet	Mean Survival Rate per	Average	Standard Error	Standard Deviation	Minimum	Maximum
front	5	Control 1	100	100	29	0	100	100
front	5	Control 2	100					
front	5	Diet 1	100					
front	5	Diet 2	100					
front	5	Diet 3	100					
front	6	Control 1	100					
front	6	Control 2	100					
front	6	Diet 1	100					
front	6	Diet 2	100					
front	6	Diet 3	100					
front	7	Control 1	100	97	28	10	67	100
front	7	Control 2	100					
front	7	Diet 1	100					
front	7	Diet 2	100					
front	7	Diet 3	100					
front	8	Control 1	100					
front	8	Control 2	100					
front	8	Diet 1	100					
front	8	Diet 2	100					
front	8	Diet 3	100					
front	9	Control 1	67	95	27	11	67	100
front	9	Control 2	100					
front	9	Diet 1	100					
front	9	Diet 2	100					
front	9	Diet 3	100					
front	10	Control 1	100					
front	10	Control 2	100					
front	10	Diet 1	100					
front	10	Diet 2	100					
front	10	Diet 3	100					
back	5	Control 1	100	91	26	17	50	100
back	5	Control 2	75					
back	5	Diet 1	100					
back	5	Diet 2	100					
back	5	Diet 3	100					
back	6	Control 1	50					
back	6	Control 2	100					
back	6	Diet 1	100					
back	6	Diet 2	100					
back	6	Diet 3	100					
back	7	Control 1	100	95	27	11	67	100
back	7	Control 2	67					
back	7	Diet 1	100					
back	7	Diet 2	100					
back	7	Diet 3	100					
back	8	Control 1	100					
back	8	Control 2	100					
back	8	Diet 1	100					
back	8	Diet 2	100					
back	8	Diet 3	100					
back	9	Control 1	100	95	27	11	75	100
back	9	Control 2	75					
back	9	Diet 1	100					
back	9	Diet 2	100					
back	9	Diet 3	100					
back	10	Control 1	100					
back	10	Control 2	100					
back	10	Diet 1	100					
back	10	Diet 2	75					
back	10	Diet 3	100					

Table C28. SBR1 kinetic data for various water quality parameters.

Time [min]	VSS	AVG	STDEV	StdERROR	TAN	NO2	NO3	sTOC	sCOD	avg,sCOD	STDEV	StdERROR
0	467	443	20.3	11.7	3.5	1.038	50.8		overrange dilution (201 read > 150 detection)			
	432											
	432											
10	450	448	3.2	1.9	3.2	0.7	48.8		540	546	8.5	6
	450								552			
	444											
23	467	493	30.5	17.6	3.7	0.9	47.6	224	496	498	2.8	2
	526								500			
	486											
38	491	479	10.8	6.2	3.3	0.896	53.2	176	456	458	2.8	2
	470								460			
	478											
54	525	527	13.3	7.7	2.85				436	434	2.8	2
	515								432			
	542											
100	626	627	9.1	5.3	2.075	0.876	50.4	119	316	309	9.9	7
	636								302			
	618											
144	672	640	27.4	15.8	1.525	0.884	58	108	262	256	8.5	6
	627								250			
	622											
188	731	751	19.0	11.0	0.7	0.848	46.4	69	183	183	0.0	0
	768								183			
	755											
217	781	763	23.9	13.8	0.6	0.808	55.2	38	119	117	2.8	2
	773								115			
	736											

Table C29. SBR2 kinetic data for various water quality parameters.

Time [min]	VSS	AVG	STDEV	StdERROR	TAN	NO2	NO3	sTOC	sCOD	avg,sCOD	STDEV	StdERROR
0	466.7	443.3	20.3	11.7	3.5	1.038	50.8		overrange dilution (201 read > 150 detection)			
	431.6											
	431.6											
17	490.9	488.6	49.4	28.5	3.55			231	508	512	5.7	4
	438.1								516			
	536.8											
42	525.5	524.2	27.9	16.1	3.1			211	436	446	14.1	10
	495.7								456			
	551.4											
59	530.4	496.3	41.6	24.0	2.65			193	400	394	8.5	6
	450.0								388			
	508.3											
106	560.0	589.8	25.8	14.9	2.025	0.904	57.2	121	318	316	2.8	2
	605.1								314			
	604.3											
149	636.7	686.0	47.0	27.2	1.475	0.88	41.2	112	266	258	11.3	8
	690.9								250			
	730.4											
195	831.6	807.9	29.0	16.8	0.575	0.832	50.4	75	172	170	2.8	2
	816.7								168			
	775.5											
222	768.6	773.8	5.8	3.3	0.6	0.78	48.8	43	120	120.5	0.7	1
	772.7								121			
	780.0											

Table C30. SBR3 kinetic data for various water quality parameters.

Time [min]	VSS	AVG	STDEV	StdERROR	TAN	NO2	NO3	sTOC	sCOD	avg,sCOD	STDEV	StdERROR
0	495	449.92785	53.4983906	30.8873102	3.5	1.012	52.8	12.8	63	64	1.4	1
	464								65			
	391											
63	453	421.988304	37.621388	21.7207185	3.5				55	59.5	6.4	5
	380								64			
	433											
110	409	446.416546	38.3497373	22.1412312	3.3							
	486											
	444											
222	392	444.038462	47.5836662	27.4724425	3.4	1.064	48.8	8.3	55	54.5	0.7	1
	485								54			
	456											

Table C31. SBR4 kinetic data for various water quality parameters.

Time [min]	VSS	AVG	STDEV	StdERROR	TAN	NO2	NO3	sTOC	sCOD	avg,sCOD	STDEV	StdERROR
0	495	449.92785	53.4983906	30.8873102	3.5	1.012	52.8	n/a	63	64	1.4	1
	464								65			
	391											
66	440	452.960373	17.3929641	10.0418325	3.6				69	59.5	13.4	9.5
	473								50			
	446											
115	416	441.69697	24.0394533	13.8791849	3.5							
	464											
	445											
222	451	457.547592	29.7453228	17.1734701	3.4	1.05	49.1	n/a	58	60.5	3.5	2.5
	432								63			
	490											