Acute Toxicity of Ammonia and Nitrite to White Shrimp (L. vannamei) at Low Salinities

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ABSTRACT

The Pacific white leg shrimp, Litopenaeus vannamei, is a potential species for low salinity inland aquaculture. Due to several independent variables, such as species, age, size, salinity and pH, that must be taken into account, there are gaps in the literature pertaining to the toxicity of ammonia and nitrite to shrimp. This study was conducted to investigate the individual and combined effects of ammonia and nitrite on L. vannamei postlarvae (25-45 days old) at 10 ppt salinity, 28 C and a pH of 7.8. The independent variables were salinity, total ammonia as nitrogen (TAN) and nitrite-N (NO_2-N), separately and combined. The TAN experiments were conducted at 18 and 10 ppt salinity while the NO_2-N test was conducted at 10 ppt salinity. Combined TAN and NO_2 tests were also conducted at 10 ppt salinity. The LC50 values for TAN at 18 ppt salinity, TAN at 10 ppt salinity, and NO_2-N at 10 ppt were observed to be 42.92, 39.72 mg/L (2.26 and 2.09 mg/L unionized ammonia-N), and 153.75 mg/L, respectively. When NO_2-N was adjusted to the LOEC level and TAN concentrations were varied, synergistic effects were observed, with an LC50 calculated to be 28.2 mg/L TAN (1.49 mg/L unionized ammonia-N). However, when the ammonia level was adjusted to the LOEC and nitrite was varied, antagonistic effects were observed with an LC50 calculated to be 163.3 mg/L NO_2-N. The results suggest that further investigations into the combined effects of ammonia and nitrite at varying concentrations and lower salinities will be important in developing "standard operating procedures" for the shrimp industry.

Chapter 1: Introduction

Pacific white leg shrimp (Litopenaus vannamei) are one of the most intensively cultivated shrimp species in the world (Pe´rez Farfante 1997). In the wild, they are found throughout tropical Pacific waters, from Mexico to Peru. In aquacutural settings, their ability to thrive in low salinity seawater has been observed, making them an especially good species for inland aquaculture (Pan et al. 2007). The reduction of salinity for inland aquaculture firms is a major goal. Prepared salts can be purchased and applied or ion supplements can be developed to mimic natural seawater conditions. No matter how the water is salinated, it is expensive. Recirculating aquaculture systems (RAS) reuse 90% of their water daily, but there is a direct proportionality between the salinity and cost of the 10% that is discharged. If shrimp can be cultivated at lower salinities, extensive costs to the producing firm are avoided.

Reducing the salinity of the water can lead to problems. The initial, and most apparent problem, is finding a species that can tolerate a shift in the iso-osmotic balance between the internal and external environments. Many marine species would experience an influx of water due to the osmotic pressure driven by the higher concentration of ions within the shrimp as compared to the surrounding water. Another problem that can result when decreased salinities are applied is decreased resiliency of shrimp to toxins, such as ammonia and nitrite.

Nitrogenous wastes products, such as ammonia and nitrite, can become concentrated in aquaculture systems (Frias-Espericueta et al. 1999). Ammonia is the resulting waste product of the cultured shrimp. It can also accumulate in the water due to the decomposition of organic solids such as excess feed and feces (Lin and Chen 2003).

For intensive RAS, the most common removal technique involves utilizing nitrifying bacteria to convert ammonia-nitrogen to nitrate-nitrogen (Ebeling et al. 2006). Of the resulting species, ammonia is more toxic than nitrite, which in turn is more toxic than nitrate. The total ammonia concentration as nitrogen (TAN) is comprised of two forms, unionized ammonia (NH_3) and ionized ammonium (NH_4^+) (Armstrong et al. 1978). The form of the TAN is dependent on the pH, salinity, and temperature (Bower and Bidwell 1978). Of the two, unionized ammonia is the more toxic (Smart 1978). The small uncharged particle can easily cross the lipid membrane of aquatic organism's gill cells.

The purpose of this study was threefold. The toxicity of ammonia was first studied at 18 and 10 ppt salinity. Secondly, nitrite was studied individually at 10 ppt. The background tests helped to provide reference data when developing and analyzing combined TAN and NO_2 tests. These studies were directed to validate data of previous studies as well as to generate new data at specific environmental parameters and ages. The third objective was to conduct two experiments at differing combinations of ammonia and nitrite in order to better understand any combined effects resulting from exposure to elevated levels of both agents.

Chapter 2: Literature Review

2.1 Introduction

The control of ammonia and nitrite in aquaculture systems is the second most important factor impacting survival and growth of cultured organisms, following dissolved oxygen (Ebeling et al. 2006). The build up of nitrogenous waste products from feed decomposition and organism excretion can lead to reduced productivity as well as the collapse of an entire aquaculture system. The cycle of nitrogen in intensive recirculating aquaculture systems (RAS) can be easily characterized using a systematic approach that analyzes the inputs, outputs, and recycled materials.

Accumulation of nitrogen in RAS originates from feed being added to the system. The metabolism pathway of protein ultimately leads to the production of ammonia (NH_3) which is excreted (Hargreaves 1998). Total ammonia as nitrogen (TAN) is the measured combination of ammonia (NH_3) and ammonium (NH_4^+), the unionized and ionized versions. Once the TAN has been measured, the concentrations of the two separate species can be calculated based on the pH, temperature, and salinity of the water (Bower and Bidwell 1978).

Of the nitrogen excreted, two forms can be found, dissolved and particulate. According to Folke and Kautsky (1989), of the 75% of nitrogen added as food that becomes excrement, 62% can be classified as dissolved. Dissolved nitrogenous compounds can be taken up by aquatic organisms through the gills during respiration.

Removal processes of dissolved nitrogen compounds, can vary. For intensive RAS, the most common practice involves utilizing nitrifying bacteria to convert ammonia-nitrogen to nitrate-nitrogen (Ebeling et al. 2006). The nitrification process is characterized by two oxidation steps, resulting in the transformation of NH_3 (ammonia) to NO_2 (nitrite) and finally NO_3 (nitrate). This process is essential in the natural world to reduce the toxicity of nitrogenous wastes. Conversion of ammonia to nitrite is accomplished by ammonia oxidizing bacteria (AOB). AOB are also commonly knows as Nitrosomonas bacteria. Nitrite oxidizing bacteria (NOB) metabolically oxidize nitrite to nitrate. NOB are also commonly known as Nitrobacter bacteria. The rate limiting step of this process is the oxidation of ammonia (Vadivelu et al. 2007). Incomplete nitrification occurs when a lack of NOB productivity is present, leading to increased concentrations of nitrite.

Volatilization is not a major factor in the removal of ammonia in recirculating aquaculture systems. Significant loss of ammonia due to volatilization only occurs at pHs above 9, when most TAN is in the unionized form. This is relevant for pond aquaculture systems and should be taken into account in nitrogen mass balances due to variation in cyclical pond characteristics (Hargreaves 1998).

2.2 Exposure to Ammonia and Nitrite

2.2.1 Toxic properties of ammonia

The toxic properties of ammonia are based on the irritative properties of the compound (CDC 2004). Unlike mammals that convert nitrogenous wastes to other forms such as urea, fish and crustaceans excrete ammonia in an unaltered state. This is possible due to the fact that in natural conditions ammonia is instantly diluted to safe levels by the surrounding water. Fish and crustaceans also lack the ability to convert ammonia to the less toxic, carbamoyl phosphate compound. Due to this, aquatic species are especially prone to toxic effects of ammonia at highly concentrated levels. The unionized form of ammonia is the more toxic species to aquatic organisms due to its ability to gain entry through the gills. The uncharged, lipid soluble molecule can readily pass through cell membranes (Boardman et al. 2004), whereas the ionized form does not readily cross hydrophobic microphores in the gill membrane (Svobodova 1993).

The documented physiological changes in aquatic organisms due to ammonia exposure vary. One initial effect of ammonia relates to site specific irritation. The gills of tilapia that had been exposed to chronic ammonia tests were analyzed by Caglan, et al (2005). The authors concluded that ammonia was responsible for gill hyperplasia as well as lamella fusion.

The result of hyperplasia and lamella fusion is restricted water flow over the gills, leading to respiratory stress on the organism. Similar results were found, as well as epithelial pitting of the gills, when rainbow trout were tested and examined using scanning electron microscopy (Kirk and Lewis 1993). Authors have not investigated the histological effects of ammonia on the gills of shrimp.

2.2.2 Effects of Ammonia on Survival and Growth

The lethal effects of ammonia in aquacultural systems are well documented. Several species of shrimp have been used to conduct toxicity experiments in order to determine the lethal concentrations to 50% of a sample population (LC50). Lethal toxicity tests can be acute or chronic depending on the time of exposure. In most cases, acute tests are performed over a period of 2-7 days, while chronic tests are longer than 7 days. Concentrations leading to 50% mortality vary depending on the organism being tested.

The comparison of previous research and data is problematic due to the parameters that can be varied within toxicity tests. Examples of these parameters are age/size of the shrimp, salinity of the water, length of exposure to toxins, temperature and pH. The temperature and pH parameters are especially important because they are the two most influential factors in the proportioning of TAN. Another obstacle is that toxicity data for ammonia is not standardized; some literature presents data in TAN while others provide data in ammonia-N without necessary conversion factors based on the parameters used.

Previous studies have shown that 48 h median lethal concentrations (LC50) for ammonia-N to varying species of shrimp, to range from 30 and 110 mg/L TAN at full strength seawater depending on size and age (Chen *et al.* 1990a, Chen *et al.* 1990b, Ostrensky and Wasielesky 1995, Frias-Espericueta et al. 1999, Kir and Kumlu 2006). For Penaeus monodon and Metapenaeus macleayi juveniles, LC50's were determined using 96 hr acute tests. The results showed the respective LC50's to be 1.69 and 1.39 mg/l NH_3-N (Allan et al. 1990). Other authors, through studies with various genera and species, have concluded that the toxicity of ammonia to specific species is dependent on time and concentration.

A study using Penaeus semisulcatus post larvae (PLs) found that the tolerance to ammonia-N decreased with decreasing salinity. Specifically, the shrimp tested at 40 ppt salinity were tolerant to ammonia-N levels 2.9 times higher than those at 15 ppt over 48 h (LC50s of 32.5 and 11.2 mg/L TAN, respectively) (Kir and Kumlu 2006).

