Effect of Ammonia Nitrogen on Production and Haemolymph of Pacific White Shrimp (*Litopenaeus vannamei*) Cultured in Low Salinity Ponds in Greene County, Alabama

by

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Abstract

Water temperature, pH, and concentration of total ammonia nitrogen were measured on 12 sampling date in summer 2013 in eight shrimp ponds on an inland, low-salinity shrimp farm in Alabama. Shrimp haemolymph samples also were collected for analysis of total ammonia nitrogen concentration, total hemocyte counts, and phagocytosis activity.

The ponds had large inputs of feed (6,100 to 10,377 kg/ha) resulting in large organic nitrogen waste loads. Survival and production were quite variable ranging from 45.5% to 104% and from 3,265 to 6,550 kg/ha, respectively. Total ammonia nitrogen concentration, however, was not particularly great, exceeding 1.0 mg/L only twice. This apparently was the result of good feed management and high rates of aeration that favored ammonia loss to the atmosphere and prevented low dissolved oxygen concentration that can stress nitrifying bacteria. There were no correlations ($P>0.05$) between total ammonia nitrogen concentration or un-ionized ammonia nitrogen concentration and shrimp survival, production, or feed conversion ration. Moreover, a comparison of ammonia concentration in ponds of this study with data from the literature on the effects of un-ionized ammonia on fish and shrimp also suggested that the ammonia

nitrogen concentration measured in the ponds would not likely have adverse impacts on shrimp survival or production.

No relationships between ammonia nitrogen concentrations in pond water and concentration of ammonia in shrimp haemolymph, total hemocyte counts or phagocytosis activity was found.

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Introduction

Low salinity, inland culture of Pacific white shrimp *Litopenaeus vannamei* is a growing endeavor in several countries including the United States (Roy *et al.* 2010). Inland shrimp farms in Alabama are filled with well water from a saline aquifer (0 to 5ppt salinity). This water is naturally low in magnesium and particularly potassium, and supplementation with mineral salts (potassium chloride and potassium magnesium sulfate) is necessary for adequate survival and growth of the shrimp (McNevin *et al.* 2004).

Potassium and magnesium supplementation greatly improves survival and production, but there is still a lot of variation from year to year in survival and production. Studies by Prapaiwong and Boyd (2012a, 2014) did not find correlations between common water quality variables or trace element concentrations and shrimp survival and production.

Generally, ammonia is the most toxic form of nitrogenous waste and the most common toxic waste found in closed culture ponds and other aquaculture environments. Ammonia nitrogen accumulates in ponds from the waste excretion of aquatic animals and decomposition of uneaten feed. There are two forms of ammonia nitrogen; un-ionized ammonia (NH_3) and ionized ammonia or ammonium (NH_4^+) that exist in a temperature and pH-dependent equilibrium. The un-ionized form is more harmful because of its

higher permeability than the ionized form through membranes. The proportion between these two forms of ammonia nitrogen varies, and the proportion of the un-ionized form increases with increasing of pH and temperature, and declines slightly with increasing salinity (Boyd 1990).

There are many reports suggesting that a high concentration of ammonia will disrupt growth and likely impair the health or physiological mechanisms of aquatic animal, leading to stress, death, and production loss in extreme cases (Cheng *et al.* 2003 and Chen and Liu 2004). The accumulation of ammonia nitrogen in ponds is of concern to farmers.

Pacific white shrimp is a well-known aquaculture species. It is cultured primarily in the tropics and subtropics; but because of its euryhaline tolerant characteristics, it can be cultured anywhere that there is salinity above about 2 ppt and water temperature above 25 ^{o}C for at least 5 to 6 months (Roy *et al.* 2010). It is one of the aquaculture species with the most rapidly increasing production, and farm stocks have been improved genetically for rearing in low salinity water. The high economic value and demand for *L. vannamei* in local and global markets provide producers incentive to culture Pacific white shrimp in inland areas. However, low salinity water may stress this species to some extent, because of the low concentration of ions and possible ionic imbalance with respect to marine or estuarine water. Low salinity water also may favor ammonia accumulation in shrimp's haemolymph because there is fewer ions that in seawater to interfere with ammonia diffusion across membranes.

The specific objectives of this study were as follows;

(1) Monitor total ammonia nitrogen [nitrogen in ammonia (NH_3-N) plus nitrogen in ammonium (NH_4-N)] in ponds at a low-salinity shrimp farm.

(2) Evaluate the concentration of ammonia in haemolymph as a practical indicator of shrimp stress under pond culture conditions.

(3) Determine if physio-immunological changes in shrimp haemolymph could be detected in response to total ammonia nitrogen concentration in water.

Literature Review

Ammonia Nitrogen

The main source of ammonia nitrogen in the water of feed-based aquaculture ponds is nitrogenous waste from protein metabolism by aquatic animals and degradation of uneaten feed and feces by microorganisms. Ammonia nitrogen also is introduced to ponds in nitrogen fertilizer such as ammonium sulfate, ammonium phosphate, urea that hydrolyzes into ammonia nitrogen, and in runoff from watershed.

Ammonia (NH_3) and ammonium (NH_4^+) coexist in equilibrium with temperature and pH. This equilibrium often is expressed by the hydrolysis of NH_3:

$$NH_3 + H_2O = NH_4^+ + OH^-; \qquad pK_b \text{ (at } 25\ ^\circ C) = 4.74$$

where pK_b is a negative base-10 logarithm of the base dissociation constant.

The ionized form (NH_4^+) is not appreciably toxic to aquatic organisms, but the gaseous, un-ionized form (NH_3) is potentially toxic. The common analytical procedures for ammonia nitrogen in water measure both NH_3 and NH_4^+ and results typically are reported as total ammonia nitrogen (TAN) in which the concentration is given in terms of the total amount of nitrogen present in NH_3 plus NH_4^+.

Values for pK_a — the acid dissociation constant — of NH_3 can be found in Bates and Pinching (1949) for different temperature between 0 °C and 50 °C. It is often more convenient to work with the expression in which NH_4^+ acts as an acid:

$$NH_4^+ = NH_3 + H^+$$

The pK_a for the reaction above at 25°C may be obtained from the relationship

$$pK_a = 14 - pK_b$$

The ratio of NH_3-N to NH_4-N can be calculated from the expression:

$$\frac{(NH_3)(pH)}{(NH_4^+)} = pK_a$$

$$\frac{(NH_3)}{(NH_4^+)} = \frac{pK_a}{pH}$$

It follows that the ratio of NH_3-N to TAN is:

$$(NH_3\text{-N})/TAN = [(pK_a/pH) \div (1 + pK_a/pH)]$$

Trussell (1972) and Emerson *et al.* (1975) made tables allowing convenient acquisition of the proportion of NH_3-N from TAN concentration, pH, and water temperature. A modification of Trussell's table is presented (Table 1).

Greater salinity decreases the proportion of un-ionized ammonia at a give pH and temperature (Boyd and Tucker 1998), but the effect is not great, e.g., at pH 8 and 25°C, the contribution of NH_3-N to ammonia nitrogen at 0, 5, 10, 15, 20 and 30 ppt salinity are 4.90, 4.93, 4.78, 4.63, 4.48 and 4.20%, respectively. Thus, the data from Table 1 may be used directly for water of 5 ppt salinity or less found in inland shrimp ponds in Alabama.

In low ammonia concentration water, most of the metabolic wastes, especially ammonia, are transported in the blood to the gill where they diffuse directly into the water. An increase in NH_3 concentration in the water decreases the gradient may resulting in an accumulation of NH_3 in the blood and leading to physiological stress or death of aquatic animals (Boyd 2013).

The toxicity of ammonia nitrogen to aquatic animals results almost entirely from NH_3, because ammonium (NH_4^+) is relatively non-toxic (Boyd and Tucker 1998). Thus, ammonia toxicity is highly dependent upon pH, because the NH_3 form becomes more prevalent at higher pH (Armstrong *et al.* 1978). Of course, in pond culture, water pH typically fluctuates daily with lowest values in the early morning hours and highest values in the afternoon. In low alkalinity waters with dense phytoplankton blooms and in high alkalinity waters, pH may be high throughout the day (Boyd and Tucker 1998).

There has been much research on ammonia toxicity to aquaculture species under controlled conditions in the laboratory. Toxicity data have commonly been reported as the lethal concentration of ammonia (as NH_3-N) to 50% of the test organisms (LC50) — the duration of tests have varied, but many were for 96 hr. Typical 96-hr LC50s found in the literature are presented in Table 2 for several aquaculture species (Boyd 2013).

The LC50s for NH_3 typically are less than 1.0 mg/L for coldwater species and from 1.0 to 3.0 mg/L for warmwater species. There is not much difference in the 96-hr LC50 range for freshwater and marine species. Some of the reported variation in LC50s resulted from species differences in susceptibility to ammonia. However, much of the variation was the result of different conditions in the toxicity test — especially water temperature, pH, and salinity.

A study on rainbow trout reported LC50 of 0.32 to 0.66 mg/L at temperature of 10 to 13°C, but at 16 to 19°C, LC50s were 0.89 to 0.93 mg/L (Thurston and Russo 1983). This reveals that NH_3 is more toxic at lower temperature. This is somewhat unusual, because the LC50 of many toxins decrease with increasing water temperature indicating a greater toxicity in warmer water.

The pH is not only important in determining the percentage of ammonia nitrogen in NH_3 form; it also affects toxicity of NH_3. In a study of channel catfish, the LC50 at pH 6.0 was 0.74 mg/L, but at pH 8.8, the LC50 was 1.91 mg/L (Sheehan and Lewis 1986). In rainbow trout, the LC50 increased from 0.13 mg/L at pH 6.5 to 0.66 mg/L at pH 8.9 (Thurston and Russo 1981). Although there is a smaller proportion of NH_3 at lower pH, NH_3 is more toxic at lower pH.

Increasing salinity lessens the toxicity of NH_3. In Pacific white shrimp, the LC 50 increased from 1.2 mg/L at 15 ppt salinity to 1.6 mg/L at 35 ppt salinity (Lin and Chen 2001). Similar results were reported for other species of shrimp and fish.

The effect of dissolved oxygen concentration on NH_3 toxicity is unclear. One study did not find an effect of dissolved oxygen concentration, but another study revealed that NH_3 was more toxic to black tiger prawn at a dissolved oxygen concentration of 2.3 mg/L than at 5.7 mg/L (Allan *et al.* 1990).

In aquaculture, the producer usually is concerned more over sublethal effects of a toxin than about the LC50. A number of studies have revealed that chronic exposure to NH_3 produces physiological changes, causes gill lesions, reduces growth, and increases susceptibility to diseases. A study with channel catfish revealed that growth decreased linearly over the NH_3-N concentration range of 0.048 to 0.989 mg/L; growth reduction was 50% at 0.517 mg/L, and no growth occurred at the highest concentration (Colt and Tchobanoglous 1978). Tilapia growth also was shown to decline progressively at NH_3-N concentration above 0.068 mg/L (El-Shafai *et al.* 2004). A NH_3-N concentration of 0.45 mg/L reduced the growth of each of five species of penaeid shrimp by about 50% (Boyd 2013). Rainbow trout exposed continuously at a NH_3-N concentration up to 0.073 mg/L

did not show reduction in growth, but histopathological lesions were noted at 0.04 mg/L and protozoan infections increased above 0.02 mg/L (Boyd 2013).

