An Engineering Analysis of the Stoichiometry of Autotrophic, Heterotrophic Bacterial Control of Ammonia-Nitrogen in Zero-Exchange Marine Shrimp Production Systems

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ABSTRACT

After dissolved oxygen, ammonia-nitrogen buildup from the metabolism of feed is usually the limiting factor to increasing production levels in intensive aquaculture systems. Currently, large fixed-cell bioreactors are the primary strategy used to control inorganic nitrogen in intensive recirculating systems. This option utilizes chemosynthetic autotrophic bacteria, ammonia-oxidizing bacteria (AOB), and nitrite-oxidizing bacteria (NOB). Zero-exchange nitrification management systems have been developed based on heterotrophic bacteria and promoted for the intensive production of marine shrimp and tilapia. In these systems, the heterotrophic bacterial growth is stimulated through the addition

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of an organic labile carbonaceous substrate. At high organic carbon to nitrogen (C/N) feed ratios, heterotrophic bacteria assimilate ammonia-nitrogen directly from the water, replacing the need for an external fixed film biofilter. As a result, build-up of suspended solids may become the second limiting factor after dissolved oxygen. This paper reviews two nitrogen conversion pathways used for the removal of ammonia-nitrogen in aquaculture systems; autotrophic bacterial conversion of ammonia-nitrogen to nitrate nitrogen, and heterotrophic bacterial conversion of ammonia-nitrogen directly to microbial biomass. The first part of this study reviews these two ammonia removal pathways, presents a set of balanced stoichiometric relationships, and discusses their impact on water quality. In addition, microbial growth energetics are used to characterize production of volatile and total suspended solids for autotrophic and heterotrophic systems. A critical verification of this work was that only a small fraction of the feed's carbon content is readily available to the heterotrophic bacteria. For example, feed containing 35% protein (350 g/kg feed) has only 109 g/kg feed of labile carbon. In the paper's second part, the results of a study on the impact C/N ratio on water quality is presented. In this experimental trial sufficient labile organic carbon in the form of sucrose (sugar) was added daily at 0%, 50%, and 100% of the system feeding rate to three prototype zero-exchange systems. The system was stocked with marine shrimp (*Litopenaeus vannamei*) at modest density (150 /m^2) and water quality was measured daily. Significant differences were seen between the three strategies in the key water quality parameters of ammonia-nitrogen, nitrite-nitrogen, nitrate-nitrogen, pH, and alkalinity. The control (0%) system exhibited water quality characteristics of a mixed autotrophic/heterotrophic system while the other two systems receiving supplemental organic carbon (50% and 100%) showed water quality characteristics of pure heterotrophic systems.

INTRODUCTION

The three pathways for the removal of ammonia-nitrogen in traditional aquaculture systems are: photoautotrophic (algae), autotrophic bacterial conversion from ammonia-nitrogen to nitrate nitrogen, and heterotrophic bacterial conversion from ammonia-nitrogen directly to microbial biomass, a more recent management method. Traditionally, pond aquaculture has used photoautotrophic algae-based systems (greenwater systems) to control inorganic nitrogen buildup. In intensive recirculating

aquaculture production systems, large fixed-cell bioreactors are routinely used that rely on the nitrification of ammonia-nitrogen to nitrate-nitrogen by ammonia oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) (Timmons and Ebeling 2007). In intensive recirculating systems, the growth of heterotrophic bacteria and the accumulation of organic carbon and nitrate are minimized intentionally through the rapid removal of solids from the system and through water exchange. It has been demonstrated that for zero-exchange pond production systems, the inorganic nitrogen build-up can be controlled by the manipulation of the organic carbon/nitrogen (C/N) ratio in such a way to promote the growth of heterotrophic bacteria (Avnimelech 1999, 2009). McIntosh (2001) demonstrated that heterotrophic bacteria assimilated the ammonia-nitrogen directly from the water column, producing cellular protein in a marine shrimp pond system. As an additional benefit, for some aquaculture species (marine shrimp and tilapia), this bacterial biomass can be an important source of feed protein, thus reducing the cost of production and improving the overall production economics (McIntosh 1999, Moss 2002).

In the last few years, research has demonstrated that low water exchange marine shrimp production systems can be technically feasible (Ebeling and LaFranchi 1990, Santos and Ebeling 1990). Large-scale pond production systems for marine shrimp have been demonstrated that are zero-exchange and are dominated by photoautotrophic algae (Hopkins *et al*. 1996, Avnimelech *et al*. 1994). Management of these systems has been improved by supplementing the shrimp feed with additional feeding of organic labile carbonaceous substrate to support and enhance microbial metabolism (Avnimelech 1999, 2009; McIntosh 1999). Several attempts have been made to develop technology for recirculating marine shrimp production systems at high densities (Weirich 2002, Otoshi 2003, Davis and Arnold 1998, Van Wyk 1999), although it should be noted that in addition to algae and bacterial biomass each of these also incorporated some form of fixed-film biofiltration.

In reviewing the literature on zero-exchange systems, there was usually no description of the pathways employed for ammonia removal and whether the removal was fundamentally photoautotrophic, autotrophic, or heterotrophic bacterial based, or in reality some mixture of the three. One exception was work done by Brune *et al*. (2003) who examined simplified

microbial growth fundamentals to analyze and compare conventional and heterotrophic techniques to the use of high rate photosynthetic systems. That paper presents a short review of two of these three pathways for the removal of ammonia-nitrogen and the results of a study conducted on the impact of C/N ratio on water quality. In these trials, supplemental carbon beyond that found in the feed in the form of sucrose (sugar) was added daily at 0%, 50%, and 100% of the shrimp feeding rate to three prototype zero-exchange systems. Every attempt was made to minimize photoautotrophic processes by shading the three systems with two layers of shade cloth (blocking 90% of the sunlight) and by high concentrations of total suspended solids (TSS). Although not measured at the time due to a limitation on resources, it was assumed that the role of photoautotrophic bacteria was minor in comparison to the heterotrophic and autotrophic bacteria populations. Thus, only the autotrophic and heterotrophic bacterial pathways were considered in the analysis.

Background: metabolic pathway for 1 kg feed (35% protein)

What follows is a short description of the metabolic pathway options for 1 kg of 35% protein feed and their impacts on water quality parameters. Ebeling *et al*. (2006) developed a set of stoichiometric relationships for the three pathways and discussed their impact on water quality. Based on these relationships, the fate of nitrogen can be determined for aquaculture systems without organic carbon supplementation and with varying degrees of added organic carbon.

Autotrophic/Heterotrophic bacteria – no carbon supplementation

If we examine a simple zero-exchange system with no supplemental organic carbon addition, the solids remain in the production tank and all of the organic carbon from decomposing feed and fecal matter is available to the heterotrophic bacteria (Figure 1). Normally in recirculating systems, uneaten feed and fecal matter containing organic carbon is quickly removed from the production system to prevent growth and build up of heterotrophic bacteria. In recirculating systems, heterotrophic bacteria are detrimental; in zero-exchange systems heterotrophic bacteria can be beneficial. Since the growth rate of heterotrophic bacteria is significantly higher than that of autotrophic bacteria (Table 1) it is assumed that the heterotrophic bacteria will initially dominate the metabolism of ammonia-nitrogen until the organic carbon source

Given: 1 kg of feed @ 35% protein
Ammonia-nitrogen production:

$1\ kg_{feed}$ * [0.35 g protein/g feed * 0.16 g nitrogen/g protein * 0.90 excreted]

$= 50.4\ g\ NH_3\text{-}N$

Heterotrophic System: Organic Carbon from Feed

$1\ kg_{feed}$ * 0.36 kg BOD/kg feed * 0.40 kg VSS_H/ kg BOD =

$= \mathbf{144\ g\ VSS_H}$

$0.124\ g\ N_H/g\ VSS_H$ → **$17.9\ g\ N_{VSS}$**

$0.531\ g\ C_H/g\ VSS_H$ → **$76.5\ g\ C_{VSS}$**

$+ 47.1\ g\ C_{CO2} = 123.6\ g\ C_{labile}$

$108.2\ g\ C_{feed}$ **$15.3\ g\ C_{alk}$**

Excess Ammonia-nitrogen:

$50.4\ g\ NH_3\text{-}N - 17.9\ g\ N_{VSS} = \mathbf{32.5\ g\ N_A}$

Autotrophic System: Inorganic Carbon from Alkalinity

32.5 g N * 0.20 g_{VSS}/ g N

$= \mathbf{6.5g\ VSS_A}$

$0.124\ g\ N_A/g\ VSS_A$ → **$0.80\ g\ N_{VSS}$**

$0.531\ g\ C_A/g\ VSS_A$ → **$3.45\ g\ C_{VSS}$**

$+ 55.8\ g\ C_{alk}$

Figure 1. Zero-exchange system with no carbon supplementation, organic carbon for the heterotrophs from the feed and inorganic carbon for the autotrophs from alkalinity.

becomes the limiting factor. The remaining ammonia-nitrogen not assimilated by the heterotrophic bacteria will then be assimilated by the autotrophic bacteria using alkalinity as an inorganic carbon source.