Elevated ammonia levels can also lead to reduced growth of species raised in intensive aquaculture systems. Wickens (1976) showed that a concentration of 0.45 mg/l NH_3-N led to a 50% decrease in growth of five species of penaid shrimp. The author also concluded that a concentration of above 0.10 mg/L NH_3-N breached maximum acceptable levels for reduced growth over a three week chronic test (Wickins 1976).

2.2.3 Toxic properties of nitrite

Nitrite toxicity is not related to site specific irritation. Instead, the toxicity of nitrite is a function of the effects on the circulatory and immune systems of aquatic organism. Nitrite enters the blood stream and inhibits the binding of oxygen to the iron molecule of hemoglobin (Hargreaves 1998). The oxidation of the iron by nitrite leads to increased levels of methemoglobin and substantially decreased levels of hemoglobin (Tilak et al. 2007). This can lead to 'brown blood syndrome' where the blood loses its reddish color and becomes brown due to a lack of oxygen. Tilak et al. (2007) also saw decreased levels of oxygen consumption in relation to the conversion of hemoglobin to methemoglobin.

The blood of shrimp, as well as other invertebrates, does not contain hemoglobin. Instead, oxygen binds to a copper based molecule at the gills and is then delivered throughout the body. The physiological and histological effects of nitrite on invertebrates is not well study, but it is possible that nitrite effects the copper of invertebrate's circulatory systems as it does the iron of vertebrate's circulatory systems.

2.2.4 Effects of Nitrite on Survival and Growth

Tseng and Chen (2004) examined the effects of nitrite stress on immune responses to Vibrio alginolyticus, a common bacterial disease in marine aquaculture systems. They found that shrimp exposed to nitrite between 5 and 22 mg/l showed significantly reduced resistance to bacterial infection. This study was conducted through analysis of haemocyte (invertebrate red blood cells) counts (Tseng and Chen 2004). In another study Macrobrachium malcolmsonii juveniles were subjected to nitrite stresses in the presence of the bacteria A. hydrophila. The authors concluded that increased nitrite stress led to a reduction in immune response to *A. hydrophila* (Chand and Sahoo 2006).

In aquacultural systems, an increase in ammonia concentration is followed by a decrease in ammonia that is indirectly proportional to a rise in nitrite, as NH_3 is oxidized to NO2. The acute lethal affects of nitrite on aquatic organisms is not as pronounced as ammonia at low concentrations, yet its toxicity is still of concern. A study that explored the acute effects of nitrite on L. vannamei shrimp over 48 h revealed LC50s of 142.2, 244.0, and 423.9 mg/L nitrite-N for 15, 25, and 35 ppt salinity respectively (Lin and Chen 2003). Gross et al. (2004) also explored the acute effects of nitrite to L. vannamei in low salinity waters. When reared in water with 2 ppt salinity, the 48 h LC value was determined to be approximately 15 mg/L NO_2-N (Gross *et al.* 2004), significantly lower than seen in the Lin and Chen (2003) experiments.

2.2.5 Ammonia and Nitrite Combined

Few studies have been conducted with shrimp PLs that investigate the combined effects of ammonia and nitrite on shrimp, especially at lowered salinities. Alcaraz et al. (1997, 1999a, 1999b) studied the combined acute effects of ammonia and nitrite on Penaeus setiferus PLs including factors such as temperature tolerance, survival, and varying dissolved oxygen (DO) all at 30 ppt salinity. It has been suggested that the ratio of ammonia to nitrite is inversely proportional to the critical thermal maximum shrimp can tolerate (Alcaraz et al. 1997). Acute survival studies have suggested synergistic effects at 48 h exposure and antagonistic effects beyond 72 h (Alcaraz et al. 1999a). Decreased respiration rates have also been seen in P. setiferus exposed to ammonia and nitrite (Alcaraz et al. 1999b). Other studies conducted using Penaeus monodon PLs have suggested antagonistic effects at 48 and 72 h with synergistic effects after 96 h (Chen and Chin 1988).

2.3 Factors affecting toxicity of Ammonia and Nitrite

2.3.1 pH

The prevalent form of ammonia found in aquatic environments is heavily dependent on the pH. The pKa of the ammonium ion is around 9.25. As the pH increases, NH_4^+ releases a hydrogen ion resulting in ammonia, NH_3 (Allan et al. 1990). The toxicity of ammonia in relationship to pH is something that has been widely tested in shrimp. Many experiments have shown that an increase in pH up to 9 leads to reduced LC50's for multiple species of shrimp (Magallon Barajas et al. 2006). According to Magallon et al. (2006), most marine aquacultured organisms should be raised in water with a pH between 7 and 8 to avoid ammonia toxicity.

2.3.2 Salinity

For most marine organisms, increased salinity levels leads to greater resilience to elevated ammonia and nitrite concentrations. Test shrimp P. semisulcatus, showed an increased tolerance to ammonia when reared in 40 ppt salt, as compared to 15 ppt. LC50 levels were 2.5 times higher in 40 ppt salt water versus 15 ppt (Kir and Kumlu 2006). The same study revealed that there is a correlation between better growth and ammonia tolerance in higher salinity waters.

The effects of nitrite in relation to salinity are similar to those of ammonia. Litopenaeus vannamei were used to determine LC50's of nitrite at varying salinity levels (15, 25, 35 ppt). Despite L. vannamei's apparent high tolerance to nitrite, those reared at 15 ppt salt showed LC50's that were 200-300 mg/l nitrite-N lower than those reared in 35 ppt salt (Lin and Chen 2003). These results are similar to other studies with different aquatic species. Another study showed decreases in the tolerance of juvenile black sea bass to nitrite in reduced salinity tanks (Weirich and Riche 2006).

As marine aquaculture facilities continue to move inland, away from natural seawaters, the cost for maintaining salt levels upwards of 40 ppt rises. The cost benefit ratio of rearing marine species in lower salt concentrations and the effects of ammonia is something that requires more interdisciplinary research.

2.3.3 Dissolved Oxygen (DO)

Dissolved oxygen levels are one of the most important factors for the conversion of ammonia to nitrate. Nitrifying bacteria require adequate levels of oxygen to oxidize waste products. Low oxygen levels can also result in a shift to aerobic heterotrophic bacteria that are better competitors for oxygen than nitrifying bacteria (Hargreaves 1998). Hargreaves (1998) also mentions that the conversion of nitrite to nitrate requires higher levels of oxygen than the conversion of ammonia to nitrite. Therefore, in poorly oxygenated waters, a build-up of nitrite and ammonia can occur, leading to toxic effects on organisms.

Another indirect effect of low dissolved oxygen is an increased respiration rate in aquatic organisms that can lead to increased uptake of dissolved nitrogenous compounds (Thurston et al. 1981). Reduced DO levels have been shown to significantly increase the acute toxicity of ammonia in P. monodon due to increased respiration rates (Allan et al. 1990).

2.3.4 Temperature

Temperature, as well as pH, plays a role in the partitioning of unionized and ionized ammonia in aqueous environments. As temperature increases, the fraction of unionized ammonia in TAN increases (Bower and Bidwell 1978). It has also been shown that an increase in temperature is coupled with increased respiration rates in the freshwater shrimp, M. rosenbergii (Niu et al. 2003). Increased oxygen consumption could be linked to an increase in ammonia/nitrite uptake. An increase in temperature has also been found to increase nitrogenous excretion in crustaceans (Regnault 1986), possibly due to increased metabolic rates.

2.3.5 Age

Generally, as the age of an organisms increases, the tolerance of the organism to toxins increases. Juvenile or larvae-aged species are used in most bioassays to determine the lowest observed effect concentration (LOEC) of a toxic substance. Experiments wth L. vannamei revealed that post larvae 15-20 days old showed a higher LC50 than those which were younger (Magallon Barajas et al. 2006). The stabilization of ammonia and nitrite levels for younger organisms is especially important for transport and breeding situations.

2.4 Conclusions

The toxicity of ammonia and nitrite is heavily dependent on environmental factors, including pH, dissolved oxygen, salinity, and temperature. For aquacultural purposes, these factors play an important role in the development, growth, and survival of species exposed to ammonia and nitrite. Intensive culture in RAS allows for significant control over these factors and therefore the susceptibility of organisms to nitrogenous based toxins.

The manipulation of environmental factors affecting toxicity becomes the most important facet of culturing aquatic species based on location and available resources. If one factor cannot be controlled as well as others, extra measures can be taken to reduce toxicity through other means. For inland systems, the cost of salinating systems must be weighed against the growth responses and toxicity of ammonia and nitrite in low salinity water.

At the most basic level, a system should be designed with the knowledge that:

1. An increase in pH leads to increased ammonia toxicity,
2. A decrease in salinity leads to increased ammonia and nitrite toxicity for most shrimp species,
3. A decrease in DO leads to increased ammonia and nitrite toxicity,
4. An increase in temperature leads to increased ammonia and nitrite toxicity,
5. Younger individuals are more susceptible to toxic effects.

2.5 Research Needs

In order to maximize growth and survival of aquatic species through the minimization of ammonia and nitrite toxic effects, more research is needed to evaluate the combined effects of factors that affect their toxicity. By testing combinations of factors more information can be derived, leading to a complete understanding of how the systems function. This should be coupled with the testing of other water quality factors that might have an affect on nitrogenous toxicity, such as metals and other water contaminants.

Research has been completed to develop a general idea as to how certain groups of aquacultured organisms will react to varying levels of ammonia and nitrite, but it would be beneficial for cultured species to be tested individually to determine specific parameters. Variations between shrimp species and age groups have been documented (Allan et al. 1990), showing the need for data on specific species.