Most toxicity studies were conducted at relatively constant concentration of NH_3-N. In culture systems, and especially in ponds, the NH_3-N concentration varies with time of day and water depth (Boyd and Tucker 1998). For example, in a freshwater pond the pH might be 7.4 in the early morning when water temperature is 26°C and 8.8 in the afternoon when the water temperature is 28°C. At an ammonia nitrogen concentration of 1.0 mg/L, the NH_3-N concentration in the morning would be 0.015 mg/L, but in the afternoon the concentration would be 0.306 mg/L — 20 times greater. Nevertheless, daily fluctuations of NH_3-N up to 0.37 mg/L did not effect on fish and crustacean growth (Hargreaves and Kucuk 2001).

Fish that have been exposed earlier to sublethal NH_3 concentrations were less affected by high NH_3 nitrogen concentration than were animals not previously exposed (Redner and Stickney 1979). Ammonia nitrogen concentrations often tends to increase over time in culture systems as biomass and feed input increase (Boyd and Tucker 1998); this may allow the culture species to acclimate to greater ammonia nitrogen concentration.

The safe concentration to aquatic life for long-term exposure of common toxins often is estimated by multiplying 0.1 or 0.05 times the 96-hr LC50 (Romano and Zeng 2013). The factor 0.05 is used most commonly for NH_3-N, safe NH_3-N concentration typically would range from 0.015 to 0.045 mg/L for coldwater species and from 0.05 to 0.15 mg/L for warmwater species (Boyd 2013). Because of the great variation with NH_3-N concentration over time, differences in NH_3-N concentration at different locations

within culture system, daily pH and water temperature variation, and development of ammonia tolerance, the reliability of such a factor is questionable. Therefore, the common practice of monitoring NH_3-N in culture systems – especially in ponds – is of dubious benefit, because it does not seem possible to interpret them meaningfully.

Even if the safe concentration of NH_3-N were know allowing a maximum allowable concentration of ammonia nitrogen to be established for culture systems, there is no sure method for reducing ammonia nitrogen short of using a high rate of water exchange to flush ammonia out of culture units or lowering nutrient (feed) inputs and production. There is no evidence that the common practices of inoculation with nitrifying bacteria or application of zeolite will remove ammonia nitrogen (Boyd and Tucker 1998, Li and Boyd 2014). Probably the best approach to ammonia nitrogen management is to adopt conservative stocking and feeding rates that will minimize NH_3-N input and avoid excessive phytoplankton blooms that cause high pH. Enough aeration should be applied to avoid low dissolved oxygen and encourage oxidation of ammonia nitrogen to nitrate by nitrifying bacteria. Disturbance of surface water by aeration also encourages NH_3 diffusion in to the air (Gross 2004). Pond bottoms should be fallowed between crops and acidic soil limed to encourage oxidation of organic matter between crops to lessen ammonia nitrogen release into the water during crops.

The increasing of ammonia nitrogen concentration in culture ponds will interrupt fish and crustaceans physiological mechanisms resulting in accumulation of ammonia in their haemolymph. One of the physiological indicators of stress is the hemocyte count and degree of phagocytosis that tend to increase in response to a stressor. However, Chen and Liu (2004) reported that the total hemocyte count in shrimp haemolymph was not

increased by elevated ammonia concentrations. Chen and Kou (1993) reported ammonia accumulation in haemolymph happened rapidly; around 4 to 8 hr after *P. monodon* had been introduced to various concentrations of total ammonia nitrogen. So, the concentration of ammonia in the haemolymph was suggested as an indicator of stress in crustacean culture species for practical purposes.

Materials and Methods

Study ponds

Eight ponds were selected from twenty ponds at Greene Prairie Aquafarm located about 3-km north of Forkland on Alabama Highway 43 in southeastern Greene County for use in this study. The selected ponds range from 0.5 to 1.9 ha in water surface area with average depths of 1.19 to 1.77 m. The water supply is a well that penetrates into a saline aquifer (3.7 ppt salinity). Mechanical aeration was used to circulate the water in ponds to prevent thermal stratification and low nighttime dissolved oxygen concentration.

Ponds were stocked with 23-31 postlarvae/m^2 between May 13 - 27, 2013 and shrimp were fed with pelleted feed that contained 35% crude protein. Daily feed input averaged around 1.5 to 2.0% of shrimp body weight per day and was applied twice daily at 0800 and 1600 hr, by a truck-mounted mechanical feeder. The farm manager provided the data on stocking density, mean size, shrimp survival and production.

Routine Water Analysis

Pond waters were collected weekly between 1000 and 1100 hr from June 20 to September 5, 2013. A 500 ml sample of water was collected from a depth of 0.5 m below a pier in each pond. Samples were confined in plastic bottles, placed on ice in an insulated chest, and transported to the E.W. Shell Fisheries Center, Auburn University, AL. The total ammonia nitrogen concentration was measured using the salicylate-

hypochlorite method (Bower and Holm-Hansen 1980). Absorbance was read on a Spectronic® 401 spectrophotometer at 630 nm. The NH_3-N concentration was calculated according to Boyd (1990). The pH was measured with a Orion Star™ pH meter at the farm, and the water temperature of each pond was recorded using HOBO Pendant Temperature Data Loggers that were attached on the tops of cement blocks (20 cm high) placed on the bottom near the sampling piers.

In each pond on August 23, 2013, water temperature and dissolved oxygen concentration were determined at the surface and 10 cm above the bottom at 3-hr intervals for 24 hr using a YSI 556 MPS Multiprobe system (Yellow Springs Instrument Company, Yellow Springs, OH, USA), and pH was measured using Orion Star™ pH meter. The bottom water for pH analysis was collected by a bottom-water sampling bottle described by Boyd and Tucker (1992).

Haemolymph Extraction

After the shrimp had been stocked for 80 days, ten shrimp from each pond were randomly sampled weekly and their haemolymph samples were extracted from the third pereiopod using a 1.0-ml disposable syringe with 25-gauge needle. Two syringes for use in measuring phagocytosis activity were filled with 0.5 ml of M-199 cell culture medium. Three syringes for use in determination of total hemocyte count were filled with 0.5 ml of anticoagulant (100 mM glucose, 30 mM trisodium citrate, and 26 mM citric acid). The five syringes for obtaining haemolymph for ammonia analysis did not require anticoagulant or other additives (R.P. Henry, Auburn University, personal

communication) All extracted samples were transferred into 1.0 ml tubes and kept on ice all the time.

Haemolymph Ammonia Nitrogen

Samples were analyzed within 24 hr after the haemolymph had been extracted. Before analyses, the samples were deproteinized with 80%v/v ethyl alcohol and completely mixed with a vortex mixer. This procedure prevented protein nitrogen from mixing with ammonia nitrogen. The samples were centrifuged at 10,000 x g for 1 min. The top layer of clear solution at the top was taken out for ammonia analysis by phenol-hypochlorite method described by Solórzano (1969).

Total Hemocyte Count

Twenty microliters of extracted haemolymph were placed in a hemocytometer for counting. The number of cells counted was recorded and converted to an estimate of cells per milliliter using the equation below:

$$N = (n)(D)(10,000)$$

where N = number of cells per milliliter; n = numbers of cells counted per mm^2; D = dilution factor.

Phagocyte Activity

The procedure for determination of phagocytosis was adapted from Itami *et al.* (1994) for use in this study, because there was no procedure for preventing hemocytes from dying during transport of the samples. The procedure follows:

1) 200 µl of extracted haemolymph were smeared on a cover glass and allowed to air dry for 20 min.

2) The cover glass was washed three times with shrimp saline for 5 min.

3) a 2 ml aliquot of heat-killed yeast solution was dropped on a cover glass and left to stand for 1 hr.

4) The cover glass was rinsed with shrimp saline five times and let air dry.

5) The cover glass was stained with Giemsa stain dye for 5 min.

6) The cover glass was rinsed with distilled water and let air dry.

7) A permanent slide was made using permount solution.

The slides were observed under a microscope and 200 hemocytes were randomly counted for each cover glass. Cells were categorized into two groups; hemocytes that ate yeast cell and hemocytes that did not eat the yeast cell. The phagocytosis percentage was calculated with the equation below;

$$\% \text{ Phagocytosis} = \frac{\text{Number of phagocytic hemocyte}}{\text{Total number of hemocyte}} \times 100$$

Data Analysis

The relationships between total ammonia, haemolymph ammonia, and total hemocyte count were analyzed by regression using SigmaPlot statistical software version 10.0 (Aspire Software International, Ashburn, VA, USA) and SAS software version 9.3 (SAS Institute Inc. 2011)

Results and Discussion

Production Data and Nitrogen Input

Production results for the eight ponds are summarized (Table 3). Stocking density was quite similar among ponds. But, crop duration ranged from 115 to 148 days and survival varied from 45.5 to 104% — obviously there was an error in counting postlarvae at stocking or in counting harvested shrimp when survival exceeded 100%. The production averaged 5,001 kg/ha, but ranged from 3,265 to 6,550 kg/ha. The variation in production and survival in 2013 was fairly typical of that observed in previous years (C.E. Boyd, School of Fisheries Aquaculture and Aquatic Science, Auburn University). Despite the high variability in survival, the farmer was able to adjust feed input well, and the average feed conversion ratio (FCR) of 1.50 (range = 1.20 to 1.79) was very good.

The feed contained 35% crude protein (5.6% nitrogen). Feed input averaged 7,363 kg/ha (6,110 to 10,377 kg/ha); thus, nitrogen input was high ranging from 328 to 579 kg/ha with an average of 412 kg/ha. Pacific white shrimp have nitrogen content of 2.86% (live weight basis) (Boyd and Teichert-Coddington 1995). The shrimp biomass at harvest in the ponds contained from 93.3 to 187 kg/ha of nitrogen (average = 143 kg/ha). The nitrogen load to ponds from feeds (feed nitrogen – shrimp nitrogen) ranged from 235to 392 kg/ha (average = 269 kg/ha). Based on the average nitrogen load, an average pond depth of 1.5 m, and assuming that all of the nitrogen not removed in shrimp biomass at harvest became ammonia nitrogen, the load of total ammonia nitrogen to ponds would

represent a concentration of 17.9 mg/L. Of course, ammonia nitrogen is lost to the air by diffusion and it is oxidized to nitrate by nitrifying bacteria (Boyd and Tucker 1998), and such a high concentration would not be expected near the end of the crop.