For this analysis, marine shrimp are being grown. For every kg of feed at 35% protein, approximately 50.4 g of ammonia-nitrogen will be generated (Timmons and Ebeling 2007, Brune *et al*. 2003). This was estimated based on the chemical composition of protein (0.16 g nitrogen per g of protein) and that 90% of the nitrogen is being excreted by the shrimp, (Brune *et al*. 2003) or:

$$1\ kg_{feed} * [0.35\ g\ protein/g\ feed * 0.16\ g\ N/g\ protein * 0.90\ excreted] = 50.4\ g\ NH_3\text{-}N$$

By comparison, for finfish only 60 to 70% of the nitrogen is excreted into the water column. One of the difficulties in this analysis was determining the fraction of the organic carbon that was available to the heterotrophic bacteria. It is straightforward to measure the carbon content of feed (approximately 40 to 50%), but as will be shown later, only a fraction of the organic carbon not metabolized by the shrimp is available to the bacteria. Thus, an estimate was made of the organic carbon utilized by the bacteria by estimating the organic carbon sequestered in the volatile suspended solids (VSS) generated by the bacteria and their known carbon content. It has been shown that the biochemical oxygen demand (BOD) content of typical aquaculture feeds is approximately 60% of the dry weight and approximately 0.30 to 0.36 kg BOD per kg of feed is excreted into the water column (Zhu and Chen 2001, Brune *et al*. 2003). Using a yield fraction of 0.40 kg $VSS_{H\ (Heterotrophic)}$ per kg BOD (Avnimelech 1999, Brune *et al*. 2003) and a $BOD_{excreted}$ content of 0.36 kg per kg feed, suggests that a kg of feed should generate approximately 144 g of VSS_H. Since bacterial biomass contains 53.1% C and 12.3% N based on its stoichiometry (Ebeling *et al*. 2006), this heterotrophic microbial biomass would assimilate approximately 17.9 g nitrogen and 76.5 g of organic carbon. In addition in the research trials conducted by the author, the long-term ratio of VSS to TSS for an autotrophic/heterotrophic system was found to average about 0.72. Thus, approximately 200 g of heterotrophic bacterial TSS_H are produced for every kg of feed fed into a system.

Note that since only 36% of the nitrogen is assimilated into cell mass by the heterotrophic bacteria, the remaining nitrogen (32.5 g N) is thus available to the autotrophic bacterial population. Using a yield fraction of 0.20 g $VSS_{A\ (Autotrophic)}$/g N (Table 1) produces 6.5 g VSS_A from 1 kg of feed. Using the same C/N ratios listed previously yields 0.80 g of nitrogen and 3.45 g of carbon assimilated by the autotrophic microbial biomass from 1 kg of feed @ 35% protein. Thus only 0.80 g of nitrogen is incorporated into the autotrophic bacteria, and the remaining is excreted as nitrate-nitrogen. Using the same ratio of TSS to VSS listed previously, only 9.0 g of TSS_A for every kg of feed is produced by the autotrophic bacteria. Combining the two forms of TSS yields a total of 209 g TSS produced per kg feed. It is interesting to note that only about 1.6% of the available nitrogen is actually contained in the autotrophic microbial biomass and about 36% in the heterotrophic microbial biomass. In addition, the mass of heterotrophic bacteria is more than twenty times the mass of the autotrophic bacteria produced.

It is somewhat more difficult to follow carbon consumption, since the carbon source can be either organic carbon from the feed (heterotrophic) or inorganic carbon from alkalinity (autotrophic). Using the stoichiometric relationships developed in Ebeling *et al*. (2006), the total carbon consumed by the heterotrophic process is 123.5 g C, divided between organic carbon (108.2 g C_{feed}) metabolized directly by the heterotrophic bacteria and the depletion of alkalinity, which provides the source of the remaining inorganic carbon consumed (15.3 g $C_{alkalinity}$). All of the inorganic carbon consumed by the autotrophic bacteria (55.8 g $C_{alkalinity}$) comes from alkalinity. Thus a total of 179.3 g of C per kg of feed is consumed by this pathway. This is divided between organic carbon (108.2 g C_{feed}) and alkalinity carbon (71.1 g $C_{alkalinity}$) or 293 g of alkalinity as $CaCO_3$. Thus, if feed contains on average approximately 40% to 50% carbon, then only about 25% of that organic carbon is available to the heterotrophic bacteria as labile carbon. In addition, 220 g of oxygen are consumed and 363 g of carbon dioxide are produced.

The percent protein content of feed determines the ratio of autotrophic versus heterotrophic removal of ammonia-nitrogen. This is because of the direct relationship between protein content and quantity of ammonia-nitrogen that is generated and that only a fixed quantity of labile carbon is available from the feed. Using the same procedure as outlined previously, the ratio of autotrophic and heterotrophic removal was calculated for a

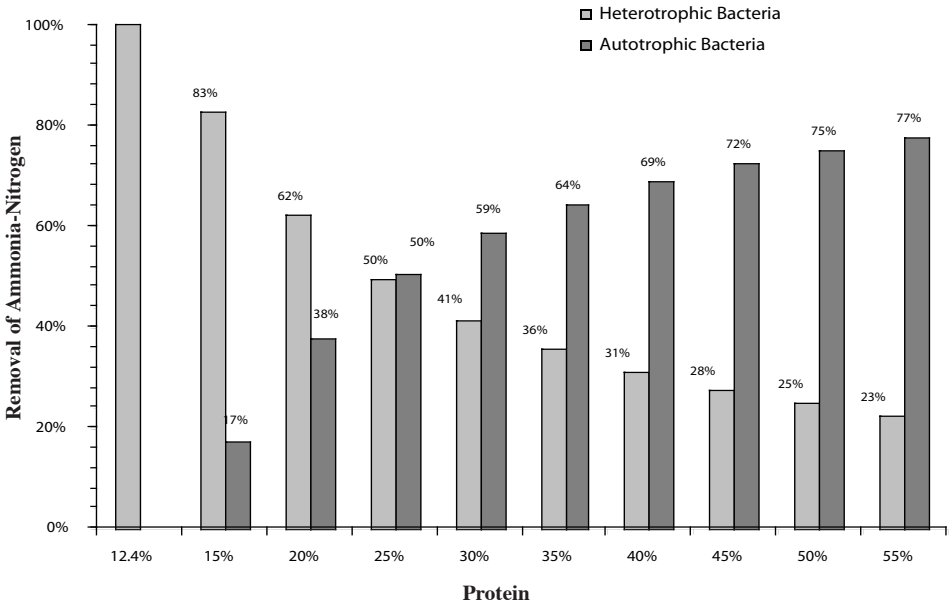

Figure 2. Percent removal of ammonia-nitrogen by heterotrophic or autotrophic processes as a function of % protein.

range of protein content in the feed (Figure 2). This figure shows that as the protein content of the feed increases, the percent removal of ammonia-nitrogen by the autotrophic pathway increases from complete removal by heterotrophic bacteria at 12.4% protein content to 75% removal of ammonia-nitrogen by the autotrophic pathway at 50% protein content.