Chapter 3: Acute toxicity of ammonia and nitrite to Pacific White Shrimp (L. vannamei) at low salinities

Acute Toxicity of Ammonia and Nitrite to Litopenaeus vannamei at Low Salinities

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ABSTRACT

The Pacific white leg shrimp, Litopenaeus vannamei, is a potential species for low salinity inland aquaculture. Due to several independent variables, such as species, age, size, salinity and pH, that must be taken into account, there are gaps in the literature pertaining to the toxicity of ammonia and nitrite to shrimp. This study was conducted to investigate the individual and combined effects of ammonia and nitrite on L. vannamei postlarvae (25-45 days old) at 10 ppt salinity, 28 C and a pH of 7.8. The independent variables were salinity, total ammonia as nitrogen (TAN) and nitrite-N (NO_2-N), separately and combined. The TAN experiments were conducted at 18 and 10 ppt salinity while the NO_2-N test was conducted at 10 ppt salinity. Combined TAN and NO_2 tests were also conducted at 10 ppt salinity. The LC50 values for TAN at 18 ppt salinity, TAN at 10 ppt salinity, and NO_2-N at 10 ppt were observed to be 42.92, 39.72 mg/L (2.26 and 2.09 mg/L unionized ammonia-N), and 153.75 mg/L, respectively. When NO_2-N was adjusted to the LOEC level and TAN concentrations were varied, synergistic effects were observed, with an LC50 calculated to be 28.2 mg/L TAN (1.49 mg/L unionized ammonia-N). However, when the ammonia level was adjusted to the LOEC and nitrite was varied, antagonistic effects were observed with an LC50 calculated to be 163.3 mg/L NO_2-N. The results suggest that further investigations into the combined effects of ammonia and nitrite at varying concentrations and lower salinities will be important in developing "standard operating procedures" for the shrimp industry.

Pacific white shrimp (Litopenaeus vannamei) are one of the most intensively cultivated shrimp in the world (Pe´rez Farfante 1997). Their ability to thrive in low salinity seawater makes them an especially good species for inland aquaculture (Pan et al. 2007). The reduction of the salinity in water used within recirculating aquaculture systems (RAS) can lead to production of shrimp at lower costs due to less salt being purchased and easier management of wastewater. The reduction of salt within this process can lead to problems for intensely grown shrimp, such as a decrease in the resiliency of shrimp to agents such as ammonia and nitrite (Chen and Lin 1992, Lin and Chen 2003).

Nitrogenous wastes products can become concentrated in aquaculture systems (Frias-Espericueta et al. 1999). Ammonia is excreted by shrimp, and it can also accumulate in the water due to the decomposition of organic solids, such as excess feed and feces (Lin and Chen 2003). For intensive RAS, the most common removal technique involves utilizing nitrifying bacteria to convert ammonia-nitrogen to nitrate-nitrogen (Ebeling et al. 2006). Of the resulting chemical species, ammonia is more toxic than nitrite, which in turn is more toxic than nitrate. The total ammonia concentration as nitrogen (TAN) is comprised of two species, unionized ammonia (NH_3-N) and ionized ammonium (NH_4^+-N) (Armstrong et al. 1978). The speciation of the TAN is dependent on the pH, salinity, and temperature (Bower and Bidwell 1978). Of the two, unionized ammonia is more toxic (Smart 1978). The small uncharged particle can easily cross the lipid membrane of aquatic organism's gill cells.

Authors of previous studies have shown 48 h median lethal concentrations (LC50) for TAN to varying species of penaid shrimp to range from 30 and 110 mg/L TAN at full strength seawater depending on size and age as summarized in Table 1 (Chen *et al.* 1990a, Chen *et al.* 1990b, Ostrensky and Wasielesky 1995, Frias-Espericueta et al. 1999, Kir and Kumlu 2006). Kir and Kumlu (2006) found that the tolerance to TAN decreased with decreasing salinity using Penaeus semisulcatus post larvae (PL). Specifically, the shrimp tested at 40 ppt salinity were tolerant to TAN levels 2.9 times higher than those at 15 ppt over 48 h (LC50s of 32.5 and 11.2 mg/L TAN, respectively) (Kir and Kumlu 2006). Chen and Lin (1992) observed that a decrease in salinity from 30 to 10 ppt led to a shift in 48 h LC50 values from 53.94 to 2.39 mg/L TAN for juvenile Penaeus chinesis.

Authors who have conducted nitrite studies at low salinities (15 ppt) have shown the 48 h LC50 of nitrite to L. vannamei juveniles to be around 143 mg/L NO_2-N (Lin and Chen 2003). Few researchers have conducted studies to investigate the combined effects of ammonia and nitrite on shrimp, especially at decreased salinities. Alcaraz et al (1997, 1999a, 1999b) studied the combined acute effects of ammonia and nitrite on Penaeus setiferus PL, including factors such as temperature tolerance, varying dissolved oxygen (DO), and survival at 30 ppt salinity. After investigating the effects of exposure to ammonia and nitrite on the critical thermal maximum (CTM) shrimp can tolerate, the authors suggested that the ratio of ammonia to nitrite is inversely proportional to the CTM. Specifically, a mixture of 0.4 mg/L NH_3-N and 120 mg/L NO_2-N was seen to decrease the CTM by 7.6% when compared to a control (Alcaraz et al. 1997). Decreased respiration rates have also been seen in P. setiferus exposed to ammonia and nitrite (Alcaraz et al. 1999b). In relation to this study, the acute survival studies were undertaken to determine any combined effects of the two agents. The authors have suggested synergistic effects at 48 h exposure and antagonistic effects beyond 72 h due to an increase in mortality rates of 40% after 48 h (with a NO_2-N concentration of 180 mg/L) and a decrease in mortality rates from 30 to 10% after 72 h (with a NO_2-N concentration of 120 mg/L) (Alcaraz et al. 1999a).

Other investigators experimenting with Penaeus monodon PL have reported antagonistic effects at 48 and 72 h, with synergistic effects after 96 h (Chen and Chin 1988). The fact is that not enough research has been conducted to fully understand the relationship of the two agents. In order to fully understand the relationship, toxicity tests need to be undertaken that examine a broad range of toxin concentrations, the effects of varying environmental parameters, and responses of specific species.

The purpose of this study was threefold. The toxicity of ammonia was first studied at 18 and 10 ppt salinity. Secondly, nitrite was studied individually at 10 ppt. The background tests helped to provide reference data when developing and analyzing combined TAN and NO_2 tests. These studies were directed to validate data of previous studies as well as to generate new data at specific environmental parameters and ages. The third objective was to conduct two experiments at differing combinations of ammonia and nitrite in order to better understand any combined effects resulting from exposure to elevated levels of both agents.

Materials and Methods

Animals

Litopenaeus vannamei PL were obtained from Shrimp Improvement Systems (Plantation Key, Fl, USA). Shrimp were fed a grow-out diet provided by Shrimp Improvement Systems to satiation. Post larvae (13 days old) (PL13) were shipped overnight to Virginia Tech Laboratories in Blacksburg Virginia and were received at 18

C and 30 ppt salinity. The shrimp were acclimated to water in Aquatic Habitat Systems (AHABs) (Aquatic Ecosystems Apopka, FL, USA) over a period of 24 h. The temperature was increased to 28 C over the next 48 h. The parameters that were monitored in the AHAB systems are summarized in Table 2. During the second week (PL20), salinity was adjusted to the desired level for specific tests at a rate less than halving the salinity over 24 h.

Seawater

Municipal water was filtered using a reverse osmosis (RO) filter (Seachem Pinnacle Series, Madison, Ga, USA). Water was stored in a 115 L receptacle where it was heated to 28 C, aerated, and mixed constantly. Instant Ocean® (Aquarium Systems Inc., Mentor, OH, USA) was used to salinate the water in the initial experiment. Crystal Sea Marine Mix® (Marine Enterprises, Baltimore, MD, USA) was used in subsequent experiments at the request of a private shrimp company that uses the Crystal Sea Marine Mix. No difference in shrimp response to the two salt mixes was apparent.

Test Protocol

Toxicity trials were conducted in accordance with recommended EPA Methods (US EPA, 1991). Shrimp were selected arbitrarily and transferred via hand net into 1 L polyethylene beakers to a density of 8 shrimp per beaker. The beakers contained a 50:50 mix of the water from the AHAB unit and salinated RO water. The shrimp were acclimated for 24 h, and then salinated RO water was added to lower ambient levels of ammonia and nitrite. Air was delivered to each beaker via a Pasteur pipette at a rate of one bubble/sec. Test agents (NH_4Cl or $NaNO_2$) were added from 10,000 mg/L stock solutions using a micropipette. Test solutions were then mixed well. Shrimp were not fed during experiments, and no water changes were administered.

Each experimental condition was tested in triplicate and each had triplicate controls. The shrimp were monitored every 0.5 h for 2 h and then at 4 h intervals until the 48 h endpoint. Death was determined by a lack of response to a glass rod stimulus. Dead shrimp were removed immediately.

Test solutions were maintained at a temperature of 28 C by using a waterbath. The pH of the test water averaged of 7.8. The alkalinity and hardness of tests conducted at 18 ppt salinity were 10.1 mg/L $CaCO_3$ (0.2 meq/L $CaCO_3$) and 310 mg/L $CaCO_3$ (3.1 mmol/L $CaCO_3$) respectively. The alkalinity and hardness of tests conducted at 10 ppt salinity was 33.4 mg/L $CaCO_3$ (0.7 meq/L $CaCO_3$) and 179 mg/L $CaCO_3$ (1.79 mmol/L $CaCO_3$), respectively. The DO was maintained above 5.75 mg/L. Testing methods for all water quality parameters measured are shown in Table 3.

Ammonia Toxicity Trials

Shrimp (PL 42) were first tested at 18 ppt salinity. Test solutions were dosed with target concentrations of TAN at 0, 20, 40, 60, 80, 100 mg/L (actual measured TAN values were 0.3, 19.5, 38.2, 57.8, 75.6, 95.4 mg/L).

Shrimp (PL28) were then tested at 10 ppt salinity. Test solutions were dosed with target concentrations TAN at 0, 10, 20, 30, 40, 50, 60, 70, 80 mg/L (actual measured TAN values were 0.3, 10.3, 20.5, 30.0, 38.7, 50.2, 58.3, 69.4, 78.9 mg/L).

Nitrite Toxicity Trials

Shrimp (PL39) were tested at 10 ppt salinity. Test solutions were dosed with concentrations of nitrite at 0, 90, 110, 130, 150, 170, 190 mg/L NO_2-N (actual data point averages were 0, 90, 115, 133, 149, 170, 187 mg/L NO_2-N).

Ammonia and Nitrite Toxicity Trials

Shrimp (PL45) were tested at 10 ppt salinity with a constant dosage of NO_2-N. The dosage chosen, 133 mg/L NO_2-N, was the lowest observed effect concentration (LOEC) determined from the previous NO_2-N test (Table 4). The TAN concentrations selected were the values determined in the previous TAN study at 10 ppt salinity (LC20, LC40, LC50, LC60, and LC80).