Water quality

The total ammonia nitrogen concentrations for the eight ponds between June 17 and September 9, 2013 are plotted (Fig. 1). The total ammonia concentration varied considerably among ponds on all dates. The greatest variation occurred on June 20 and August 15; on these dates, the total ammonia nitrogen concentrations varied from 0.05 to 1.54 mg/L and from 0.05 to 0.86 mg/L, respectively. Mean concentrations (±standard errors) were 0.340±0.175 mg/L and 0.383±0.123 mg/L, respectively, on those dates. The average total ammonia nitrogen concentration for individual ponds ranged from 0.07 to 0.65 mg/L for the 12 sampling dates.

The 96 total ammonia nitrogen measurements were plotted as a histogram (Fig. 2). The distribution was highly skewed to the left — towards lower total ammonia nitrogen concentration. More than 70% of the samples fell within the concentration range of 0.0 to 0.2 mg/L. Only two samples exceeded 1.0 mg/L, and only 16 samples had concentrations above 0.5 mg/L. The total ammonia nitrogen data are presented for each date as box plots (Fig. 3) in order to provide additional information on the variation among ponds.

There was no correlation between average total ammonia nitrogen concentration in ponds and shrimp survival ($r = 0.26649$; $P>0.05$), production ($r = 0.27752$; $P>0.05$), and FCR ($r = -0.36849$; $P>0.05$). Thus, average total ammonia nitrogen concentration did

not seem to be a cause of the variation in survival production, and FCR observed among the ponds.

At the time of sampling to obtain water for total ammonia nitrogen analyses, water temperature ranged from 28.06 to 32.81 °C (average = 30.00±0.12 °C) and pH varied from 7.4 to 9.5 (average = 8.28±0.04) in pond bottom water. These determinations were made during summer, and water temperature is lower in spring, when shrimp are stocked and in fall when they are harvested (Prapaiwong and Boyd 2012b). The results of the measurements of pH, temperature and dissolved oxygen in the eight ponds over a 24-hr period in late August are presented in Figs. 4-6.

During 24-hr sampling period, water temperature ranged from 30 to 37 °C and water temperatures were somewhat higher at the surface than at the bottom in the afternoon (1200-2100 hr) despite mixing by the aerators. The pH values ranged from 7.5 to 9.6 during the 24-hr period with the lowest values occurring in the morning and the highest values occurring between 1200 and 2100 hr in surface water. Only in pond S1 and S3 did pH of bottom water exceed 9.0. The observation of lower pH in bottom water is important to note because shrimp spend most of their time on or near the bottom.

The dissolved oxygen concentration tended to be above saturation (8.0 to 8.5 mg/L) in the surface water between 1200 and 2100 hr. However, because of mechanical aeration in the ponds, the dissolved oxygen concentration was usually above 4 mg/L at night even in bottom water. Thus, if results for the single August sampling date are representative of other days, it is unlikely that shrimp were stressed by low dissolved oxygen concentration. Of course, the combination of low dissolved oxygen concentration and high NH_3-N concentration no doubt would be more harmful to shrimp than either

high NH_3-N concentration or low dissolved oxygen concentration alone (Boyd and Tucker 1998).

The temperature, pH, and total ammonia nitrogen concentration for each pond and sampling date were used to estimate NH_3-N concentration (Fig. 7). Like the total ammonia nitrogen concentrations, NH_3-N concentrations varied greatly. However, maximum concentrations usually were below 0.10 mg/L, and the greatest concentration was only 0.16 mg/L. Also like total ammonia nitrogen concentration, average NH_3-N concentration for each sampling date did not tend to increase as the growing season progressed. This was surprising, because feed input (and nitrogen input) increased with increasing shrimp biomass as the growing season progressed. Also, greater feed input favored greater phytoplankton productivity, and more phytoplankton photosynthesis would favor higher afternoon pH. The fact that no season trend in total ammonia nitrogen and NH_3-N concentration were observed attests to aerated ponds having a great capacity to assimilate nitrogenous waters.

Despite the large input of feed, the water of the selected ponds contained relatively low concentrations of total ammonia nitrogen – most were below 0.2 mg/L, two were above 1 mg/L, and only 16 exceeded 0.5 mg/L. A concentration of 1.0 mg/L or less usually is considered ideal in fish culture ponds (Boyd and Tucker 1998). Prapaiwong and Boyd (2012a) also found that the ponds at Greene Prairie Aquafarm had low concentrations of total ammonia nitrogen. They attributed the low concentration of total ammonia nitrogen to ammonia diffusion to the air because of splashing by aeration and high rate of nitrification. Nevertheless, Prapaiwong and Boyd (2012a) suggested that

when pH was high, the ponds might have high enough NH_3-N concentration to stress shrimp.

In laboratory toxicity experiments, the LC50 at 48 hr for ammonia nitrogen and ammonia (NH_3-N) of young white shrimp *L. schmitti* exposed at pH 8.0 in 5 ppt salinity water was 32.63 and 0.89 mg/L, respectively (Barbieri, 2010). Toxicity of total ammonia to penaeid shrimp increased as pH increased and the salinity decreased (Chen and Sheu, 1989 and Noor-Hamid *et al.*, 1994). Chen *et al.*, (1990) suggested ambient ammonia concentration should be below 4.26 mg/L for adolescent *P. monodon.* However, Boyd and Tucker (1998) suggested the safe concentration for continuous exposure of aquaculture species to NH_3-N is probably 0.05 times the 48-hr LC50. Based on LC50 tests, Schuler *et al.* (2010) suggested that *L. vannamei* should not be exposed to more than 0.10 mg/L of NH_3-N for long periods.

The United States Environmental Protection Agency (1999, 2009) developed criterion chronic concentration (CCC) that represent the maximum allowable total ammonia nitrogen concentration for freshwater fish at different pHs and temperatures. The toxicity of ammonia to most species of warmwater fish and shrimp are similar (Boyd and Tucker 1998), and the CCC concept would seem applicable to shrimp. At the temperature of the shrimp ponds in this study, the CCC values would be about 2.0 mg/L at pH 7.5, 1.0 mg/L at pH 8.0, 0.4 mg/L at pH 8.5, and 0.2 mg/L at pH 9.0.

Sometimes during present study total ammonia nitrogen concentrations in ponds were greater than the criterion chronic concentration (Figs. 8 and 9). Growth rate data provided by the farm manager revealed that growth of shrimp in the eight ponds of this study tended to decline at times when the total ammonia nitrogen concentration increased

above the criterion chronic concentration. Shrimp may adjust to prolonged growth at normal rates, but being exposed to high ammonia seems to adversely affect growth.

The NH_3-N concentration also occasionally exceeded the concentration of 0.1 mg/L recommended as the maximum safe concentration for long-term exposure for *L. vannamei* (Schuler *et al.* 2010). However, these limits are for continuous exposure to the ammonia concentration recommended. In ponds, the pH fluctuates daily – between about 7.5 - 8.0 to 8.5 - 9.5 in the ponds of the present study. Because of the fluctuation in NH_3-N concentration, shrimp would not be exposed to the highest concentration observed in this study for more than a few hours each day. Moreover, the total ammonia concentration varied over time, and the highest concentration in a pond usually did not persist for more than one or two sampling dates.

Hargreaves and Kucuk (2001) conducted a study in which channel catfish, *Ictalurus punctatus,* were exposed to a constant total ammonia concentration, but the pH was allowed to fluctuate from 7 to 9.5 according to the pattern typically observed in ponds. Under these conditions, daily exposure for 30 days to a daily maximum NH_3-N concentration of 0.91 mg/L did not affect survival or growth of channel catfish as compared to the control that contained a very low total ammonia nitrogen concentration. Channel catfish have a recommended maximum NH_3-N concentration based on 0.05 times LC50 of 0.113 mg/L (Hargreaves and Kucuk 2001) – similar to the value of 0.1 mg/L for *L. vannamei.* Assuming that the response of *L. vannamei* to daily fluctuating NH_3-N concentration also is similar to that of channel catfish, shrimp in ponds of the present study should not have been adversely affected by the observed concentrations of NH_3-N.

Relationship of Total Ammonia Nitrogen to Haemolymph Ammonia Nitrogen and Total Hemocyte Count

Shrimp haemolymph contained more ammonia than was measured in the pond water, and haemolymph ammonia tend to increase as the growing season progressed (Fig. 10, Table 4). However, there was no correlation between total ammonia nitrogen concentration in water and ammonia concentration in haemolymph or in total hemocyte count (Figs. 11 and 12).

Generally, crustaceans lose their metabolic ammonia via diffusion across the gill epithelium, moreover, crustaceans can counter balance ammonia against the gradient of total ammonia in ambient water, but at high ambient concentration, ammonia accumulates in haemolymph (Mangum *et al.,* 1976, Chen and Cheng 1993 and Chen and Kou 1993). The normal level of haemolymph ammonia varies with species and metabolic rate at the ambient environment conditions. Mangum *et al.* (1976) and Armstrong *et al.* (1978) reported concentrations of ammonia in the haemolymph of crustaceans ranged from 2 to 18 mg/L.

Generally, the concentration of ammonia in aquatic organism's blood is much higher than in ambient water. This present study, the haemolymph of shrimp in most samples presented high ammonia concentration compared to ambient water. Haemolymph ammonia was about 60 times greater on average than total ammonia nitrogen in ambient water.

In this study, the variation of haemolymph ammonia nitrogen in shrimp was quite high (Fig. 11; Table 4). This high degree of variation might be associated with the feeding activities of the individuals. The highest haemolymph ammonia may have been

from the shrimp that had been eating soon before sampling, while lower haemolymph ammonia concentration may have been from shrimp that just start eating or were not eating. However, shrimp were collected a few hours after they were fed in the morning and at least 90% of the shrimp randomly selected for haemolymph extraction had full guts at the time of sampling. Nevertheless, there was no correlation between total ammonia nitrogen in ambient water with the haemolymph ammonia concentration. Mugnier and Justou (2004) reported that exposure of shrimp to high ammonia concentration increased the variability of many immunological parameters.

Chen and Lin (1992) reported that excretion of ammonia by *P. monodon* was reduced when the concentration of ambient total ammonia nitrogen exceeded 10 mg/L, and Chen and Cheng (1993) reported negative ammonia excretion when *P. monodon* was exposed to ambient ammonia concentration more than 4.5 mg/L. The excretion of ammonia from shrimp in the present study should not have been negatively affected, because the ambient ammonia concentrations were low.

Low salinity water and high temperature tended to increase ammonia excretion by *P. japonicus* (Chen and Lai 1993, Chen and lee 2003). Greene Prairie Aquafarm used low salinity water and the pH and water temperature also tended to steadily increase during the culture period. These conditions may have accelerated ammonia excretion by the shrimp.

The number of hemocytes is suggested as an immunological indicator for stress in crustaceans (Mugnier *et al.* 2008). Even though ammonia can decrease the efficiency of immune response in shrimp, this study found no trend in total hemocytes count with respect to total ammonia nitrogen concentration. However, total hemocyte count tended

to increases, as the shrimp grew larger. The study showed no correlation between total ammonia nitrogen and total hemocyte count under the prevailing culture conditions (Fig. 12; Table 5). There is some research to support the finding of no significant difference of total hemocyte count from shrimp exposed to different levels of ammonia (Chen and Liu 2004, Cheng and Chen 2002) and other environmental stress (Johansson *et al.* 2000). Although previous studies and the present study found no correlation between haemolymph ammonia to total hemocyte counts, some research has shown that increasing ammonia concentration can decrease total hemocyte count (Le Moullac and Haffner 2000).