Heterotrophic bacteria – carbon supplementation

Consider next a zero-exchange system where organic carbon is added to make up the difference between what is available from the feed and the total demand by the heterotrophic bacteria for complete conversion of all available nitrogen (Figure 3). From the above analysis, 32.5 g of nitrogen needs to be consumed by the additional heterotrophic bacteria from the supplemental organic carbon source. From Table 1, 8.07 g VSS_H per g of N are produced, thus an additional 262 g VSS_H are generated by the supplemental carbon. This additional VSS_H requires 225 g of carbon, divided between organic carbon (197 g $C_{S\ (Substrate)}$) metabolized by the heterotrophic bacteria and the depletion of inorganic carbon (28 g $C_{alkalinity}$). Thus the total VSS_H generated is 406 g per kg feed. The research described later in this paper found a TSS to VSS ratio of 81%,

which then suggests a total TSS_H production of 500 g for every kg of feed. Thus a total of 349 g of C per kg of feed is consumed by this pathway, with the heterotrophic bacteria metabolizing all available organic carbon from the feed (109 g C_{feed}) and the supplemental organic carbon (197 g C_S) added to the system. In this case sucrose ($C_{12}H_{22}O_{11}$) at 42% carbon was used requiring 470 g sucrose per kg feed. Concurrently, inorganic carbon as alkalinity was depleted (43.3 g $C_{alkalinity}$) or 180 g of alkalinity as $CaCO_3$. In addition 220 g of oxygen are consumed and 486 g of carbon

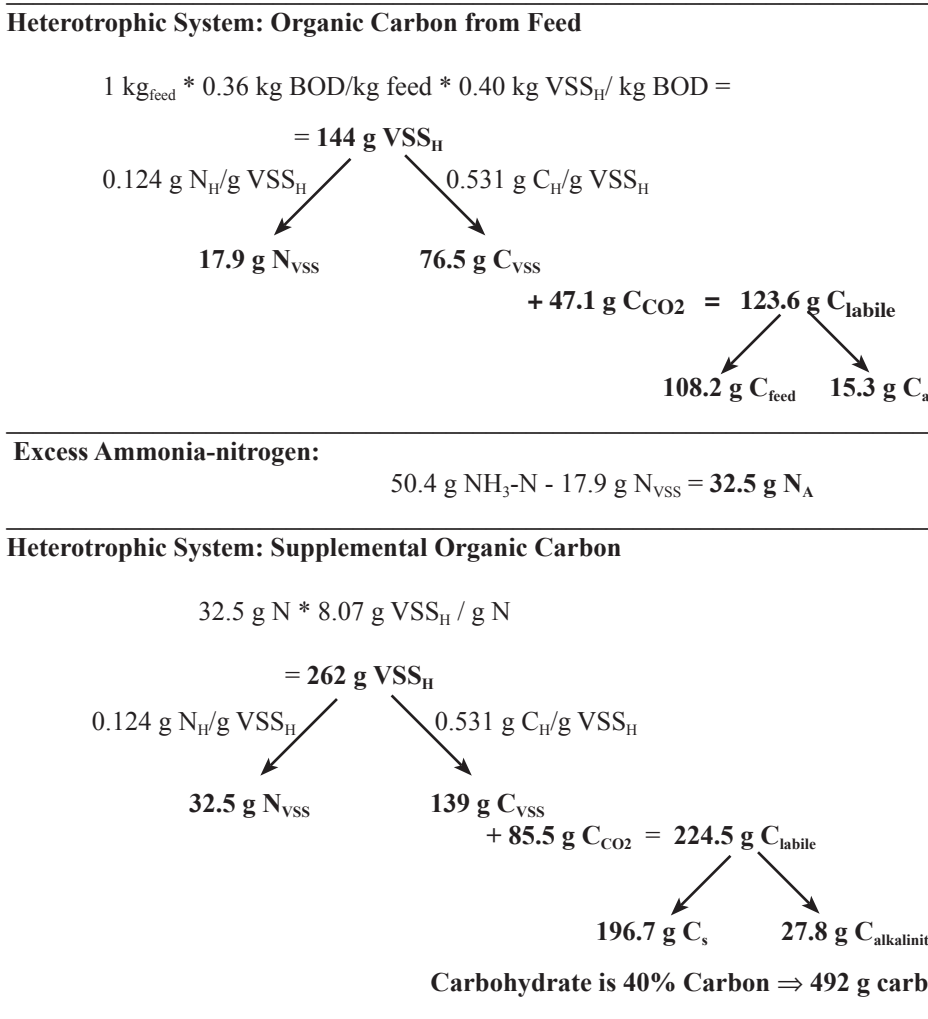

Figure 3. Zero-exchange system with supplemental carbon addition of approximately 50% carbohydrate addition for 35% protein feed yielding a C/N ratio of approximately 13.0.

	Autotrophic	Heterotrophic
C/N Ratio:	4.28 g C/g N	4.28 g C/g N
Yield (Y)		
g VSS/g BOD*: (range)	0.16 g VSS_A / g BOD (0.1 – 0.3)	0.4 g VSS_H / g BOD (0.4 – 0.8)
g VSS/g N:	0.20 g VSS_A /g N	8.07 g VSS_H / g N
g VSS/g C:	0.12 g VSS_A /g C	1.33 g VSS_H / g C_s
g VSS/g sucrose:	----	0.56 g VSS_H / g sucrose
Consumption		
g O_2/g N:	4.18 g O_2 / g N	4.71 g O_2 / g N
g C/g N:	1.69 g C/ g N	6.07 g C_S/ g N
g Alk ($CaCO_3$)/g N:	7.05 g Alk/ g N	3.57 g Alk/ g N
Production		
g CO_2/g N:	5.85 g CO_2/ g N	9.65 g CO_2/ g N
g NO_3-N/g N:	0.976 g NO_3-N/g N	----------
Kinetic Rates*		
μ, specific growth rate (range)	1 day^{-1} (0.4 – 2.0)	5 day^{-1} (2 – 10)
k_d, endogenous respiration (range)	0.05 day^{-1} (0.03 – 0.06)	0.05 day^{-1} (0.025 – 0.075)

C_s is carbon in substrate, i.e. carbohydrates or labile carbon in feed
*Metcalf and Eddy 2003.

Table 1. Comparison of autotrophic and heterotrophic bacterial in terms of production and consumption based on the stoichiometry (modified from Ebeling et al. 2006).

dioxide are produced, while 237 g of oxygen (50.4 g NH_3-N x 4.71 g oxygen per g of nitrogen produced) are consumed and 486 g of carbon dioxide are produced.

MATERIALS AND METHODS

The two pathways for nitrogen removal are very different in terms of substrate utilization, bacterial biomass generated, and by-products produced. The difficulty in practical application is that both may be present to some degree depending upon the availability of inorganic and organic carbon. The ability to control the C/N ratio by feed formulation, solids removal, or addition of organic carbon allows the aquaculture producer to manage what type of system is used. To examine this potential, a study was conducted where supplemental organic carbon in the form of the carbohydrate (sucrose) was added daily at 0%, 50% and 100% of the shrimp feed rate to three prototype zero-exchange systems. These systems had been operated for several months as marine shrimp juvenile production systems and all had well-developed and stable bacterial communities. The three systems were stocked with 675 *Litopenaeus vannamei* marine shrimp at a density of 150/m^2 with an initial average weight of 3.60 g.