Shrimp (PL47) were then tested at 10 ppt salinity with a constant dosage of TAN. The dosage chosen, 30.0 mg/L TAN, was the LOEC determined from the previous TAN test (Table 5). The NO_2-N concentrations selected were the values determined in the previous NO_2-N study (LC20, LC40, LC50, LC60, and LC80).

Statistical Analysis

Toxicity data were analyzed in accordance with EPA recommended guidelines for analysis of toxicity data (Toxcalc™ statistical analysis software, Tidepool Scientific®, McKinleyville, CA, USA). Among the tests used were the Shapiro-Wilk's Test to determine normality of data, the Dunnett's Hypothesis Test (1 tail, 0.05 level of significance), and the Maximum Likelihood-Probit Test to develop dose-response curves for each experiment.

Results

The 48 h LC values were calculated from the TAN and NO_2-N at 10 ppt and then used to determine dosages for combined ammonia-nitrite tests are provided in Tables 4 and 5. The LC50 values of all tests are reported in Tables 6 and 7.

Ammonia Toxicity Trials

In tests at 18 ppt salinity, no deaths were recorded in the control over 48 h. At 20 and 40 mg/L TAN, mortalities of 4.2 and 12.5% were observed, respectively, over 48 h. Mortality levels of 46 and 96% were recorded for shrimp exposed to 60 mg/L TAN over 24 and 48 h, respectively, while shrimp exposed to 80 mg/L exhibited 67 and 100% mortality over the same time intervals. All shrimp tested at 100 mg/L were dead after 12 h.

When the salinity was lowered from 18 ppt to 10 ppt, no significant reduction ($p>0.05$) in LC50 values was observed after 48 h. No shrimp mortalities were recorded for the control and 10 mg/L TAN over 48 h. At 20, 30 and 40 mg/L TAN mortality rates were observed to be 4.2, 25, and 46%, respectively, after 48 h. After 24 h, the mortality rates for 50, 60, 70, and 80 mg/L were observed to be 13, 25, 46, and 71% respectively. Mortality rates for the same experimental conditions after 48 h were observed to be 67, 88, 92 and 100%, respectively, (See Fig. 1).

Nitrite Toxicity Trials

In tests with NO_2-N at 10 ppt salinity, no deaths were observed in the control and at 90 mg/L NO_2-N at 48 h. For 110, 130, and 150 mg/L NO_2-N mortality rates after 48 h were observed to be 8, 25, and 50%, respectively. After 24 h, the mortality rates for 170 and 190 mg/L were observed to be 33 and 29%, respectively. Mortality rates for the same experimental conditions after 48 h were observed to be 67 and 79%, respectively, (See Fig. 2).

Ammonia and Nitrite Toxicity Trials

The LOEC calculated for NO_2-N at 10 ppt salinity was 133 mg/L NO_2-N. This concentration was administered in all trials, except for the control, along with varying TAN concentrations equal to the LC20, LC40, LC50, LC60, and LC80 of the TAN test at 10 ppt salinity. A mortality rate of 4.2% occurred in the control tests after 48 h. This was identified to be the result of cannibalism, as only part of the mort was found. The mortality rates at 29.1 (LC20), 36.2 (LC40), 39.7 (LC50), and 43.73 (LC60) mg/L TAN were observed to be 33, 42, 79, and 93%, respectively, after 24 h. After 48 h, the same experimental conditions showed mortality rates of 63, 88, 96 and 100%, respectively. All shrimp tested at 54.2 mg/L TAN (LC80) were dead after 12 h, (See Fig. 3).

The LOEC calculated for TAN at 10 ppt salinity was 30.0 mg/L. This concentration was administered in all tests, except for the control, along with varying NO_2-N concentrations equal to the LC20, LC40, LC50, LC60, and LC80 values of the TAN test at 10 ppt salinity. No deaths were observed in the control and 128 mg/L NO_2-N (LC20) experiments after 48 h. The mortality rates at 146 (LC40), 154 (LC50), 162 (LC60), and 185 mg/L NO_2-N (LC80) were observed to be 8, 17, 25, and 42%, respectively, after 48 h, (See Fig. 4).

Discussion

Ammonia Toxicity Trials

The investigators of prior research on the toxicity of ammonia to shrimp have conducted experiments that vary the salinity levels of test solutions, but the information available is not complete. Conditions, such as the species and age of shrimp, as well as the temperature and pH levels of test solutions are important in understanding the toxicity of ammonia to cultured shrimp and the effects such knowledge can have on "Standard Operating Procedures." The 48 h NOEC, LOEC, and LC50 (20.5, 30.0, and 35.9 mg/L TAN, respectively) observed during this experiment are products of certain specified conditions (PL42, 28 C, and 7.8 pH). Compared to Chen and Lin (1992), who found a 48 h LC50 of 2.39 mg/L TAN for juvenile P. chinensis at 10 ppt salinity, L. vannamei exhibit a higher tolerance to ammonia levels. This is also true when compared to P. semisulcatus as studied by Kir and Kumlu (2006), who reported a 48 h LC50 of 11.2 mg/L TAN at 15 ppt salinity.

Nitrite Toxicity Trials

Authors who have conducted nitrite studies at low salinities (15 ppt) have shown the 48 h LC50 of nitrite to L. vannamei juveniles to be around 143 mg/L NO_2-N with a 95% confidence interval of 137.6 to 148.4 (Lin and Chen 2003). This is similar to the data collected in this study due to overlapping confidence intervals. The calculated LC50 with 95% Fiducial Limits was for this study were 154 mg/L NO_2-N and 146 to 163, respectively. This difference could be accounted for due to the stock of shrimp purchased or another unnoted variance in water parameter.

Ammonia and Nitrite Toxicity Trials

Understanding the combined effects of TAN and NO_2-N on L. vannamei at a pH of 7.8 and temperature of 28 C allows for better control during the production of shrimp in RAS. Prior research has shown conflicting results as to the synergistic or antagonistic effects of elevated TAN and NO_2-N levels; one suggested that synergistic effects take place up to 48 h and antagonistic beyond, while the other suggested antagonistic effects being followed by synergistic effects after 96 h (Alcaraz et al. 1999a; Chen and Chin 1988). Both studies related the exposure time to varying synergistic or antagonistic effects. A variance of synergistic and antagonistic effects related to the dominant toxin present was observed during this study.

The data collected from this study suggested a synergistic effect ($p<0.05$) when NO_2-N levels were adjusted to the 48 h LOEC concentrations (138 mg/L NO_2-N determined from previous test) and TAN concentrations were varied, as seen when Figs. 1 and 3 are compared. The LC50 at 48 h calculated from the TAN test at 10 ppt salinity was 11.5 mg/L TAN higher than when TAN was tested with adjusted NO_2-N (39.72 vs. 28.24 mg/L TAN respectively). These results suggested that the elevated NO_2-N levels might weaken the shrimp, resulting in a lower tolerance to elevated TAN levels.

When TAN levels were held constant at the determined 48 h LOEC level and NO_2-N levels were varied between determined LC20 and LC80 values, antagonistic characteristics ($p<0.05$) were observed, as seen when Figs. 2 and 4 are compared. The LC50 value during this test was calculated to be 33 mg/L NO_2-N higher when compared to the single toxin NO_2-N experiment at 10 ppt salinity, a shift from 154 to 187 mg/L NO_2-N. When compared to other studies involving combined ammonia and nitrite, this study differed due to the species tested as well as the salinity of test solutions. These variables inhibited a direct comparison, because both variables have been shown to effect tolerance to ammonia and nitrite (Chen and Lin 1992, Lin and Chen 2003). Alcaraz et al.

(1999a) and Chen and Chin's (1988) experiments with P. setiferus and P. monodon were conducted at 25 ppt salinity and natural seawater, respectively. The approach to the studies also varied due to exposure concentrations.

Salinity

The reduced salinity concentration used in these tests was chosen to begin the analysis of the effects of low salinity on the tolerance of L. vannamei to combined elevated TAN and NO_2-N levels. It was assumed that the concentrations of essential nutrients incorporated into the salts used would be directly proportional to the amount of salt used. When the salinity was lowered from 18 to 10 ppt for individual TAN experiments, no significant difference ($p>0.05$) was observed. This was possibly due to a variance in the age of the shrimp.

Application to Wild Populations

Although this research was conducted in a controlled laboratory, the results can be applied to wild populations of L. vannamei. The life cycle of L. vannamei includes an oceanic planktonic stage, an estuarine post larval stage and finally, and adult oceanic stage that involves maturation and reproduction (Valles-Jimenez *et al.* 2004). The effects of low salinities on PL explored during this study can be related to estuarine environments, where the salinity of the water varies according to tides and precipitation. Elevated nitrogenous waste product levels are possible in such regions due to industrial processes and wastes, as well as natural decomposition of organic matter. Stagnant water, due to slack tides, or natural pools are examples of areas where concentrated ammonia and nitrite could be observed.

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Species	Age	Size	48 h LC50 (mg/L TAN)	Author
P. monodon	Juvenile	4.87 g	88	Chen *et al.* 1990a
P. chinensis	PL	0.36 g	51.1	Chen *et al.* 1990b
P. paulensis	Juvenile	5.45 g	43.1	Ostrensky and Wasielesky 1995
P. semisulcatus	PL	.028 g	32.5	Kir and Kumlu 2006
L. vannamei	Juvenile	0.99 g	92.5	Frias-Espericueta et al. 1999
L. vannamei	Juvenile	3.8 g	110.6	Frias-Espericueta et al. 1999

Table 1: 48 h LC50 values for varying species and ages of shrimp

AHAB Water Quality Parameters	
pH	7.9 ± 0.2
Temp	29 ± 1.1 C
DO	> 5.5 mg/L
Salinity	28 ppt
NH_3	< 0.8 mg/L NH_3-N
NO_2	< 1.0 mg/L NO_2-N

Table 2: Water quality maintained in AHAB systems

Parameter	Equipment	Method
Ammonia	Hach Spectrophotometer DR2800	Hach – Nessler 8038
Nitrite	Hach Spectrophotometer DR2800	Hach - 8507/8153
Alkalinity	Hach Digital Titrator 16900	Hach – 8203
Hardness	Hach Digital Titrator 16900	Hach – 8213
DO	YSI 85	
Salinity	YSI 85	
Temperature	YSI 85	
pH	YSI PH10	

Table 3: Methods for monitoring water quality.