Phagocyte Activity

The experiment was done at the farm site to evaluate efficiency of the procedure from Itami *et al.* (1994) as a practical tool for shrimp health assessment. The culture medium used to preserve the cells could not keep the cells available until they arrived at the laboratory. The procedure described for this method can provide only a small amount of slide area that shows a clear picture under microscope (Fig. 13). However, the usable slide area showed hemocyte phagocytes of the yeast cells. The amount of yeast cells was high and covered most of the usable slide area making it difficult to determine whether or not the hemocytes actually were phagocytes yeast cells. Also, the yeast cells used in this study seem to have a similar size to the hemocytes. The lack of centrifugation of samples made the slides look blurred and cloudy, and it was difficult to count the hemocytes. Contamination with small particulates matter also was normally found in the slides. A

portable centrifuge would help to isolate only hemocyte from the medium used for hemocyte extraction.

Conclusions

Ponds for this investigation had large inputs of feed and high shrimp production. However, the analyses suggested that total ammonia nitrogen concentrations in low salinity ponds for culture of *L. vannamei* were not great enough to be expected to have negative impacts on shrimp survival and production, but have some negative impact on shrimp growth.

The conclusions above is supported by studies of shrimp haemolymph. The concentration of ammonia in haemolymph has been suggested in the literature an index to detect ammonia stress in shrimp. In the eight ponds studied here, no relationship between haemolymph ammonia concentration, total hemocyte count or phagocytosis activity related to total ammonia nitrogen or NH3-N concentration in ponds were found.

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Table 1. Decimal fractions of ammonia nitrogen existing as un-ionized ammonia (NH_3-N) at various pH values and water temperatures.

	Temperature (°C)								
pH	16	18	20	22	24	26	28	30	32
7.2	0.004	0.005	0.006	0.007	0.008	0.009	0.011	0.012	0.015
7.6	0.011	0.013	0.015	0.017	0.020	0.023	0.027	0.031	0.036
8.0	0.028	0.033	0.038	0.043	0.049	0.057	0.065	0.075	0.087
8.4	0.069	0.079	0.090	0.103	0.117	0.132	0.149	0.169	0.194
8.8	0.157	0.178	0.200	0.223	0.248	0.276	0.306	0.339	0.377
9.2	0.319	0.352	0.386	0.420	0.454	0.489	0.526	0.563	0.603

Table 2. Examples found in literature for 96-hr LC50s for NH_3-N to selected aquaculture species.

Common name	96-hr LC50 (mg/L)	Common name	96-hr LC50 (mg/L)
Freshwater		Marine	
Channel catfish	0.74-3.1	Stripe bass	0.64-1.1
Tilapia	2.88	Spotted sea trout	1.72
Fathead minnows	0.32-0.93	Southern white shrimp	0.69-1.20
Freshwater prawn	0.50-0.80	Pacific white shrimp	1.20-2.95
Rain bow trout	0.20-3.4	Black tiger prawn	1.04-1.69
Cutthroat trout	2.0-2.5	School prawns	1.39

Table 3. Pond information and data on stocking and harvest in eight ponds at Greene Prairie Aquafarm, Alabama in 2013.

Pond	Area (ha)	Stocking density (No. m^{-2})	Crop Duration (days)	Feed applied (Kg/ha)	Total Production (Kg/ha)	Mean size (g)	Survival (%)	FCR
N1	0.5	26.01	134	6,325	3852	29.4	50.4	1.64
N2	0.6	25.34	127	5,867	3961	34.4	45.5	1.48
N5	1.3	24.82	140	8,353	6550	25.5	103.6	1.28
N9	1.6	23.14	125	5,850	3265	27.5	51.4	1.79
S1	1.2	25.42	115	6,110	4037	23.4	67.9	1.51
S3	1.0	30.40	148	10,337	5886	23.7	81.8	1.76
S4	1.1	25.91	147	8,908	6518	24.2	104.0	1.37
S8	1.9	25.14	132	7,151	5936	25.4	93.1	1.20
Mean±SE	1.2±0.17	25.77±0.73	134±4	7,363±589	5,001±476	26.69±1.31	74.71±8.57	1.50 ±0.08

Table 4. Means and standard error for haemolymph ammonia concentration of shrimp in eight ponds at Greene Prairie Aquafarm, Alabama.

	Ammonia concentration in shrimp haemolymph (mg/L)							
Pond	7/18/2013	7/25/2013	8/1/2013	8/8/2013	8/15/2013	8/22/2013	8/29/2013	9/5/2013
N1	2.72±0.34	3.4±0.34	0.714±0.33	2.93±0.39	7.09±0.29	6.20±0.52	5.46±0.76	7.44±0.80
N2	0.94±0.12	4.13±0.97	1.393±0.00	3.21±0.23	2.23±0.70	5.69±0.51	5.48±0.39	5.35±1.02
N5	0.79±0.05	4.08±0.45	1.24±0.17	5.23±0.65	3.16±0.08	5.90±0.64	4.15±0.80	7.59±0.80
N9	0.64±0.56	4.85±0.27	0.11±0.02	3.64±0.36	3.82±0.25	4.95±0.54	6.07±0.13	6.30±0.72
S1	1.59±0.54	4.86±1.05	1.34±0.15	4.76±0.60	3.28±0.44	6.00±0.73	6.15±0.94	5.01±1.34
S3	2.05±0.52	4.20±0.61	1.39±0.53	4.89±1.17	2.26±0.56	6.08±0.34	6.98±1.85	4.14±1.37
S4	5.92±2.55	5.35±0.67	2.27±0.08	6.77±1.4	4.37±0.20	7.32±0.52	4.65±0.81	6.02±0.52
S8	1.92±0.94	4.24±0.38	1.06±0.14	4.21±0.39	3.79±0.31	7.31±0.20	5.14±0.39	5.70±0.44
Mean size (g)	11.13+1.18	13.11+1.30	15.41+1.30	17.60+1.42	19.26+1.36	21.29+1.55	22.71+1.68	24.25+1.76

Table 5. Means and standard error for total hemocyte count of shrimp in eight ponds at Greene Prairie Aquafarm, Alabama.

	Total hemocyte count (10^6 cells/ mL)							
Pond	7/18/2013	7/25/2013	8/1/2013	8/8/2013	8/15/2013	8/22/2013	8/29/2013	9/5/2013
N1	0.35±0.12	0.37±0.08	0.46±0.24	0.22±0.09	3.20±0.74	2.28±1.19	3.21±1.34	4.84±0.80
N2	0.08±0.03	0.66±0.19	0.83±0.59	1.09±0.78	3.13±0.62	2.30±0.46	3.27±0.66	2.76±1.57
N5	0.06±0.01	0.42±0.17	0.18±0.05	4.05±1.97	3.53±1.52	4.96±0.93	2.68±0.83	3.08±0.48
N9	0.17±0.08	1.64±1.23	2.30±1.33	2.50±1.03	2.65±1.05	4.47±1.32	3.72±0.83	4.26±0.39
S1	0.23±0.07	0.46±0.18	1.12±0.36	0.61±0.31	3.73±0.91	2.59±0.57	2.53±0.68	1.84±0.31
S3	0.24±0.08	2.52±0.91	1.06±0.69	1.00±0.43	0.96±0.22	1.91±0.70	2.06±0.50	3.46±0.94
S4	0.46±0.22	0.71±0.04	2.05±0.18	1.90±1.46	1.31±0.62	2.22±0.75	2.21±0.38	1.65±0.81
S8	0.05±0.12	0.34±0.1	2.58±1.30	0.64±0.36	0.88±0.24	1.15±0.38	1.02±0.21	3.24±0.94
Mean size (g)	11.13±1.18	13.11±1.30	15.41±1.30	17.60±1.42	19.26±1.36	21.29±1.55	22.71±1.68	24.25±1.76

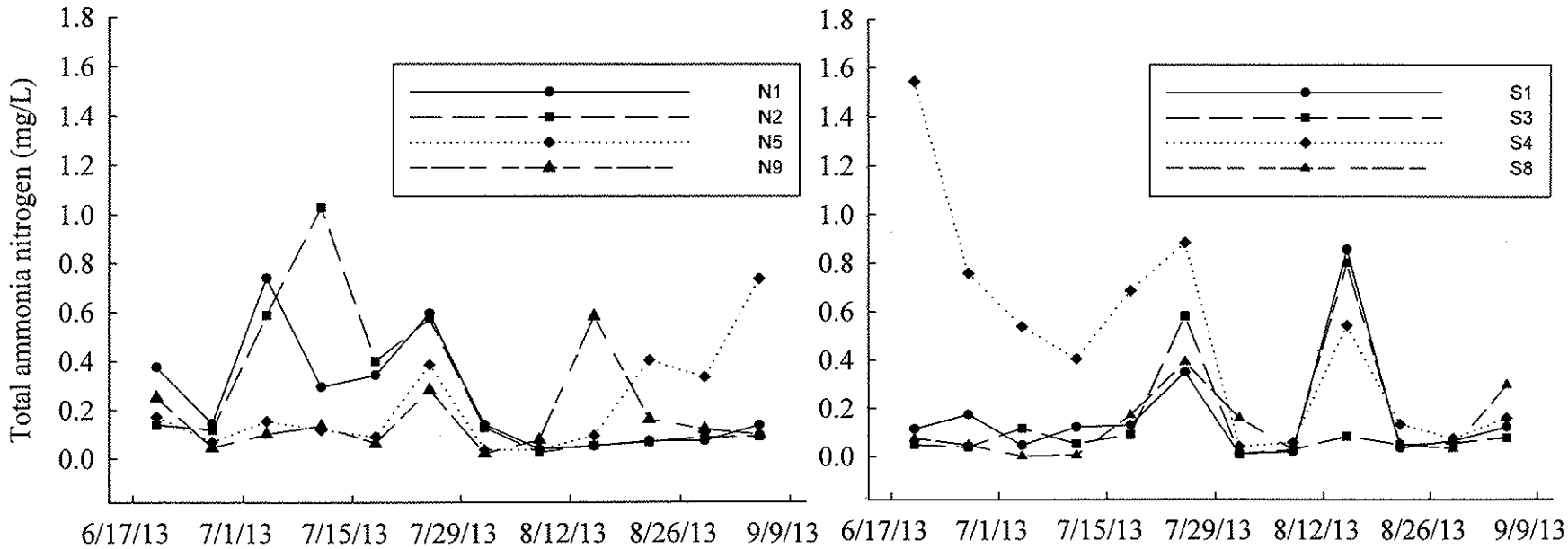

Figure 1. Concentration of total ammonia nitrogen in each of eight ponds from June 6 to September 5, 2013 at Greene Prarie Aquafarm, Alabama.