Juvenile Production System

The juvenile production system (Figure 4) consisted of rectangular fiberglass tanks, measuring 1.22 m x 3.66 m x 0.76 m (4 ft x 12 ft and 30 in). Water depth was maintained at 61 cm (24 in) with an outside standpipe. Outside standpipes, 5 cm (2 in) in diameter were used to manage water removal and control water depth. A 7.6 cm (3 in) PVC drain line pipe was used to remove water or to harvest shrimp in bulk. In addition, a 1/4 in PVC mesh screen was placed at the discharge from the tanks. Tanks were initially covered with 1/4 in PVC mesh tops, but shade cloth was added within the first week to help reduce stress on the juvenile shrimp and limit growth of photoautotrophic algae.

Two titanium, 1.8 kW, 240 VAC bayonet style heaters were mounted in each tank to maintain system temperature at approximately 30 ± 2ºC. Aeration in the tanks was provided by four 5 x 30 cm (2x12 in) air stones and two 3.66 m (12 ft) lengths of aeration hose on each side of the bottom of each tank. The aeration hose provided good mixing by creating two

counter-rotating cells along the long axis of the tank. Additional air stones were used when needed to maintain dissolved oxygen levels above target levels of 4.0 mg/L. Two automatic vibratory feeders hung above the tanks dispensed feed every 2 hours from 8 am to 10 pm. Fresh water was added as needed to make up for evaporation and other minor losses. A clarifier (Figure 4) was used to harvest suspended solids from the tank when the TSS approached 450 mg/L. Figure 5 shows the weekly average weight of a sample of approximately 50 to 100 animals. Over the first four weeks of

Figure 4. Three juvenile shrimp production tanks showing automatic feeders and solids management clarifier.

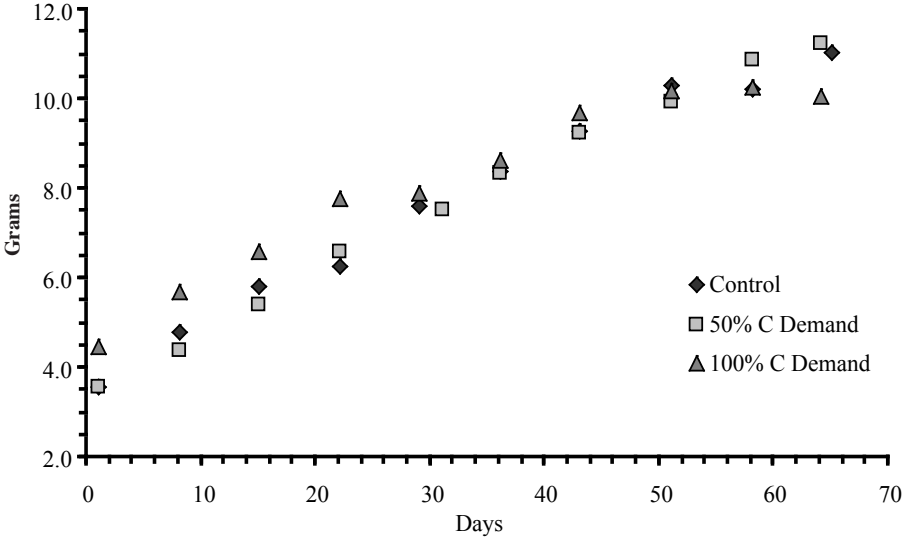

Figure 5. The mean weekly weights of the marine shrimp showing an average growth rate of 0.90 g/week.

growout, survival averaged 90% in the three tanks with an average feed conversion ratio (FCR) of 1.8. During this phase of research, the shrimp were seen primarily as 'food processors' for conversion of the feed to either small organic particles or fecal matter.

Water quality analysis

Dissolved oxygen, temperature, and salinity were measured daily between the hours of 0800 to 0900 h. At the same time, grab samples were taken and filtered through 8 - 12 μm filter paper (506-59 filter paper, Hach Company, Loveland, CO, USA) with the filtrate then used to determine dissolved constituent concentrations, TAN, nitrite-nitrogen, nitrate-nitrogen, pH, and alkalinity. In addition, daily samples were also analyzed for TSS and VSS. Weekly samples were analyzed for total organic carbon and total nitrogen. Standard methods were routinely used and, where appropriate, primary standards were analyzed along with the samples for quality assurance (Table 2).

Parameter	Method / Range
DO / Temperature	Hach Model 58 Dissolved Oxygen Meter
Salinity / Conductivity	Hach Model 33 S-C-T Meter
Nitrogen – Ammonia*	Hach Method 8038 Nessler Method 0 – 2.50 mg/L NH_3-N
Nitrogen –Nitrite*	Hach Method 8507 Diazotization Method 0 – 0.300 mg/L NO_2^--N
Nitrogen -Nitrate	Hach Method 8039 Cadmium Reduction Method 0.0 – 30.0 mg/L NO_3^- - N
Total Organic Carbon	Hach Method 10173 Direct Method 15 to 150 mg/L as C
Total Nitrogen	Hach Method 10071 Persulfate Digestion Method 0 to 25.0 mg/L - N
Alkalinity+	Standard Methods 2320B as $CaCO_3$
Total Suspended Solids	Standard Methods 2540D
Total Volatile Solids	Standard Methods 2540E

*US-EPA approved for reporting, +Adapted from Standard Methods for the Examination of Water and Wastewater (APHA, 1998)

Table 2. Laboratory methods used for analysis via titration and Hach DR/2500 colorimeter.

RESULTS

Water quality

Water quality data for the three treatments over the research period is presented in Table 3. Overall water quality in all three systems was maintained within the range for optimal shrimp growth and survival. Note the substantial difference in nitrate-nitrogen (54.7 versus 7.7 mg/L) and alkalinity (183 versus 328 mg/L) between the control system and the two systems receiving supplemental carbon. Figures 6 through 9 show the impact of the three treatments (control, sucrose at 50% and 100% of feed rate) on TAN, NO_2-N, NO_3-N, and alkalinity over the 10-week research period.

There was a substantial difference in nitrate-nitrogen, alkalinity, and pH for the three treatments. Since the control tank received no supplemental organic carbon, it should exhibit water quality that is a combination of a heterotrophic and autotrophic system. For example, Table 1 shows a lower mean pH for the control versus the two other treatments, which would be expected in an autotrophic system because of the alkalinity reduction due to H^+ production. The impact of the autotrophic bacteria is especially apparent in Figures 8 and 9, with the increase of nitrate-nitrogen and the rapid decline in alkalinity. The alkalinity became so low that sodium bicarbonate was added on day 58 to increase it above the minimum recommended level of 150 mg/L (Timmons and Ebeling 2007). In all three systems, TAN increased slowly over the research trial, but was never higher than 1.5 mg/L –N. For the control, nitrite-nitrogen was typically less than 0.1 mg/L, although it reached a maximum of 0.2 mg/L near the end of the 10 week research period.

Table 3. Average water quality for the three treatments over the study period.

Water parameter	DO (mg/L)	Temp (C°)	Salinity (ppt)	pH	TAN (mg/L)	NO_2-N (mg/L)	NO_3-N (mg/L)	Alkalinity (mg/L)
Control	6.1	29.5	4.8	7.78	1.15	0.13	54.7	183
StDev:	0.4	0.5	0.4	0.20	1.06	0.16	29.0	49
50% of Feed	5.7	29.8	4.5	8.15	1.06	0.39	7.7	328
StDev:	0.9	0.9	0.4	0.14	0.26	1.02	3.3	22
100% of Feed:	5.3	29.4	4.7	8.19	1.36	0.61	1.9	360
StDev:	1.5	0.2	0.2	0.18	0.81	1.13	0.8	24