48 h LC values for TAN			
	mg/L TAN	95% Fiducial Limits	
LOEC	30.0	-	-
LC20	29.1	24.5	32.7
LC40	36.2	32.1	39.7
LC50	39.7	35.9	43.4
LC60	43.6	39.8	47.8
LC80	54.2	49.4	61.3

Table 4: LC results for Ammonia-N at 10 ppt salinity

48 h LC values for NO_2-N			
	mg/L NO2-N	95% Fiducial Limits	
LOEC	132.8	-	-
LC20	128.0	116.4	136.1
LC40	145.5	137.0	153.7
LC50	153.7	145.6	163.3
LC60	162.5	153.8	174.6
LC80	184.6	172.2	207.1

Table 5: LC results for NO_2-N at 10 ppt salinity

LC50's (with 95% Fiducial Limits) mg/L TAN			
	Endpoints		
Experiment	12 h	24 h	48 h
Ammonia 18ppt	67.9 (62.1 - 73.7)	63.0 (57.4-68.1)	42.9
Ammonia 10ppt	N/A	69.9 (63.7-80.4)	39.7 (35.9-43.4)
Ammonia/Nitrite 10ppt[a]	52.0 (47.7-61.3)	34.2 (31.5-36.2)	28.2 (22.9-30.8)

[a] Nitrite stable at 133 mg/L NO_2-N with ammonia-N concentration varying

Table 6: LC50 results for TAN at varying salinities

LC50's (with 95% Fiducial Limits) mg/L NO_2-N		
	Endpoints	
Experiment	24 h	48 h
Nitrite 10ppt	199 (183-248)	154 (146-163)
Nitrite/Ammonia 10ppt	-	187 (175-219)

Table 7: LC50 results for NO_2-N tests with and without TAN at 30.0 mg/l

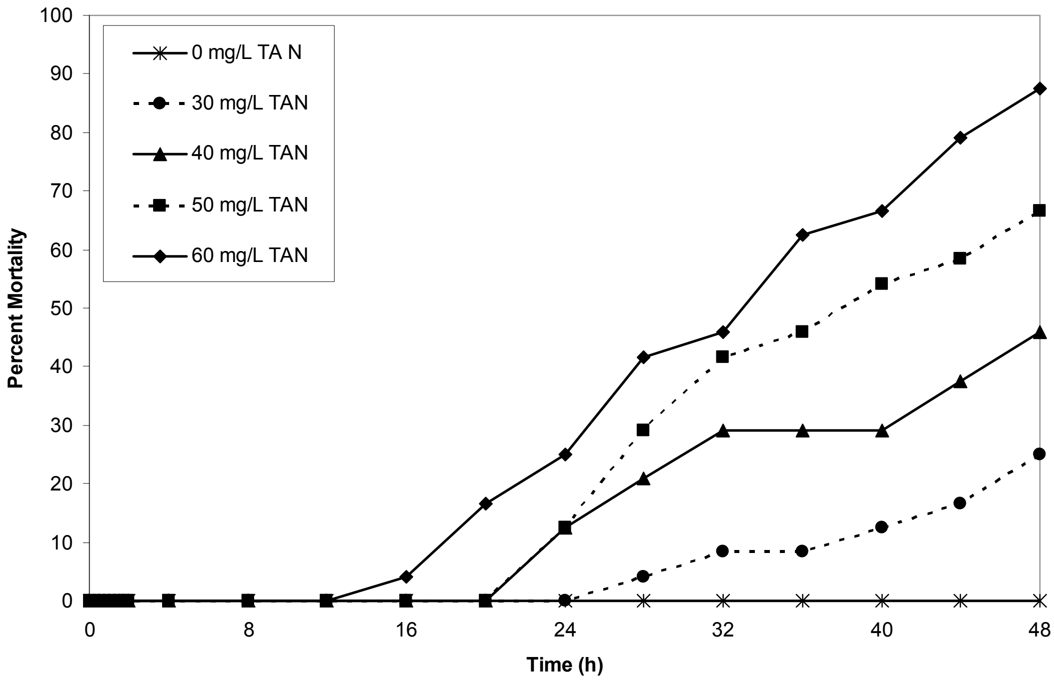

Figure 1: Percent mortality over time due to varying TAN-N concentrations at 10 ppt salinity.

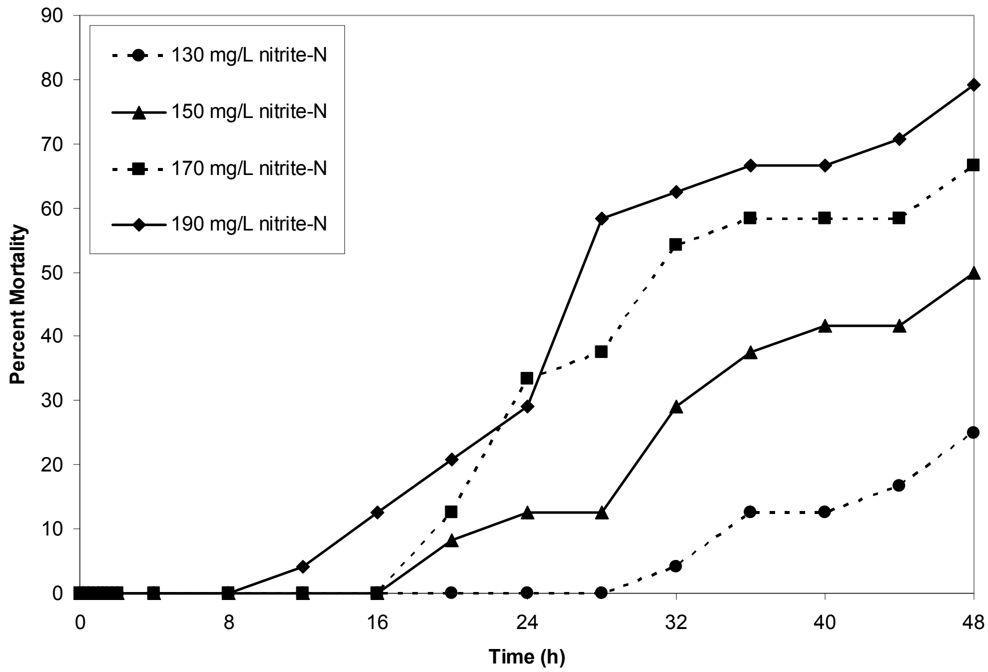

Figure 2: Percent mortality over time due to varying NO_2-N concentrations at 10 ppt salinity.

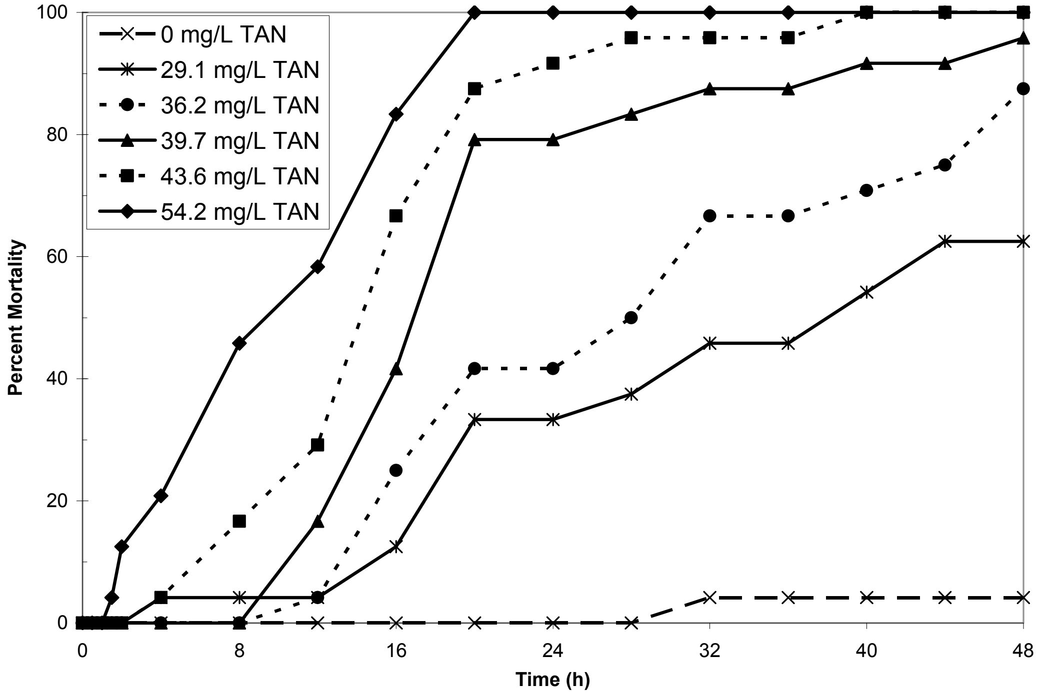

Figure 3: Percent mortality over time due to varying TAN-N concentrations with an adjusted concentration of NO_2-N at 133 mg/L and 10 ppt salinity.