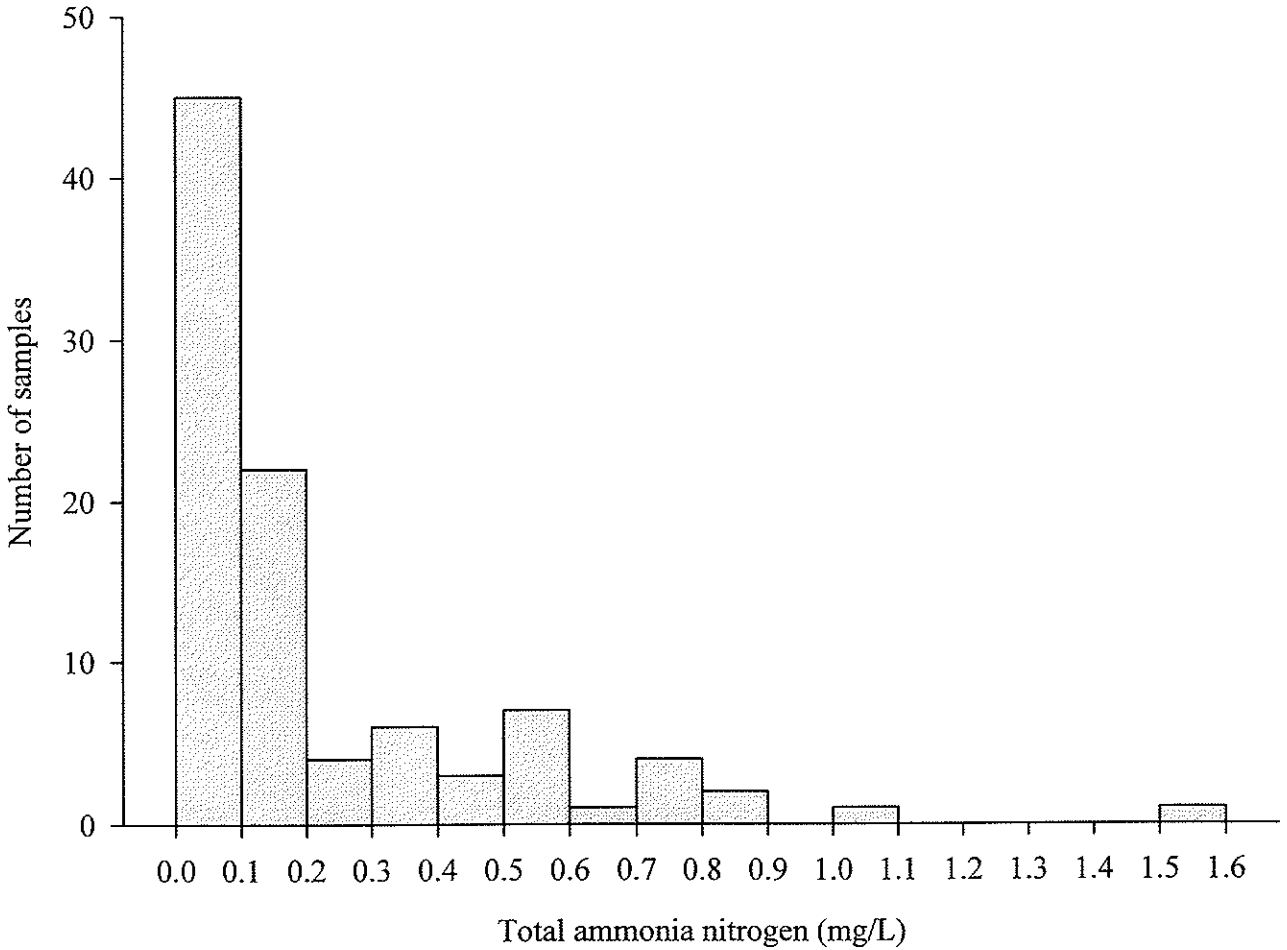

Figure 2. Distribution of total ammonia nitrogen concentration in eight ponds at 1-wk intervals at Greene Prairie Aquafarm, Alabama.

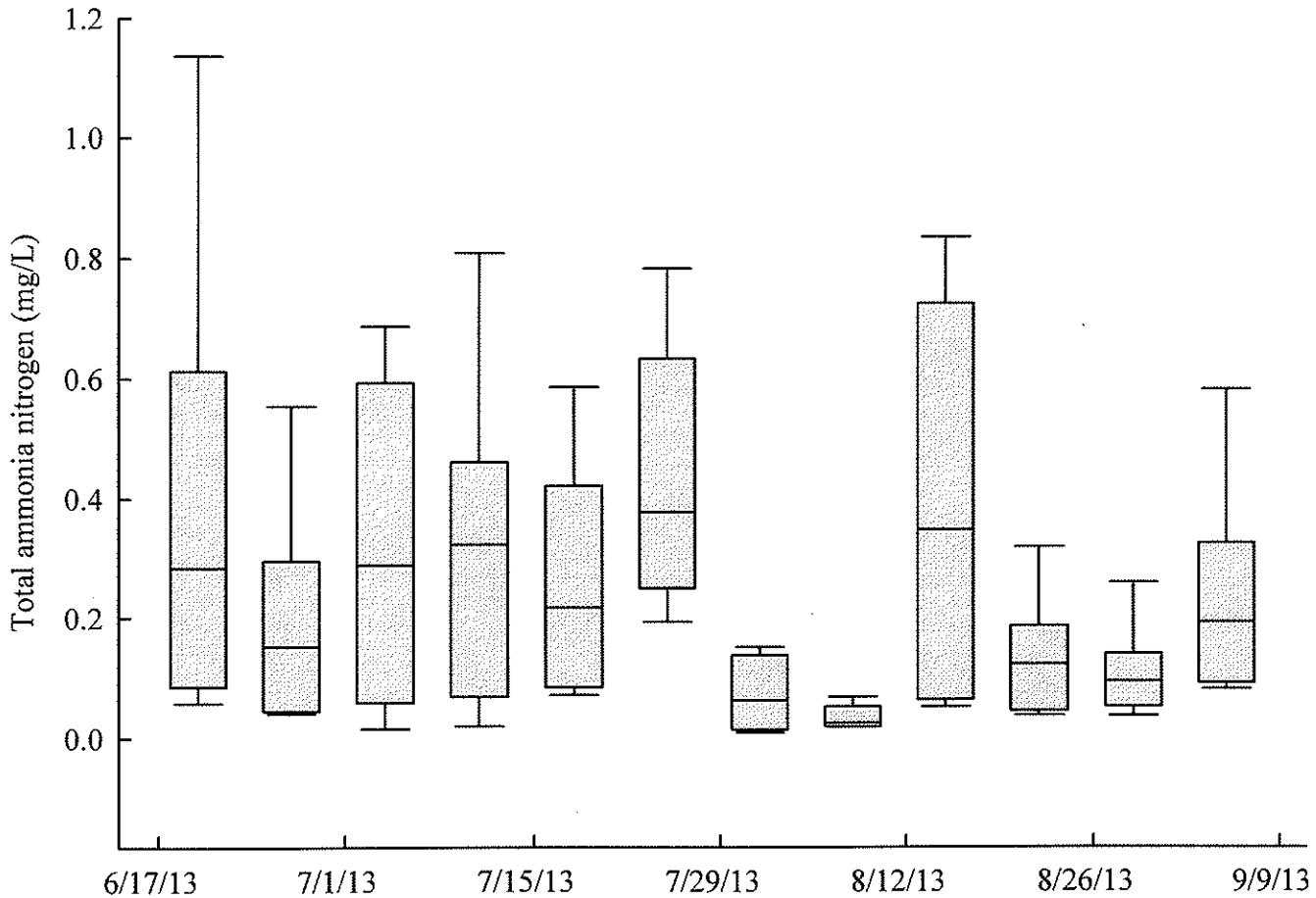

Figure 3. Variation of total ammonia nitrogen (mg/L) on 12 sampling dates (June 20 – September 5, 2013) at Greene Prairie Aquafarm. Each box represents the range of total ammonia nitrogen from eight ponds.

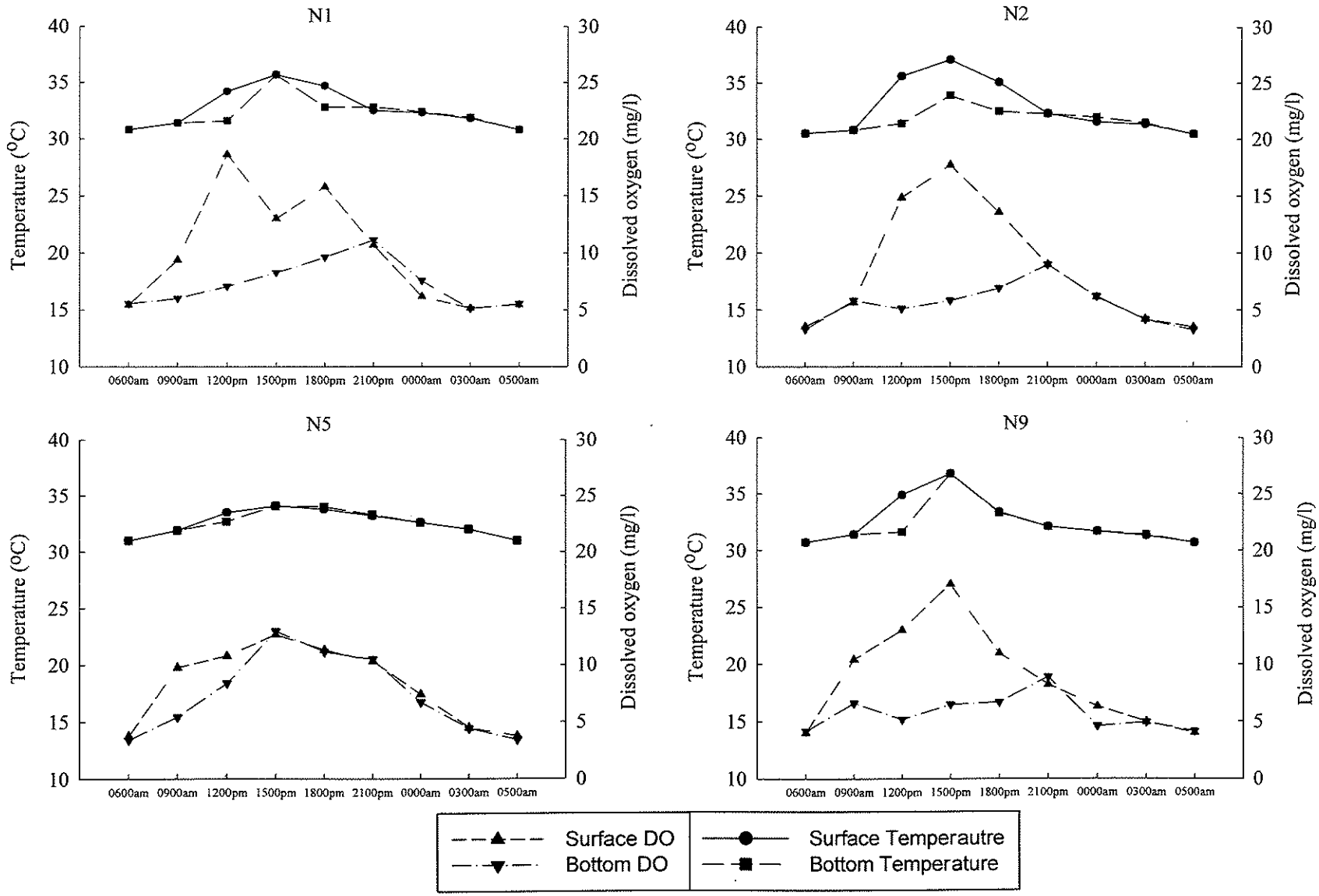

Figure 4. Daily variation for temperature and dissolved oxygen concentration for pond N1, N2, N5 and N9 at Greene Prairie Aquafarm, Alabama.