Both treatments (50% and 100% of feed as carbohydrate) exhibited similar pH values. The pH decreased slightly during the initial start-up phase, then increased and finally remained constant throughout the trial around a pH of 8.3. The direct conversion of ammonia-nitrogen to bacterial biomass in these systems is demonstrated in Figure 8, where the nitrate-nitrogen concentrations are either very low or at barely detectable limits. The limited number of autotrophic bacteria implies that very small quantities of nitrite-nitrogen or nitrate-nitrogen is produced. The higher than expected nitrite-nitrogen concentrations in the 50% of feed as sucrose (Figure 7) might be explained by a limited population of autotrophic bacteria that are inhibited by the high carbon/nitrogen ratios in the system from completing the conversion of TAN to nitrate (Zhu and Chen 2001, Michaud *et al*. 2006). Near the end of the growout, the concentration of nitrite-nitrogen was significantly reduced, although it should be noted that at no time was the concentration high enough to have any significant impact on the marine shrimp juveniles. The fact that the alkalinity (Figure 9) increased and then remained constant during the growout trial is unexplained. Theoretically, alkalinity should be consumed by the heterotrophic bacteria, although at a much lower rate than for an autotrophic system. One explanation might be the recovery

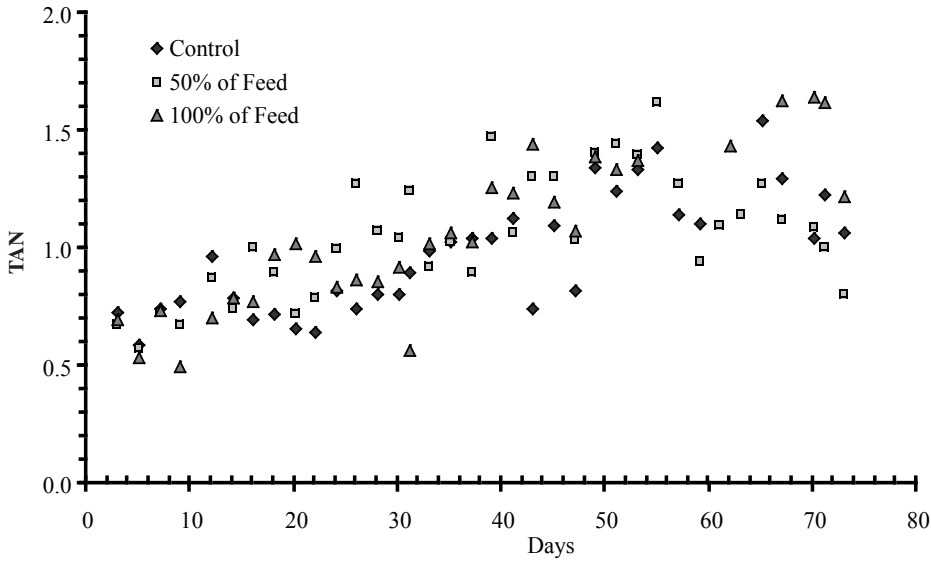

Figure 6. TAN for the three treatments (control, sucrose at 50% and 100% of feed rate) over the 10 week research period.

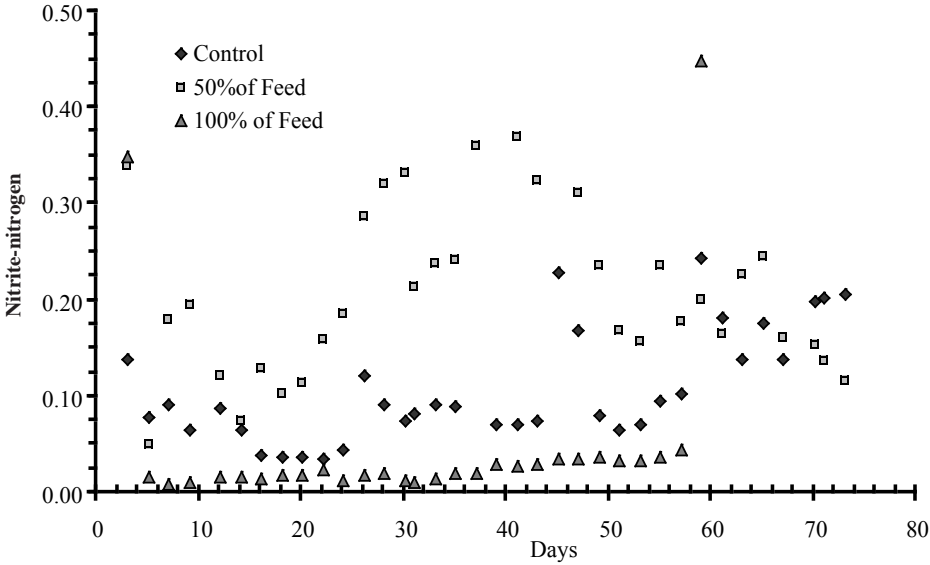

Figure 7. Nitrite-nitrogen for the three treatments (control, sucrose at 50% and 100% of feed rate) over the 10 week research period.

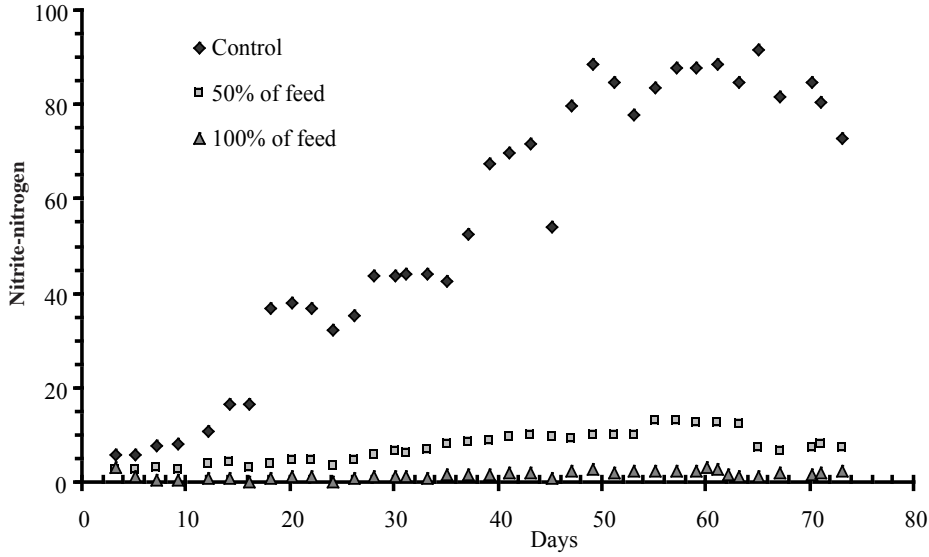

Figure 8. Nitrate-nitrogen for the three treatments (control, sucrose at 50% and 100% of feed rate) over the 10 week research period.

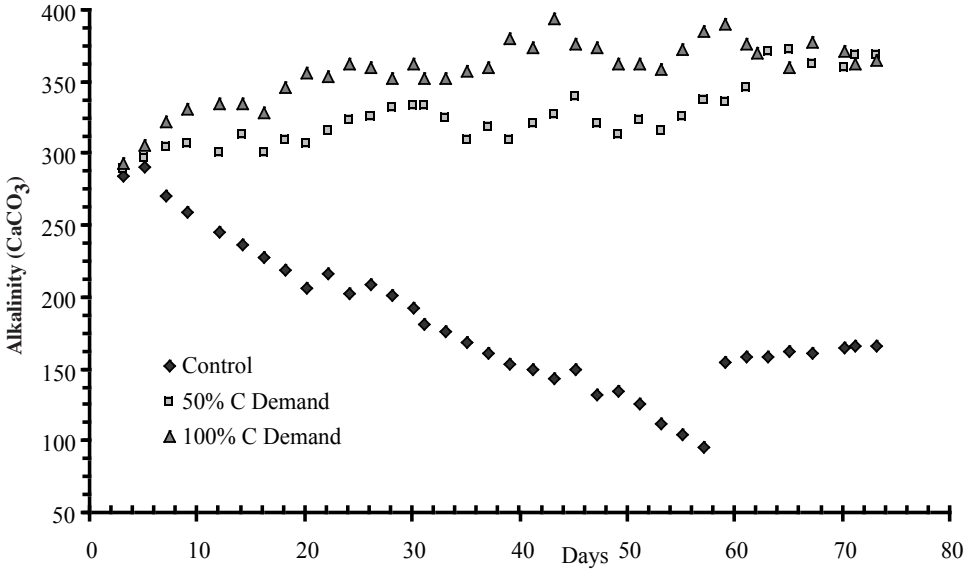

Figure 9. Alkalinity as $CaCO_3$ for the three treatments (control, sucrose at 50% and 100% of feed rate) over the 10 week research period.

of alkalinity during some limited denitrification that may have occurred. Denitrification might be occurring in the interior of the large floc particles, where oxygen would be limited and anoxic conditions would prevail, which would potentially cause denitrification.