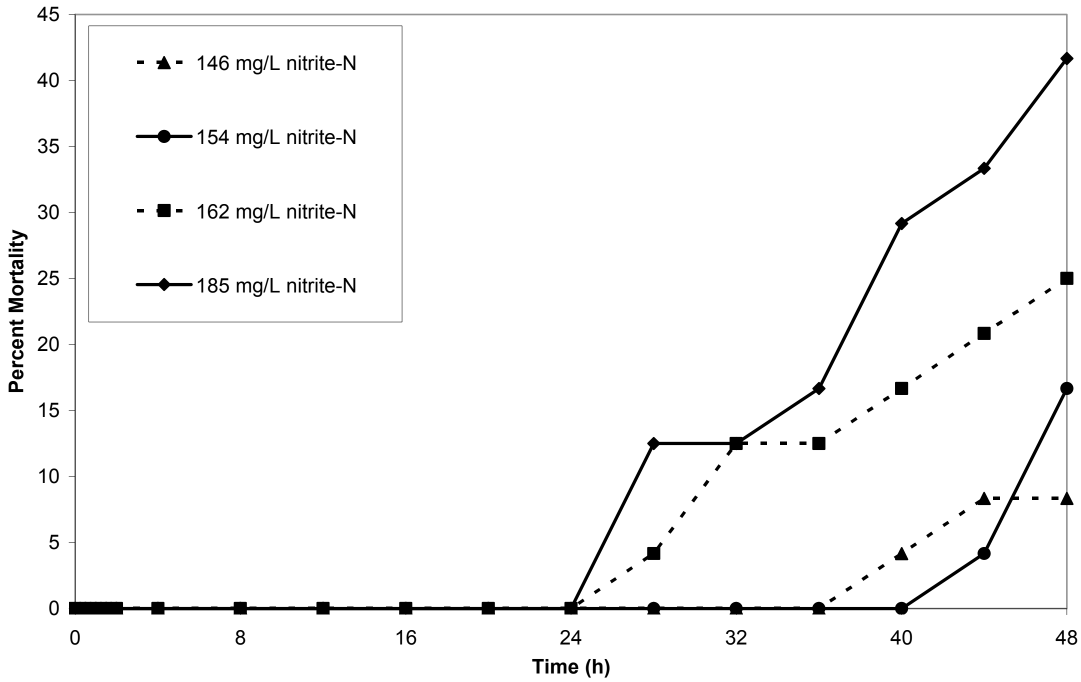

Figure 4: Percent mortality over time due to varying NO_2-N concentrations with an adjusted concentration of TAN-N at 30.0 mg/L and 10 ppt salinity.

Chapter 4: Conclusions

The objectives of this study were fulfilled. The background study conducted at 18 ppt salinity provided data that verified the protocol and system design. Experiments that addressed the individual toxicity of ammonia and nitrite at low salinities resulted in data, which not only verified previous data within the literature but also addressed new issues. The experiments involving combined adjusted levels of ammonia and nitrite led to valuable insight as to the mutualistic effects of the two agents. All of the knowledge gained can be directly applied to the “standard operating procedures” of Virginia Shrimp Farms (VSF) in Martinsville, Va in an effort to evaluate production losses due to elevated ammonia and nitrite.

Future research should focus on gathering data for younger shrimp. Since shrimp had to be shipped from Florida and then acclimated, there was a limit as to how young shrimp could be for experiments. With VSF now on-line, younger shrimp can be obtained and tested to provide VSF with valuable knowledge that encompasses not only the acute effects of ammonia and nitrite, but also any chronic effects such as reduced growth. The breeding program at VSF could also benefit from experiments that focused on larval shrimp and any problems with development due to elevated ammonia and nitrite levels.

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Appendix A:

Raw Data

Ammonia 18 ppt - Mortalities

	Dose (mg/L Ammonia-N) with Replicates																	
		0.274			19.46			38.42			57.8			75.55			95.37	
Time (h)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
0.5	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
1	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
1.5	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
2	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	8
4	8	8	8	8	8	8	8	8	8	8	7	8	8	6	7	5	5	6
8	8	8	8	8	8	8	8	8	8	7	2	6	5	4	4	1	0	0
12	8	8	8	8	8	8	8	8	8	7	2	6	5	4	4	0	0	0
16	8	8	8	8	8	8	8	8	8	5	2	6	3	4	3	0	0	0
20	8	8	8	8	8	8	8	8	8	5	2	6	2	3	3	0	0	0
24	8	8	8	8	8	8	8	8	8	5	2	6	2	3	3	0	0	0
28	8	8	8	8	8	8	8	8	8	5	2	6	1	3	2	0	0	0
32	8	8	8	8	8	8	8	7	8	5	2	5	1	2	0	0	0	0
36	8	8	8	8	8	7	7	7	8	3	2	4	0	0	0	0	0	0
40	8	8	8	8	8	7	7	7	8	1	2	2	0	0	0	0	0	0
44	8	8	8	8	8	7	7	7	8	0	1	0	0	0	0	0	0	0
48	8	8	8	8	8	7	7	6	8	0	1	0	0	0	0	0	0	0

Ammonia 10 ppt - Mortalities

	Dose (mg/L Ammonia-N) with Replicates																										
	0			10			20			30			40			50			60			70			80		
Time (h)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
0.5	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
1	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
1.5	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
2	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
4	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
12	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	7	8	8	8
16	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	6	7	5	6	6	5
20	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	7	7	6	6	5	2	3	4	4
24	8	8	8	8	8	8	8	8	8	8	8	8	7	6	8	7	7	7	6	7	5	6	5	2	2	3	2
28	8	8	8	8	8	8	8	8	8	7	8	8	6	6	7	6	6	5	4	5	5	5	4	2	2	1	0
32	8	8	8	8	8	8	8	8	8	7	7	8	6	4	7	4	5	5	4	4	5	4	4	1	2	0	0
36	8	8	8	8	8	8	8	8	8	7	7	8	6	4	7	4	4	5	3	3	3	2	3	1	2	0	0
40	8	8	8	8	8	8	8	8	8	7	6	8	6	4	7	3	4	4	2	3	3	2	2	1	1	0	0
44	8	8	8	8	8	8	8	7	8	7	6	7	5	4	6	3	4	3	1	3	1	1	2	1	1	0	0
48	8	8	8	8	8	8	8	7	8	5	6	7	4	3	6	3	3	2	1	2	0	1	1	0	0	0	0

Nitrite 10ppt – Mortalities

	Dose (mg/L Nitrite-N) with Replicates																				
	0			90			110			130			150			170			190		
Time (h)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
0.5	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
1	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
1.5	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
2	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
4	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
12	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8
16	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	7	6
20	8	8	8	8	8	8	8	8	8	8	8	8	6	8	8	8	6	7	7	7	5
24	8	8	8	8	8	8	8	8	8	8	8	8	6	8	7	5	6	5	6	6	5
28	8	8	8	8	8	8	8	8	8	8	8	8	6	8	7	4	6	5	3	4	3
32	8	8	8	8	8	8	8	8	8	7	8	8	5	6	6	3	4	4	3	4	2
36	8	8	8	8	8	8	8	7	8	6	8	7	4	6	5	3	3	4	3	3	2
40	8	8	8	8	8	8	8	7	7	6	8	7	4	5	5	3	3	4	3	3	2
44	8	8	8	8	8	8	8	7	7	6	7	7	4	5	5	3	3	4	3	2	2
48	8	8	8	8	8	8	8	7	7	6	7	5	4	5	3	2	3	3	2	2	1

AvNs 10 ppt - Mortalities

Time (h)	*Dose (mg/L Ammonia-N) with Replicates*																	
	0			29.1			36.2			39.7			43.6			54.2		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
0.5	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
1	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
1.5	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8
2	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	7	6	8
4	8	8	8	7	8	8	8	8	8	8	8	8	7	8	8	7	6	6
8	8	8	8	7	8	8	8	8	8	8	8	8	7	7	6	5	4	4
12	8	8	8	7	8	8	8	8	7	7	6	7	6	6	5	4	3	3
16	8	8	8	7	6	8	6	7	5	4	5	5	3	2	3	2	1	1
20	8	8	8	5	5	6	4	5	5	0	3	2	1	2	0	0	0	0
24	8	8	8	5	5	6	4	5	5	0	3	2	0	2	0	0	0	0
28	8	8	8	5	4	6	4	3	5	0	2	2	0	1	0	0	0	0
32	8	7	8	5	4	4	2	2	4	0	2	1	0	1	0	0	0	0
36	8	7	8	5	4	4	2	2	4	0	2	1	0	1	0	0	0	0
40	8	7	8	5	3	3	2	1	4	0	1	1	0	0	0	0	0	0
44	8	7	8	4	3	2	2	1	3	0	1	1	0	0	0	0	0	0
48	8	7	8	4	3	2	1	0	2	0	0	1	0	0	0	0	0	0

AsNv 10 ppt – Mortalities

	Dose (mg/L Nitrite-N) with Replicates																	
	0			128			146			154			162			185		
Time (h)	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
0.5	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
1	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
1.5	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
2	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
4	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
12	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
16	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
20	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
24	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8	8
28	8	8	8	8	8	8	8	8	8	8	8	8	8	7	8	6	8	7
32	8	8	8	8	8	8	8	8	8	8	8	8	7	6	8	6	8	7
36	8	8	8	8	8	8	8	8	8	8	8	8	7	6	8	6	7	7
40	8	8	8	8	8	8	8	8	7	8	8	8	7	6	7	5	6	6
44	8	8	8	8	8	8	7	8	7	8	7	8	6	6	7	4	6	6
48	8	8	8	8	8	8	7	8	7	7	7	6	6	5	7	4	5	5

Appendix B:

Toxcalc Summary Reports

Acute Shrimp Test-12 hr Survival

Start Date:	12/18/2007 18:00	Test ID:	1A	Sample ID:	ASTOCK-Ammonium Chloride Stock
End Date:	12/20/2007 18:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	AMCL-Ammonium Chloride
Sample Dat	12/18/2007	Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei
Comments:					

Conc-mg/L	1	2	3
'4 D-Control	1.0000	1.0000	1.0000
19.46	1.0000	1.0000	1.0000
38.24	1.0000	1.0000	1.0000
57.8	0.8750	0.2500	0.7500
75.55	0.6250	0.5000	0.5000
95.37	0.0000	0.0000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
'4 D-Control	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3				0	24
19.46	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.470	0.3299	0	24
38.24	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.470	0.3299	0	24
*57.8	0.6250	0.6250	0.9267	0.5236	1.2094	38.677	3	3.492	2.470	0.3299	9	24
*75.55	0.5417	0.5417	0.8275	0.7854	0.9117	8.815	3	4.235	2.470	0.3299	11	24
95.37	0.0000	0.0000	0.1777	0.1777	0.1777	0.000	3				24	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)	0.718479	0.835	-1.270504	6.1775356
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	38.24	57.8	47.01353		0.2050351	0.2116492	0.2432848	0.0267587	0.0022965	4, 10
Treatments vs 0.274 D-Control										

					Maximum Likelihood-Probit						
Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	9.4665168	1.6962854	6.1417972	12.791236	0	9.0345291	9.4877291	0.06	1.8320405	0.1056355	7
Intercept	-12.34304	3.131866	-18.4815	-6.204585							
TSCR											