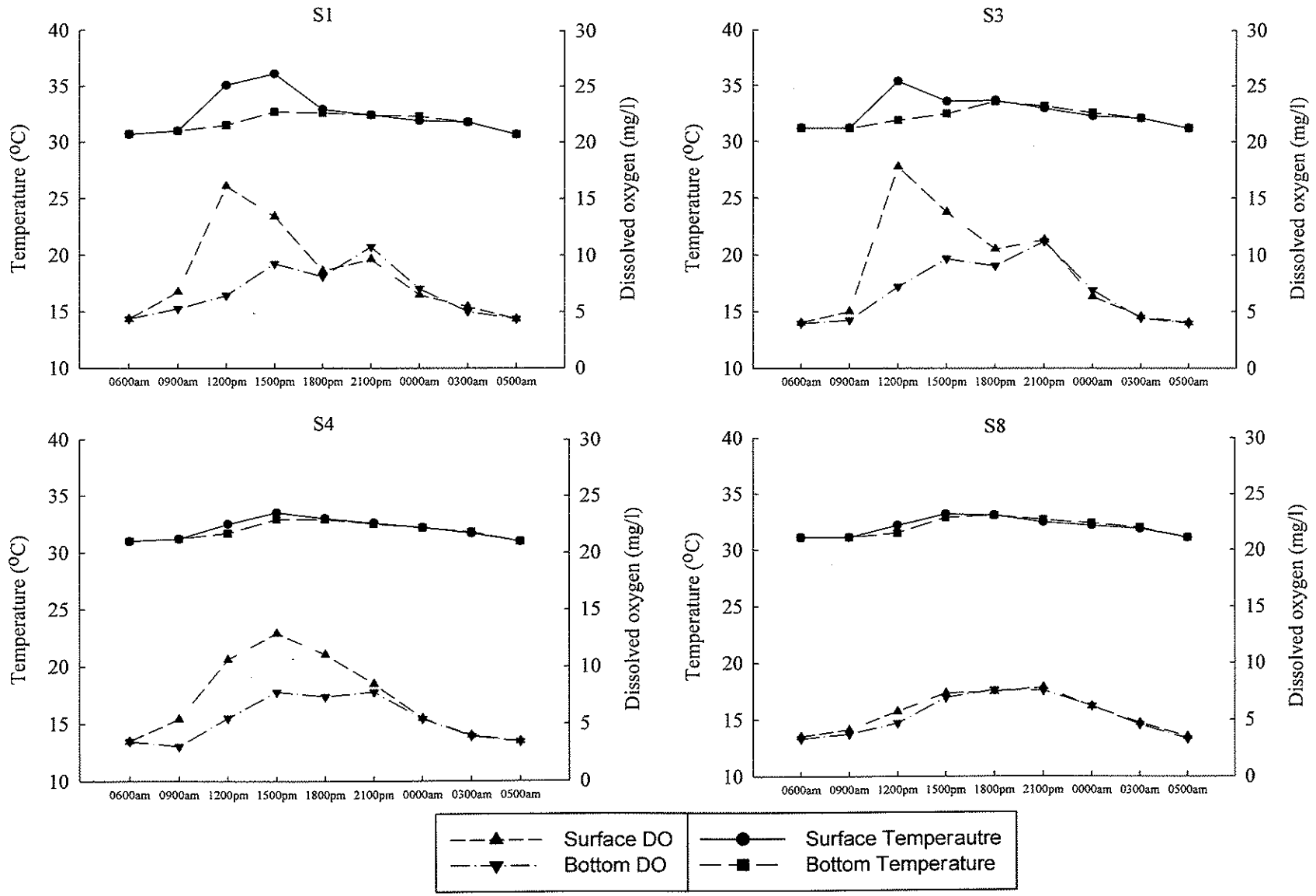

Figure 5. Daily variation for temperature and dissolved oxygen concentration for pond S1, S3, S4 and S8 at Greene Prairie Aquafarm, Alabama.

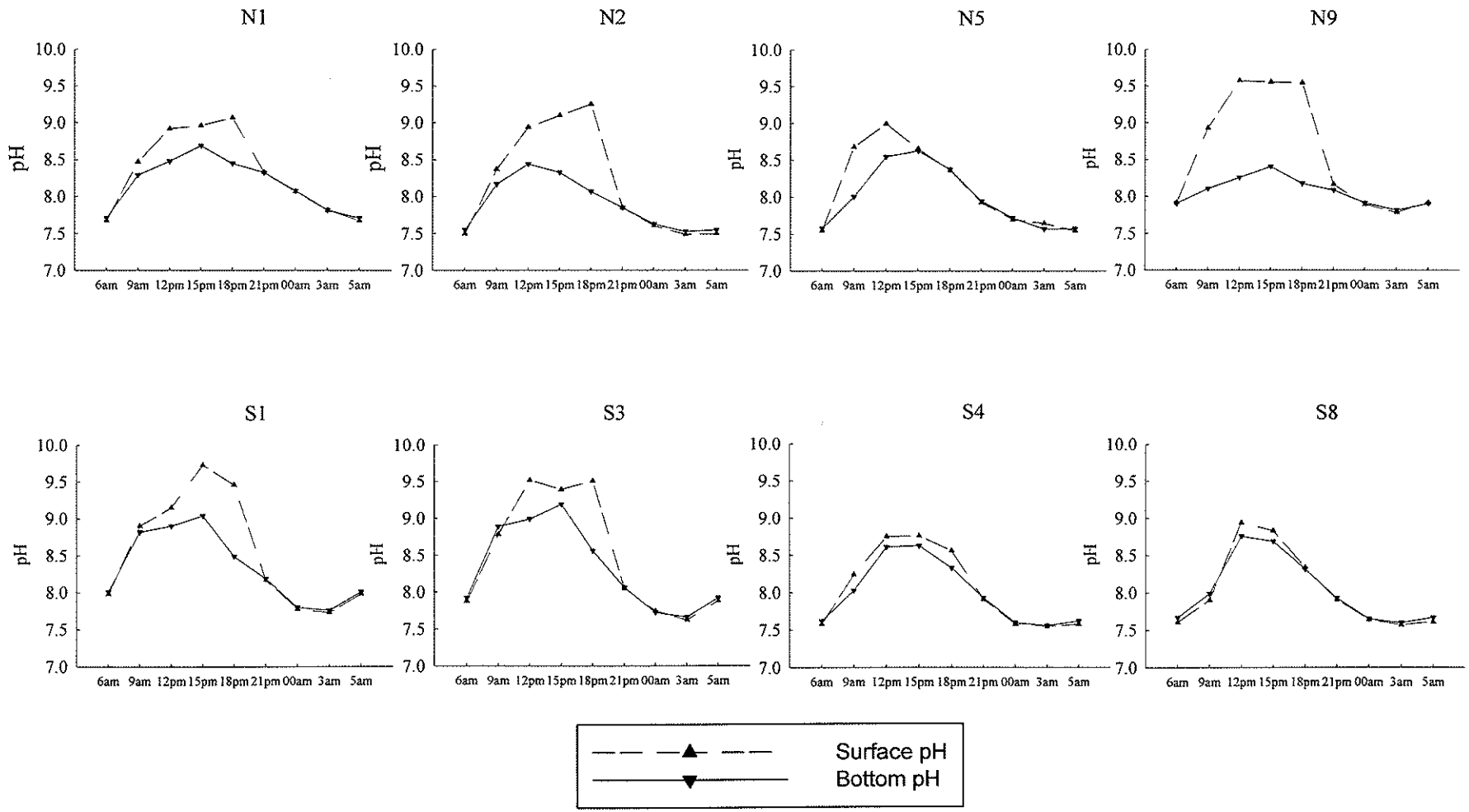

Figure 6. Daily variation in pH for eight ponds at Greene Prairie Aquafarm, Alabama.