Mathematical model

A simple model to predict VSS and TSS concentrations in the three systems was written using an EXCEL® spreadsheet (Microsoft Office, Redmond, WA, USA). The three systems were modeled as a mixed autotrophic/heterotrophic system (control) and as a pure heterotrophic system (50% and 100% of feed as sucrose). As was shown earlier, the amount of sucrose required to fulfill the carbon requirement to consume all of the ammonia-nitrogen produced by the feed is approximately 470 g sucrose / kg feed, or 47% of the feed as sucrose. As a result, the system supplemented with 50% of feed as sucrose should be a pure heterotrophic system, the system supplemented with 100% of feed as sucrose should be overdosed, and the effect on resulting TSS is unknown.

In the case of the control, the model:

- allocated the daily feed organic carbon to heterotrophic bacterial production,
- calculated VSS_H [VSS_H = feed g/m^3 day * 0.36 g BOD/g feed * 0.40 g VSS_H / g BOD],
- calculated amount of ammonia-nitrogen assimilated in the VSS_H [$TAN_H = 0.123 * VSS_H$],
- subtracted TAN_H from the daily TAN_{feed} produced [TAN_{feed} = feed g/m^3 day * (0.35 * 0.16 * 0.9)],
- allocated excess ammonia-nitrogen to autotrophic bacterial consumption [$TAN_A = TAN_{feed} - TAN_H$],
- determined VSS_A [$VSS_A = TAN_A$ * 0.20 g VSS_A/g N],
- calculated total VSS and TSS.

In the case of 50% of feed as sucrose, the model:

- allocated the daily feed carbon to heterotrophic bacterial production,
- calculated VSS_H [VSS_H = feed g/m^3 day * 0.36 g BOD/g feed * 0.40 g VSS_H / g BOD],
- calculated amount of ammonia-nitrogen sequestered in the VSS_H [$TAN_H = 0.123 * VSS_H$],
- subtracted from the daily TAN_{feed} produced [TAN_{feed} = feed g/m^3 day * (0.35 * 0.16 * 0.9)],
- allocated excess ammonia-nitrogen to additional heterotrophic bacterial production [$TAN_{H+} = TAN_{feed} - TAN_H$],
- determined VSS_{H+} [VSS_{H+} = 8.07 g VSS_H/g N * g N],
- calculated total VSS and TSS.

Finally in the case of 100% feed as sucrose, it was observed that significant quantities of TSS were produced in excess of the available nitrogen. Thus the assumption was made that somehow there was sufficient nitrogen in the water column to react with all of the available carbon from the sucrose.

In the case of 100% of feed as sucrose, the model:

- allocated the daily feed carbon to heterotrophic bacterial production,
- calculated VSS_H
  [VSS_H = feed g/m^3 day * 0.36 g BOD/g feed * 0.40 g VSS_H / g BOD],
- assumed all of the sucrose carbon was converted into bacterial biomass [VSS_{H+} = g sucrose/m^3 day * 0.56 g VSS_H/g sucrose],
- calculated total VSS and TSS.

In each case, the TSS values were estimated based on the long term average of the measured ratio of TSS to VSS determined during the course of this research period for the heterotrophic system.

The results of these models are shown in Figures 10 through 12. Figure 10 shows excellent agreement between the model and the actual measured TSS concentrations. The saw-tooth nature of the TSS data reflects the periodic harvesting of bacterial biomass using a cone-bottom clarifier. The model was restarted after each harvest of biomass from the tank using the experimentally determined TSS value for the starting point. The control tank required solids culling approximately every three weeks in order to maintain tank TSS concentrations below 450 mg/L.

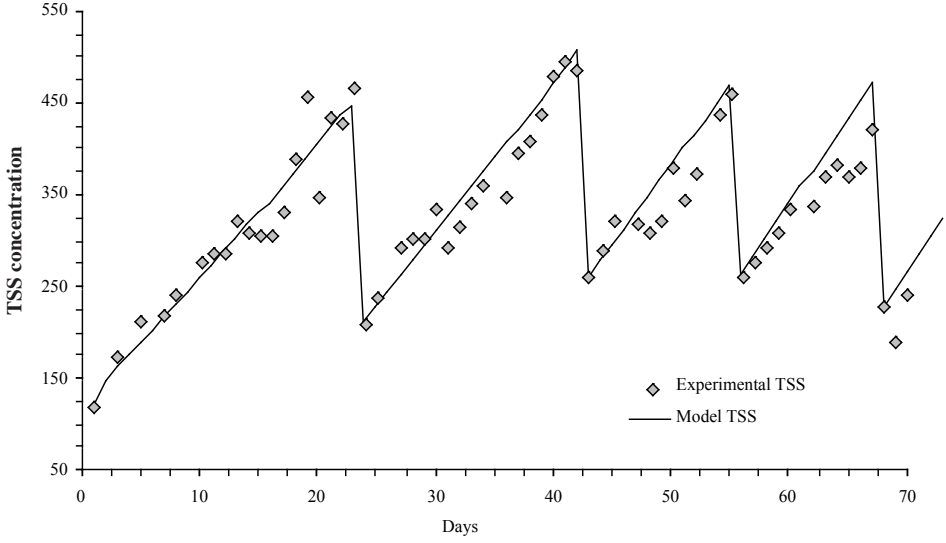

Figure 10. Predicted and measured TSS concentration for an autotrophic / heterotrophic system without carbon supplementation with periodic harvesting of excess bacterial biomass.

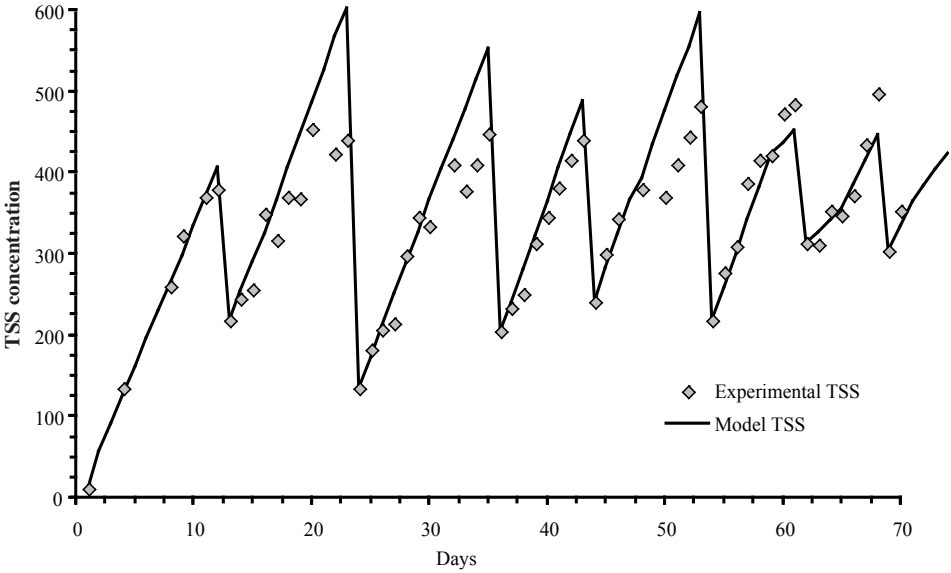

Figure 11. Predicted and measured TSS concentration for a heterotrophic system with carbon supplementation at 50% of feed rate as sucrose and periodic harvesting of excess bacterial biomass.