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	38.574086	27.579747	45.663111
EC05	3.355	45.528738	35.42465	51.890576
EC10	3.718	49.735123	40.413378	55.64469
EC15	3.964	52.790573	44.117614	58.399576
EC20	4.158	55.352271	47.252999	60.749337
EC25	4.326	57.648825	50.068192	62.9053
EC40	4.747	63.867227	57.543433	69.142906
EC50	5.000	67.926698	62.13257	73.702132
EC60	5.253	72.244194	66.626753	79.105505
EC75	5.674	80.036953	73.760266	90.266606
EC80	5.842	83.35767	76.514472	95.473808
EC85	6.036	87.402659	79.714847	102.10284
EC90	6.282	92.772188	83.77268	111.31339
EC95	6.645	101.34338	89.943337	126.83399
EC99	7.326	119.61493	102.32593	162.72616

Acute Shrimp Test-24 hr Survival

Start Date:	12/18/2007 18:00	Test ID:	1A	Sample ID:	ASTOCK-Ammonium Chloride Stock
End Date:	12/20/2007 18:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	AMCL-Ammonium Chloride
Sample Dat	12/18/2007	Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei
Comments:					

Conc-mg/L	1	2	3
'4 D-Control	1.0000	1.0000	1.0000
19.46	1.0000	1.0000	1.0000
38.24	1.0000	1.0000	1.0000
57.8	0.6250	0.2500	0.7500
75.55	0.2500	0.3750	0.3750
95.37	0.0000	0.0000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
'4 D-Control	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3				0	24
19.46	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.470	0.2551	0	24
38.24	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.470	0.2551	0	24
*57.8	0.5417	0.5417	0.8275	0.5236	1.0472	32.842	3	5.477	2.470	0.2551	11	24
*75.55	0.3333	0.3333	0.6139	0.5236	0.6591	12.739	3	7.546	2.470	0.2551	16	24
95.37	0.0000	0.0000	0.1777	0.1777	0.1777	0.000	3				24	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)	0.7532141	0.835	-1.175823	5.4787565
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	38.24	57.8	47.01353		0.1446376	0.1493033	0.4239927	0.0159952	2.6E-05	4, 10
Treatments vs 0.274 D-Control										

			Maximum Likelihood-Probit								
Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	10.035093	1.742805	6.6191949	13.450991	0	4.4211213	9.4877291	0.35	1.7988541	0.0996503	6
Intercept	-13.05167	3.1757941	-19.27622	-6.827111							
TSCR											

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	36.90055	26.932091	43.413002
EC05	3.355	43.146338	33.968398	49.0272
EC10	3.718	46.897221	38.384667	52.390174
EC15	3.964	49.610455	41.641519	54.844809
EC20	4.158	51.878367	44.387321	56.926917
EC25	4.326	53.906483	46.847521	58.825377
EC40	4.747	59.375597	53.387766	64.230817
EC50	5.000	62.929474	57.441803	68.087372
EC60	5.253	66.696067	61.461902	72.576847
EC75	5.674	73.462754	67.905632	81.739532
EC80	5.842	76.334684	70.38949	86.00127
EC85	6.036	79.82428	73.262631	91.420731
EC90	6.282	84.4425	76.883249	98.934987
EC95	6.645	91.783422	82.346691	111.54008
EC99	7.326	107.3187	93.199568	140.37364

Acute Shrimp Test-48 hr Survival

Start Date:	12/18/2007 18:00	Test ID:	1A	Sample ID:	ASTOCK-Ammonium Chloride Stock
End Date:	12/20/2007 18:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	AMCL-Ammonium Chloride
Sample Dat	12/18/2007	Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei
Comments:					

Conc-mg/L	1	2	3
'4 D-Control	1.0000	1.0000	1.0000
19.46	1.0000	1.0000	0.8750
38.24	0.8750	0.7500	1.0000
57.8	0.0000	0.1250	0.0000
75.55	0.0000	0.0000	0.0000
95.37	0.0000	0.0000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
'4 D-Control	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3				0	24
19.46	0.9583	0.9583	1.3319	1.2094	1.3931	7.961	3	0.655	2.420	0.2262	1	24
38.24	0.8750	0.8750	1.2166	1.0472	1.3931	14.225	3	1.888	2.420	0.2262	3	24
*57.8	0.0417	0.0417	0.2389	0.1777	0.3614	44.379	3	12.346	2.420	0.2262	23	24
75.55	0.0000	0.0000	0.1777	0.1777	0.1777	0.000	3				24	24
95.37	0.0000	0.0000	0.1777	0.1777	0.1777	0.000	3				24	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.9740677	0.805	0.0750676	-0.046793
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	38.24	57.8	47.01353		0.1232334	0.1272086	0.8826425	0.0131086	5.1E-06	3, 8
Treatments vs 0.274 D-Control										

			Maximum Likelihood-Probit								
Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	8.9370387	5.567157	-6.519867	24.393945	0	43.442045	9.4877291	8.4E-09	1.6326952	0.1118939	7
Intercept	-9.59146	9.329007	-35.49294	16.310016							
TSCR											

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	23.571708		
EC05	3.355	28.096103		
EC10	3.718	30.852998		
EC15	3.964	32.864317		
EC20	4.158	34.555954		
EC25	4.326	36.076457		
EC40	4.747	40.211213		
EC50	5.000	42.923503		
EC60	5.253	45.818742		
EC75	5.674	51.070069		
EC80	5.842	53.317213		
EC85	6.036	56.061631		
EC90	6.282	59.716306		
EC95	6.645	65.575893		
EC99	7.326	78.162656		

Significant heterogeneity detected (p = 8.38E-09)

Acute Shrimp Test-24 hr Survival

Start Date:	2/18/2006 13:00	Test ID:	1A10	Sample ID:	ASTOCK-Ammonium Chloride Stock
End Date:	2/20/2008 13:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	AMCL-Ammonium Chloride
Sample Dat	2/18/2008	Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei
Comments:					

Conc-mg/L	1	2	3
36 D-Control	1.0000	1.0000	1.0000
10.31	1.0000	1.0000	1.0000
20.48	1.0000	1.0000	1.0000
29.97	1.0000	1.0000	1.0000
38.65	0.8750	0.7500	1.0000
50.23	0.8750	0.8750	0.8750
58.33	0.7500	0.8750	0.6250
69.41	0.7500	0.6250	0.2500
78.92	0.2500	0.3750	0.2500

			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
36 D-Control	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3				0	24
10.31	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.580	0.2553	0	24
20.48	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.580	0.2553	0	24
29.97	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.580	0.2553	0	24
38.65	0.8750	0.8750	1.2166	1.0472	1.3931	14.225	3	1.784	2.580	0.2553	3	24
50.23	0.8750	0.8750	1.2094	1.2094	1.2094	0.000	3	1.856	2.580	0.2553	3	24
*58.33	0.7500	0.7500	1.0561	0.9117	1.2094	14.113	3	3.406	2.580	0.2553	6	24
*69.41	0.5417	0.5417	0.8275	0.5236	1.0472	32.842	3	5.717	2.580	0.2553	11	24
*78.92	0.2917	0.2917	0.5688	0.5236	0.6591	13.751	3	8.332	2.580	0.2553	17	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)	0.8129228	0.894	-0.61145	3.0770376
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	50.23	58.33	54.128698		0.1447815	0.1494518	0.2601976	0.0146821	4.7E-07	8, 18
Treatments vs .286 D-Control										

					Maximum Likelihood-Probit						
Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	6.2970073	1.1729739	3.9979784	8.5960361	0	4.48646	14.067141	0.72	1.8446505	0.1588056	3
Intercept	-6.615778	2.0900758	-10.71233	-2.519229							
TSCR											

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	29.868503	19.676519	36.572794
EC05	3.355	38.321153	28.906089	44.244252
EC10	3.718	43.765608	35.343298	49.167087
EC15	3.964	47.869524	40.33412	52.982782
EC20	4.158	51.403935	44.631968	56.43545
EC25	4.326	54.643408	48.480506	59.824314
EC40	4.747	63.740812	58.178082	71.124141
EC50	5.000	69.927907	63.711652	80.426454
EC60	5.253	76.715563	69.130388	91.7889
EC75	5.674	89.487686	78.36053	115.52961
EC80	5.842	95.127196	82.201372	126.81146
EC85	6.036	102.15085	86.849405	141.46904
EC90	6.282	111.72955	92.996338	162.47559
EC95	6.645	127.60347	102.80307	199.70237
EC99	7.326	163.71469	123.82629	294.65677

Acute Shrimp Test-48 hr Survival

Start Date:	2/18/2006 13:00	Test ID:	1A10	Sample ID:	ASTOCK-Ammonium Chloride Stock
End Date:	2/20/2008 13:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	AMCL-Ammonium Chloride
Sample Dat	2/18/2008	Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei

Comments:

Conc-mg/L	1	2	3
36 D-Control	1.0000	1.0000	1.0000
10.31	1.0000	1.0000	1.0000
20.48	1.0000	0.8750	1.0000
29.97	0.6250	0.7500	0.8750
38.65	0.5000	0.3750	0.7500
50.23	0.3750	0.3750	0.2500
58.33	0.1250	0.2500	0.0000
69.41	0.1250	0.1250	0.0000
78.92	0.0000	0.0000	0.0000

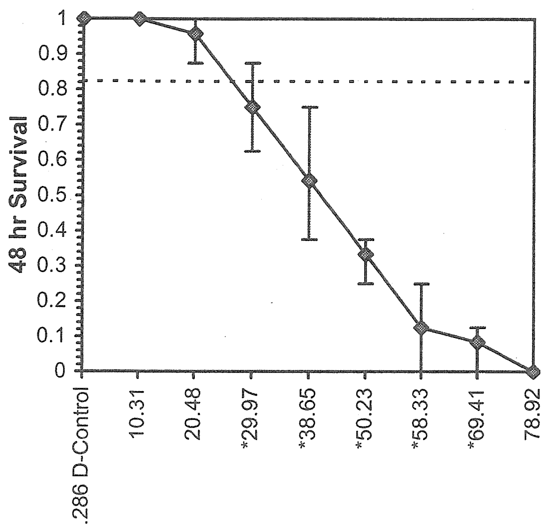
			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
36 D-Control	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3				0	24
10.31	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.560	0.2560	0	24
20.48	0.9583	0.9583	1.3319	1.2094	1.3931	7.961	3	0.612	2.560	0.2560	1	24
*29.97	0.7500	0.7500	1.0561	0.9117	1.2094	14.113	3	3.370	2.560	0.2560	6	24
*38.65	0.5417	0.5417	0.8306	0.6591	1.0472	23.836	3	5.626	2.560	0.2560	11	24
*50.23	0.3333	0.3333	0.6139	0.5236	0.6591	12.739	3	7.793	2.560	0.2560	16	24
*58.33	0.1250	0.1250	0.3542	0.1777	0.5236	48.854	3	10.390	2.560	0.2560	21	24
*69.41	0.0833	0.0833	0.3001	0.1777	0.3614	35.327	3	10.931	2.560	0.2560	22	24
78.92	0.0000	0.0000	0.1777	0.1777	0.1777	0.000	3				24	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.9453489	0.884	0.0747641	-0.096561
Equality of variance cannot be confirmed				