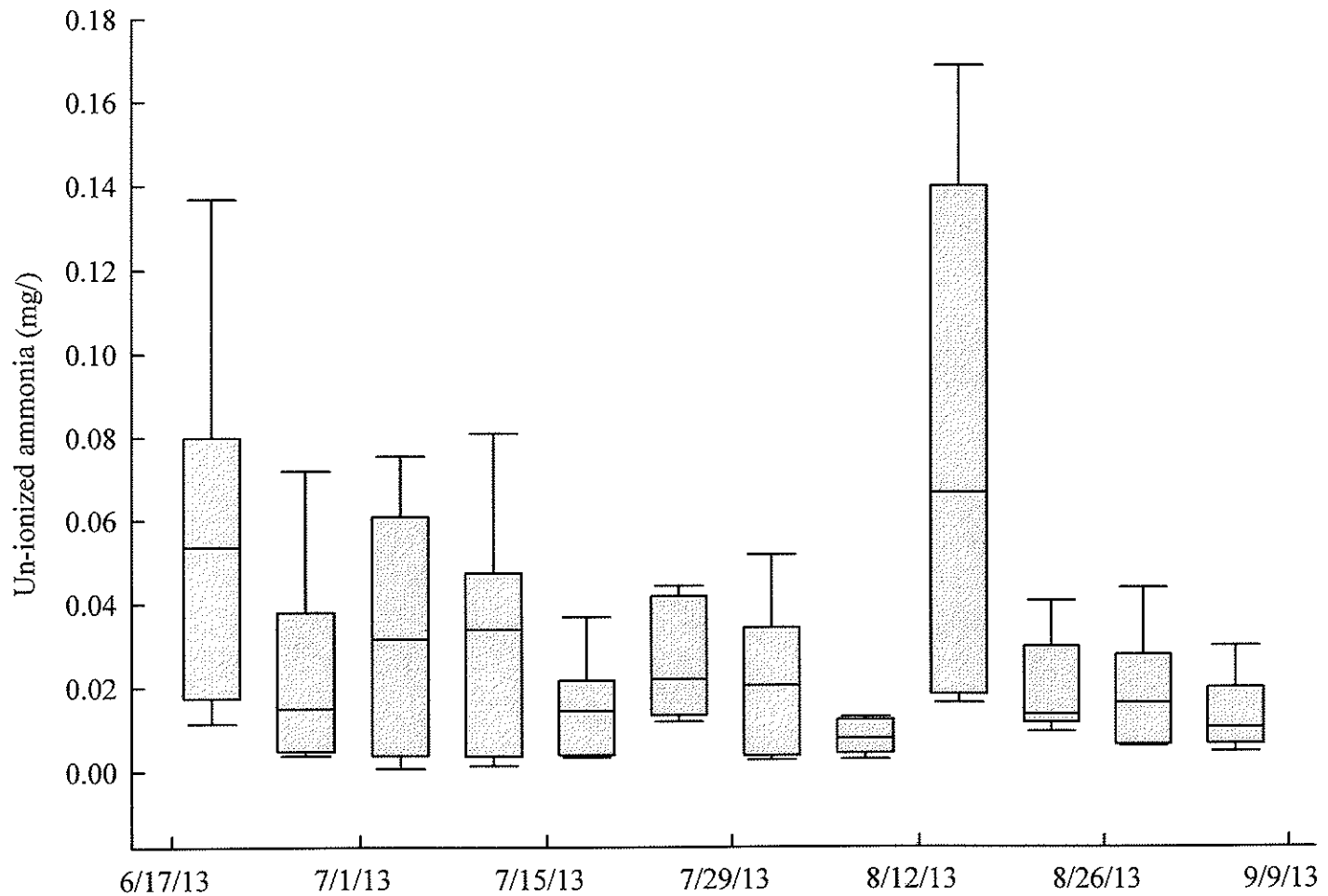

Figure 7. Variation in un-ionized ammonia nitrogen (mg NH_3-N/L) on 12 sampling dates (June 20 – September 5, 2013) at Greene Prairie Aquafarm. Each box represents the range of un-ionized ammonia nitrogen from eight ponds.

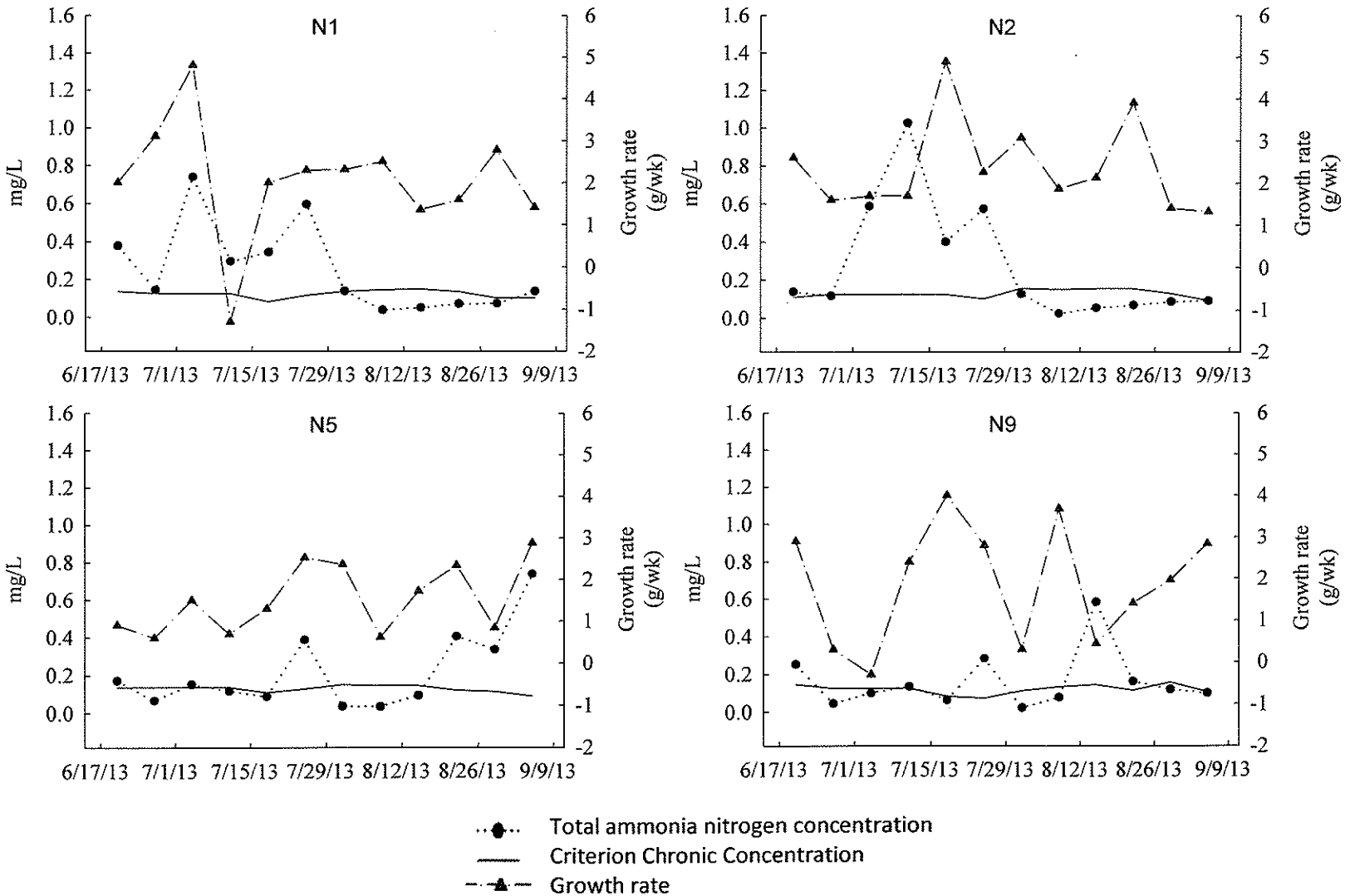

Figure 8. Total ammonia nitrogen and criterion chronic concentration and growth rate of pond N1, N2, N5 and N9 at Greene Prairie Aquafarm, Alabama from June 20 to September 5, 2013.

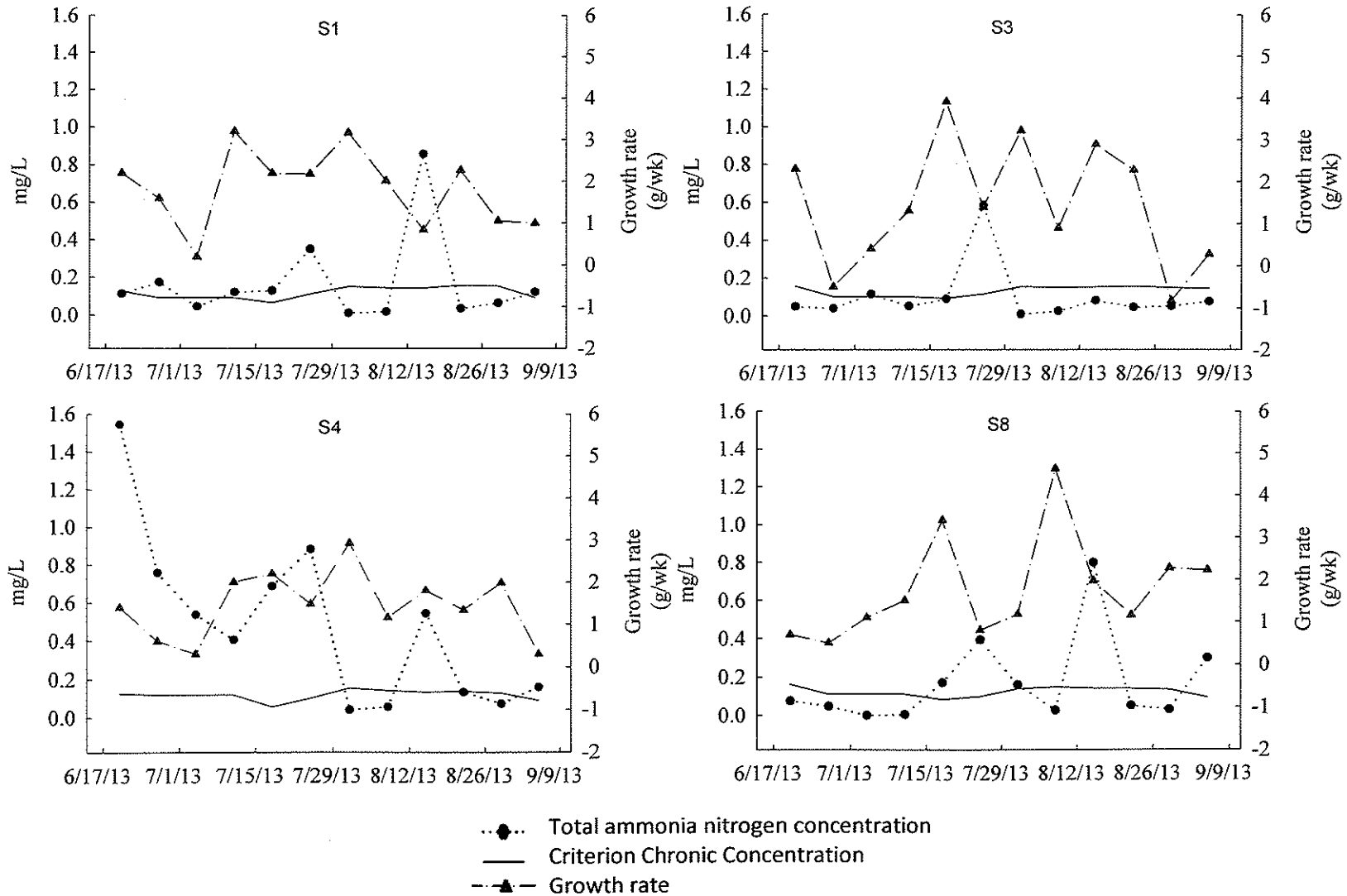

Figure 9. Total ammonia nitrogen and criterion chronic concentration and growth rate of pond S1, S3, S4 and S8 at Greene Prairie Aquafarm, Alabama from June 20 to September 5, 2013.