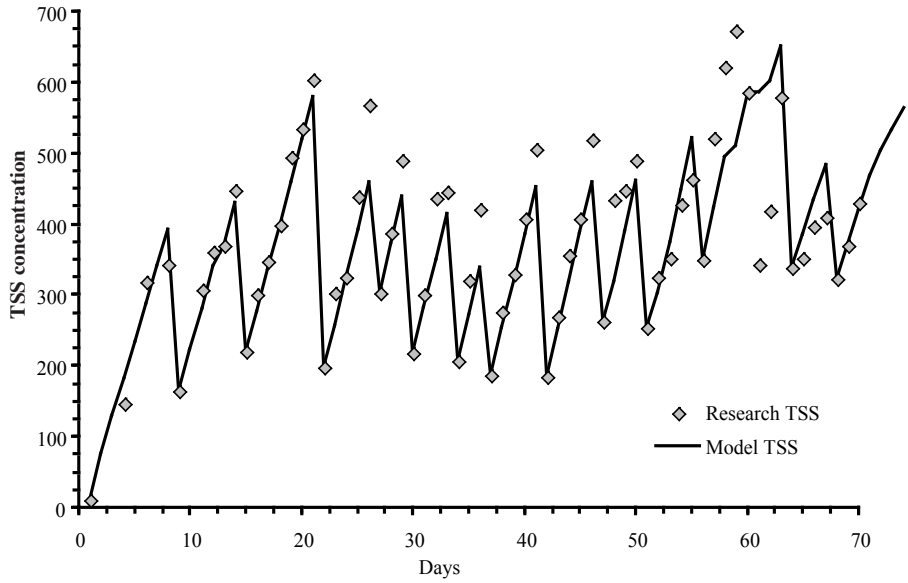

Figure 12. Predicted and measured TSS concentration for a heterotrophic system with excess carbon supplementation at 100% of feed rate as sucrose and periodic harvesting of excess bacterial biomass.

Figure 11 reflects what would occur if sufficient carbon supplementation was available to completely convert all metabolic ammonia-nitrogen to bacterial biomass. The model predictions and the observed data agree quite closely, although in some cycles the model tended to over predict TSS values as a solids harvesting event was about to occur. Due to the rapid production of biomass, the production system was culled of excess bacteria on average every ten days.

The results of excess carbon supplementation (100% of feed as carbohydrate), in this case twice what is stoichiometrically required, is shown in Figure 12. The assumption that there was sufficient nitrogen to react with all of the available carbon from the sucrose appears to be verified in this instance. The source of this nitrogen, which is beyond that provided by the shrimp feed, is unknown. One of the problems with excess carbon supplementation is the large quantity of bacterial biomass that is generated, requiring frequent (every five days) harvesting of excess biomass.

Dissolved organic carbon and total nitrogen

Figure 13 shows the dissolved organic carbon (DOC) concentration in the three treatments over the ten week research trial. As can be seen, there appears to be no major difference in the DOC between treatments and there was a consistent increase in the DOC over the growout period. This is probably the result of the gradual buildup in all the systems of humic substances, the 'tea' color seen in intensive recirculation systems that accumulates when ozone or UV is not used to remove it. Humic substances correspond to the non-biodegradable part of the dissolved organic carbon and are not available as a carbon source to the bacteria. Humic substances are hydrophobic dissolved organic matter produced by the auto-oxidation of polyunsaturated fatty acids released by fish feces, uneaten feed, and the lysis of dead bacteria.

Figure 14 shows the results of a mass balance on nitrogen for the autotrophic/heterotrophic system without carbon supplementation and with periodic harvesting of excess bacterial biomass. The amount of total nitrogen (Total Nitrogen - Model) was calculated using the VSS concentrations predicted by the previously presented model and assuming it contained 12.4% nitrogen based on the stoichiometry of bacterial biomass. Total Nitrogen - Experimental Data represents the

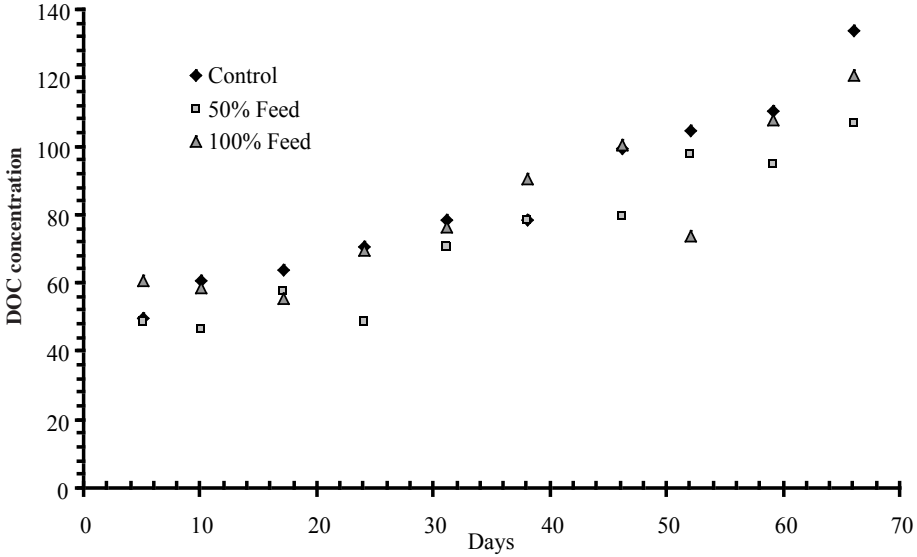

Figure 13. Dissolved organic carbon (DOC) concentrations for the three treatments (control, sucrose at 50% and 100% of feed rate) over the 10 week research period.

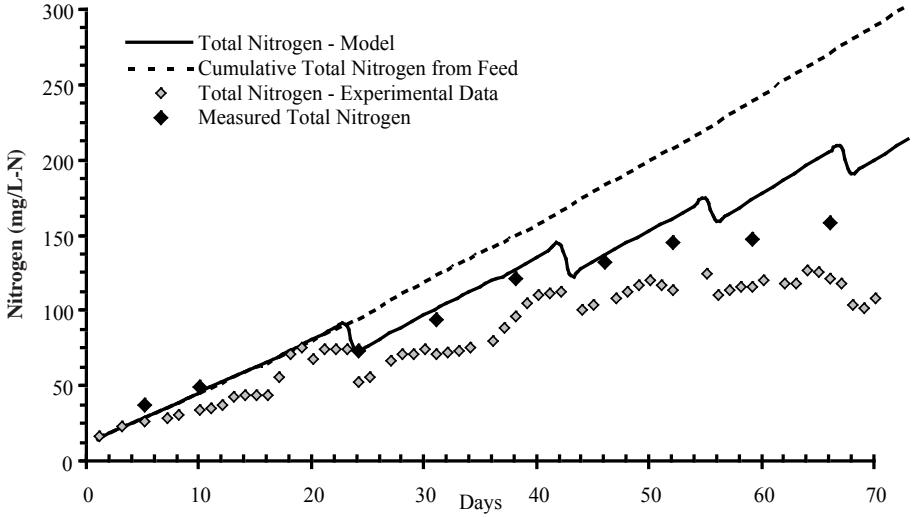

Figure 14. Mass balance on nitrogen for the autotrophic/heterotrophic system without carbon supplementation and with periodic harvesting of excess bacterial biomass.