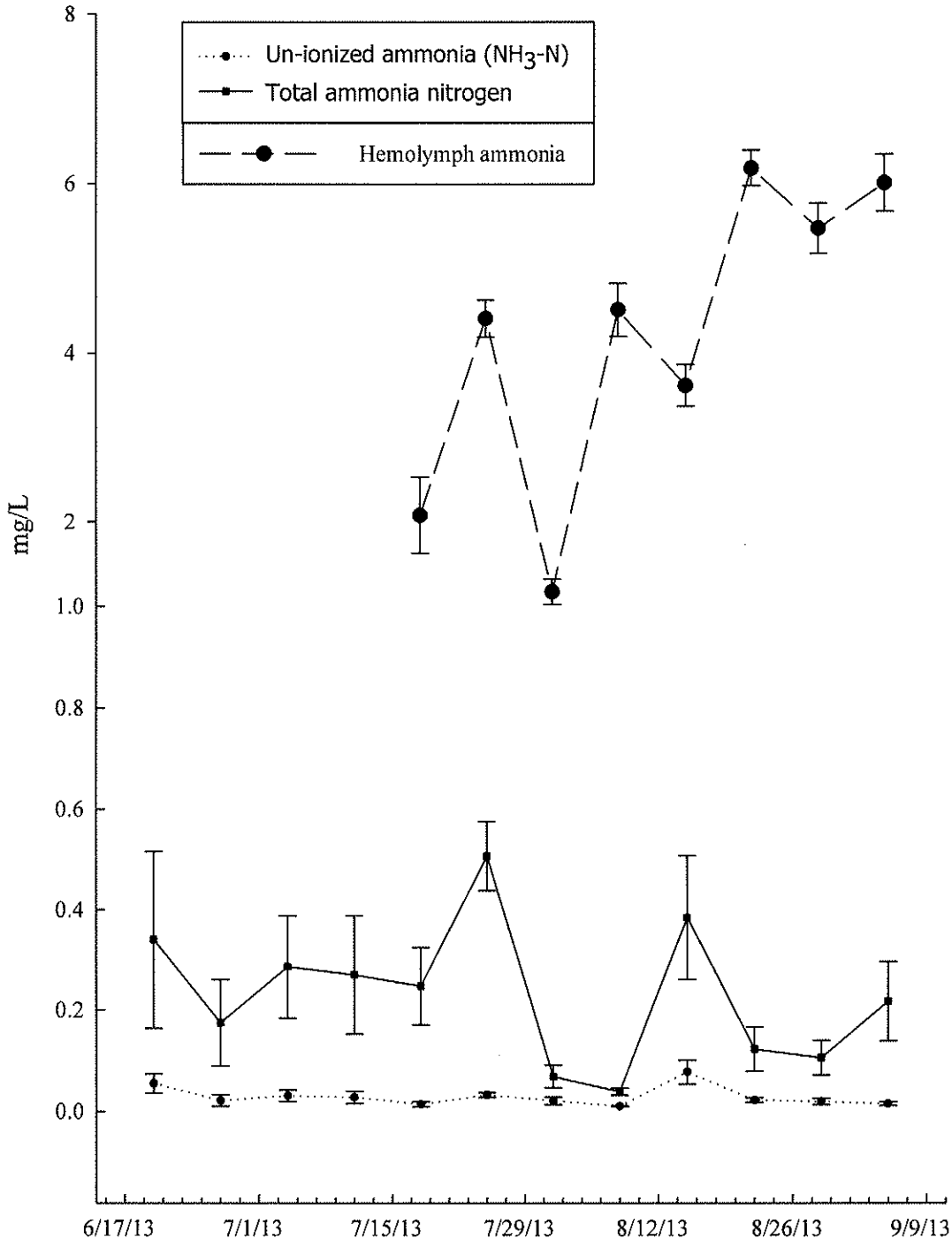

Figure 10. Pond means and standard deviations for concentration of total ammonia nitrogen, un-ionized ammonia, and haemolymph ammonia at Greene Prairie Aquafarm, Alabama.

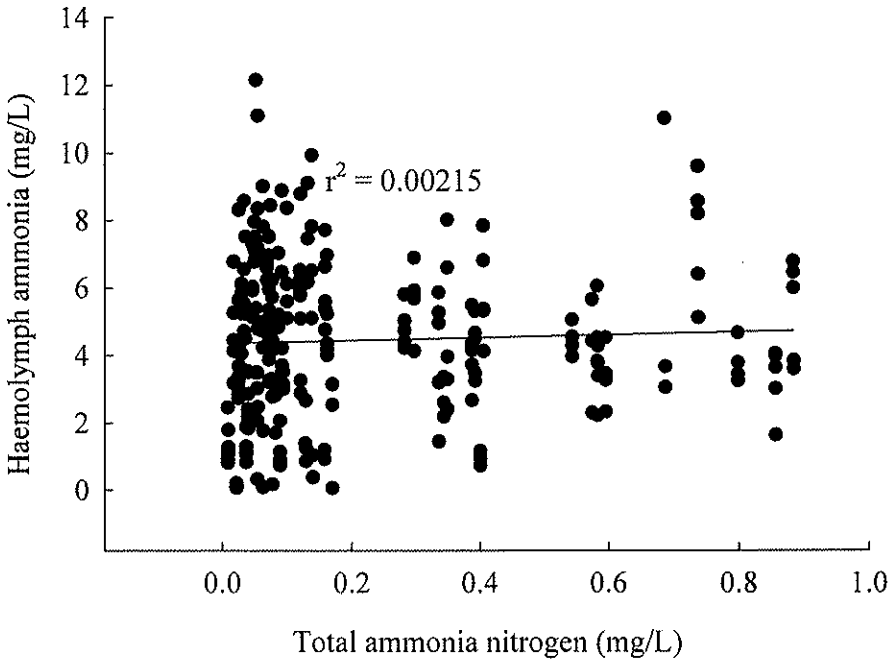

Figure 11. Correlations between concentration of total ammonia nitrogen in waters of ponds and haemolymph ammonia in blood of shrimp at Greene Prairie Aquafarm, Alabama.

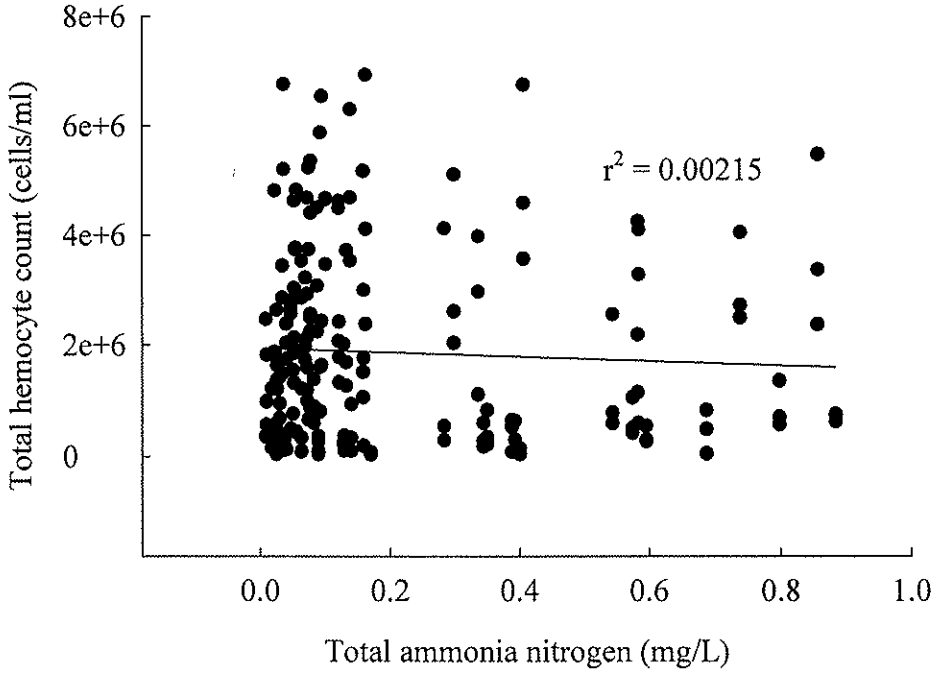

Figure 12. Correlations between concentration of total ammonia nitrogen in waters of ponds and total hemocyte count in blood of shrimp at Greene Prairie Aquafarm, Alabama.

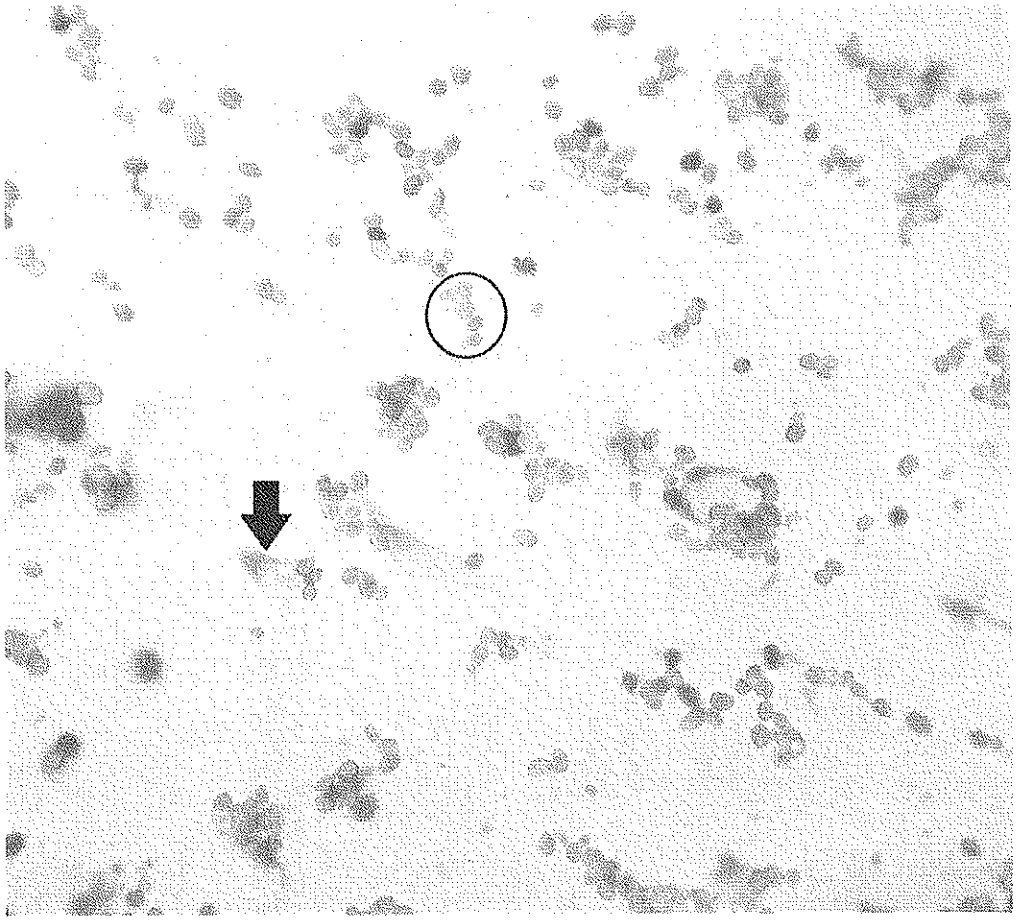

Figure 13. Phagocytosis activity. The cell to which the arrow points is a phagocytic hemocyte from *L. vannamei*, and cells in the circled area are heat-killed yeast.

Appendix

Shrimp Saline's Recipe

1. Sodium Chloride	28.4	g
2. Magnesium Chloride Hexahydrate	1.0	g
3. Magnesium Sulfate	2.0	g
4. Calcium Chloride Dihydrate	2.25	g
5. Potassium Chloride	0.7	g
6. Glucose (Dextrose)	1.0	g
7. HEPES	2.38	g