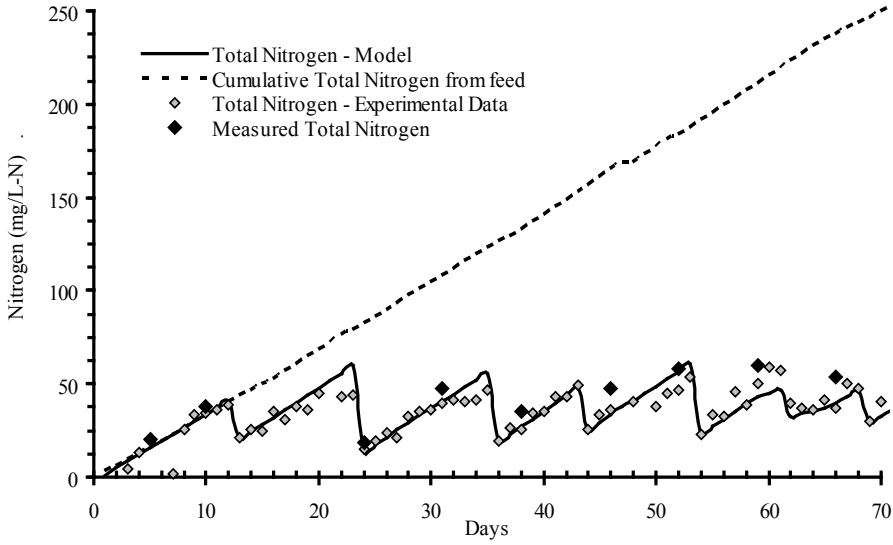

Figure 15. Mass balance on nitrogen for the heterotrophic system with carbon supplementation at 50% of feed rate as sucrose and periodic harvesting of excess bacterial biomass.

sum of the nitrogen contained in the experimentally measured VSS plus experimentally measured concentrations of TAN, NO_2-N, and NO_3-N. The Measured Total Nitrogen is the sum of the nitrogen contained in the experimentally-measured VSS plus the experimentally-measured Total Nitrogen. Finally, the total nitrogen-feed is the estimated nitrogen content of the feed (35% protein), 0.0504k g N/ kg feed.

In Figure 14, the stair step nature of total nitrogen can be seen as bacterial biomass is removed from the system even as the cumulative total nitrogen from the feed steadily increases. The experimentally measured value for total nitrogen falls below the model for several possible reasons including the difficulty in measuring nitrate-nitrogen accurately with the analysis methods employed and the impact of denitrification, especially noticeable near the end of the research period. The use of total nitrogen appears to do a better estimation of the nitrogen and also shows a reduction near the end of the research period, most likely due to denitrification. Interestingly, over the growout period almost all the nitrogen remains in the system.

Figure 15 shows the impact of carbon supplementation at 50% of the feed as sucrose on the system with excess bacterial biomass and nitrogen being periodically removed from the system. Since this is a pure heterotrophic system, there is no nitrate-nitrogen created. Thus the system's total nitrogen remains at very low levels, fluctuating within a very narrow range, even as the cumulative total nitrogen steadily increases. The system supplemented at 100% of feed as sucrose showed similar characteristics, except for a greater rate of increase in nitrogen per harvesting cycle and a need for more frequent culling of biomass.

CONCLUSIONS

The pathways for nitrogen removal are very different in terms of substrate utilization, bacterial biomass generated and by-products generated. Using simple stoichiometry for autotrophic and heterotrophic bacteria, it is possible to characterize and model the two pathways for nitrogen removal. The difficulty in practice is that each bacterial pathway may be present to some degree and the bacterial communities associated with each will compete for the same substrate, possibly resulting in dominance by one group over another. The ability to control the carbon to nitrogen ratio by feed formulation, solids removal, or addition of organic carbon allows the aquaculture producer to manage what type of system is created.

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REFERENCES

APHA **1998**. Standard Methods for the Examination of Water and Wastewater, 18th ed. American Public Health Association, American Water Works Association, Water Pollution and Control Federation: Washington, DC, USA.

Avnimelech, Y. Carbon/Nitrogen Ratio as a Control Element in Aquaculture Systems. *Aquaculture* **1999**, 176:227-235.

Avnimelech, Y. **2004**. Intensive Shrimp and Fish Ponds: Where We Are and Where We are Heading. Proceedings of the Fifth International Conference on Recirculating Aquaculture. Roanoke, VA, USA, 192-200.

Avnimelech, Y. **2009**. Biofloc Technology: A Practical Guide Book. World Aquaculture Society, 182 p.

Avnimelech, Y., Kochva, M., and Diab, S. Development of Controlled Intensive Aquaculture Systems with a Limited Water Exchange and Adjusted C to N Ratio. *Israel Journal of Aquaculture-Bamidgeh* **1994**, 46:119-131.

Brune, D.E., Schwartz, G., Eversole, A.G., Collier, J.A., and Schwedler, T.E. Intensification of Pond Aquaculture and High Rate Photosynthetic Systems. *Aquacultural Engineering* **2003**, 28:65-86.

Davis, D.A. and Arnold, C.R. The Design, Management and Production of a Recirculating Raceway System for the Production of Marine Shrimp. **Aquacultural Engineering 1998**, 17:193-211.

Ebeling, J.M. and LaFranchi, C. Results of Long-Term Weather and Water Quality Monitoring at a Shrimp Pond Research Facility in Hawaii. Presented at the World Aquaculture Society Meeting, June **1990**, Halifax, Canada.

Ebeling, J.M., Timmons, M.B.. Bisogni, J.J., **2006**. Engineering Analysis of the Stoichiometry of Photoautotrophic, Autotrophic, and Heterotrophic Control of Ammonia-Nitrogen in Aquaculture Production Systems. *Aquaculture* 257:346-358.

Hopkins, J.S., Sandifer, P.A., Browdy, C.L., and Holloway, J.D. Comparison of Exchange and No-Exchange Water Management for the Intensive Culture of Marine Shrimp. *Journal of Shellfish Research* **1996**, 13:441-445.

Metcalf and Eddy Inc. **2003**. Wastewater Engineering: Treatment and Reuse. 4th ed., McGraw Hill: New York, NY, USA, 1819 pp.

McIntosh, R.P. Changing Paradigms in Shrimp Farming: I. General Description. *Global Aquaculture Advocate* **1999**, 2(4/5):40-47.

McIntosh, R.P. High Rate Bacterial Systems for Culturing Shrimp. Proceedings from the Aquacultural Engineering Society's 2001 Issues Forum **2001**. Summerfelt, S.T. et al. (Eds.). Aquaculture Engineering Society: Shepherdstown, WV, USA, 117-129.

Michaud, L., Blancheton, J.P., Bruni, V., and Piedrahita, R. Effect of Particle Organic Carbon on Heterotrophic Bacterial Populations and Nitrification Efficiency in Biological Filters. *Aquaculture Engineering* **2006**, 34:224-233.

Moss, S.M. **2002**. Dietary Importance of Microbes and Detritus in Penaeid Shrimp Aquaculture. In Microbial Approaches to Aquatic Nutrition within Environmentally Sound Aquaculture Production Systems. Lee, C.-S. and O'Bryen, P. (Eds.). The World Aquaculture Society: Baton Rouge, LA, USA.

Otoshi, C.A., Arce, S.M., and Moss, S.M. Growth and Reproductive Performance of Broodstock Shrimp Reared in a Biosecure Recirculating Aquaculture System Versus a Flow-Through Pond. *Aquaculture Engineering* **2003**, 29:93-107.

Santos, B.H. and Ebeling, J.M. Pond Growout of Three Species of Penaeid Shrimp in Hawaii. Presented at the World Aquaculture Society Meeting, June **1990**, Halifax, Canada.

Timmons, M.B. and Ebeling, J.M. **2007**. Recirculating Aquaculture. Cayuga Aqua Ventures, LLC: Ithaca, NY, USA, 975 pgs.

Van Wyk, P.M., **1999**. Farming Marine Shrimp in Recirculating Freshwater Systems. Prepared by Harbor Branch Oceanographic Institute and Florida Department of Agriculture and Consumer Services: Contract No. 4520, 220 pgs.

Weirich, C., Browdy, C., Bratvold, D., McAbee, B., and Stokes, A., **2002**. Preliminary Characterization of a Prototype Minimal Exchange Super-Intensive Shrimp Production System. In Proceedings of the 4th International Conference on Recirculating Aquaculture. Virginia Tech: Blacksburg, VA, USA, 255-270.

Zhu, S. and Chen, S. Effects of Organic Carbon on Nitrification Rate in Fixed Film Biofilters. *Aquacultural Engineering* **2001**, 25:1-11.