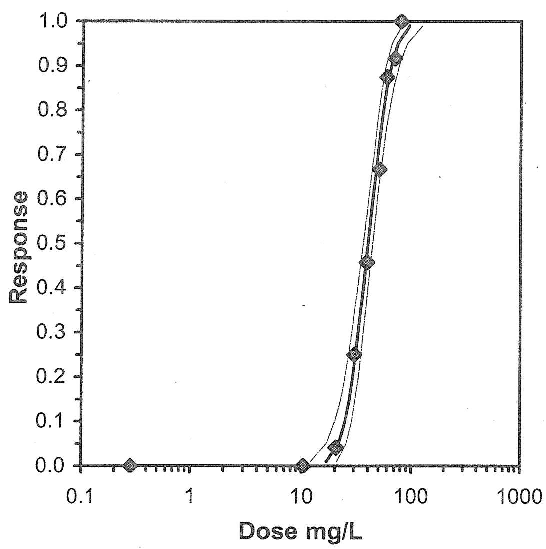
Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	20.48	29.97	24.774697		0.1453178	0.1500055	0.6175069	0.0149947	4.6E-09	7, 16
Treatments vs .286 D-Control										

			Maximum Likelihood-Probit								
Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	6.2246443	0.7915181	4.6732688	7.7760197	0	1.7708547	14.067141	0.97	1.598994	0.1606518	3
Intercept	-4.953169	1.3052026	-7.511366	-2.394972							
TSCR											

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	16.798178	12.02285	20.671275
EC05	3.355	21.614512	16.732191	25.426734
EC10	3.718	24.723534	19.924997	28.438482
EC15	3.964	27.07006	22.393104	30.7018
EC20	4.158	29.09284	24.549198	32.657297
EC25	4.326	30.948249	26.541047	34.46323
EC40	4.747	36.16541	32.134761	39.680348
EC50	5.000	39.71861	35.851698	43.434352
EC60	5.253	43.620909	39.762302	47.826006
EC75	5.674	50.974386	46.555626	56.942948
EC80	5.842	54.225301	49.350694	61.287987
EC85	6.036	58.277229	52.701905	66.923978
EC90	6.282	63.808352	57.097365	74.948916
EC95	6.645	72.986518	64.072839	88.95467
EC99	7.326	93.913054	79.060963	123.40974



Test ID: 1A10
Sample ID: Ammonium Chloride Stock
Sample Type: Ammonium Chloride
Method: Dunnett's Test



Test ID: 1A10
Sample ID: Ammonium Chloride Stock
Sample Type:Ammonium Chloride
Method: Maximum Likelihood-Probit

Acute Shrimp Test-24 hr Survival

Start Date:	2/25/2006 13:00	Test ID:	1N10	Sample ID:	NO2STOCK
End Date:	2/27/2008 13:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	NANO2
Sample Dat	2/25/2008	Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei

Comments:

Conc-mg/L	1	2	3
.0 D-Control	1.0000	1.0000	1.0000
90.4	1.0000	1.0000	1.0000
114.6	1.0000	1.0000	1.0000
132.8	1.0000	1.0000	1.0000
148.8	0.7500	1.0000	0.8750
170.2	0.6250	0.7500	0.6250
187	0.7500	0.7500	0.6250

			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
.0 D-Control	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3				0	24
90.4	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.530	0.1604	0	24
114.6	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.530	0.1604	0	24
132.8	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.530	0.1604	0	24
*148.8	0.8750	0.8750	1.2166	1.0472	1.3931	14.225	3	2.785	2.530	0.1604	3	24
*170.2	0.6667	0.6667	0.9569	0.9117	1.0472	8.173	3	6.882	2.530	0.1604	8	24
*187	0.7083	0.7083	1.0020	0.9117	1.0472	7.805	3	6.170	2.530	0.1604	7	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)	0.812308	0.873	0.1292139	3.8688993
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	132.8	148.8	140.57254		0.0787503	0.0812906	0.1152028	0.0060258	5.3E-06	6, 14
Treatments vs 0.0 D-Control										

					Maximum Likelihood-Probit						
Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	10.666049	2.82458	5.1298719	16.202226	0	3.4863561	9.4877291	0.48	2.2988409	0.0937554	6
Intercept	-19.51955	6.2716133	-31.81191	-7.227187							
TSCR											

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	120.42958	84.162104	136.1086
EC05	3.355	139.51695	113.17352	151.41443
EC10	3.718	150.89975	131.47534	161.55176
EC15	3.964	159.09982	144.12602	170.34319
EC20	4.158	165.93361	153.48587	179.47277
EC25	4.326	172.02987	160.57534	189.35794
EC40	4.747	188.4032	175.19671	222.60015
EC50	5.000	198.99444	182.94834	247.59741
EC60	5.253	210.18107	190.51142	276.17018
EC75	5.674	230.18553	203.17396	332.13813
EC80	5.842	238.64234	208.30931	357.58024
EC85	6.036	248.89272	214.40439	389.80585
EC90	6.282	262.41783	222.26366	434.635
EC95	6.645	283.82777	234.34984	510.94288
EC99	7.326	328.8128	258.61624	692.61406

Acute Shrimp Test-48 hr Survival

Start Date:	2/25/2006 13:00	Test ID:	1N10	Sample ID:	NO2STOCK
End Date:	2/27/2008 13:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	NANO2
Sample Dat	2/25/2008	Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei
Comments:					

Conc-mg/L	1	2	3
.0 D-Control	1.0000	1.0000	1.0000
90.4	1.0000	1.0000	1.0000
114.6	1.0000	0.8750	0.8750
132.8	0.7500	0.8750	0.6250
148.8	0.5000	0.6250	0.3750
170.2	0.2500	0.3750	0.3750
187	0.2500	0.2500	0.1250

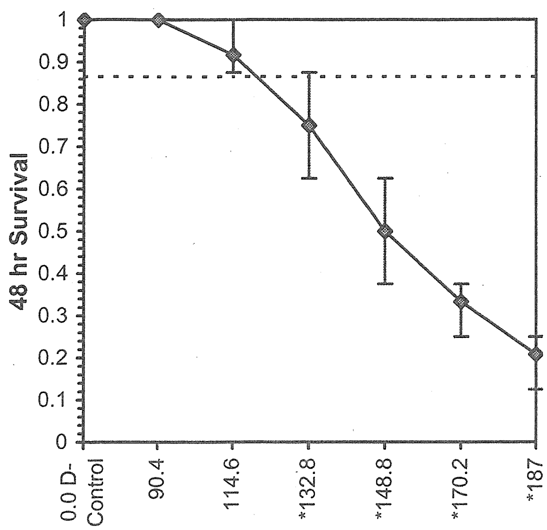
			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
.0 D-Control	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3				0	24
90.4	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.530	0.1980	0	24
114.6	0.9167	0.9167	1.2706	1.2094	1.3931	8.345	3	1.564	2.530	0.1980	2	24
*132.8	0.7500	0.7500	1.0561	0.9117	1.2094	14.113	3	4.306	2.530	0.1980	6	24
*148.8	0.5000	0.5000	0.7854	0.6591	0.9117	16.086	3	7.765	2.530	0.1980	12	24
*170.2	0.3333	0.3333	0.6139	0.5236	0.6591	12.739	3	9.956	2.530	0.1980	16	24
*187	0.2083	0.2083	0.4695	0.3614	0.5236	19.949	3	11.801	2.530	0.1980	19	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.9499284	0.873	0.0501515	-0.278105
Equality of variance cannot be confirmed				

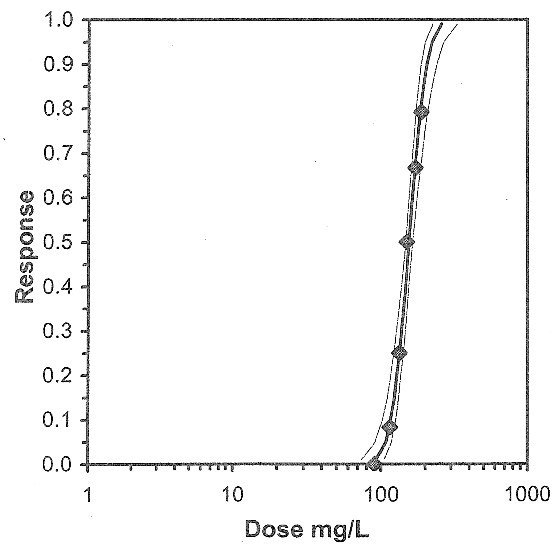
Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	114.6	132.8	123.36482		0.1033895	0.1067246	0.430958	0.009187	1.8E-08	6, 14
Treatments vs 0.0 D-Control										

			Maximum Likelihood-Probit								
Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	10.588202	1.6844889	7.286604	13.889801	0	0.6462051	9.4877291	0.96	2.1868099	0.0944447	3
Intercept	-18.15439	3.6658691	-25.33949	-10.96928							
TSCR											

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	92.704019	73.927552	104.86185
EC05	3.355	107.51328	91.339047	117.85791
EC10	3.718	116.35206	102.08593	125.62159
EC15	3.964	122.7225	109.91433	131.2994
EC20	4.158	128.03337	116.43496	136.14286
EC25	4.326	132.77242	122.19708	140.60052
EC40	4.747	145.50656	136.95838	153.66547
EC50	5.000	153.74814	145.58119	163.33087
EC60	5.253	162.45654	153.8232	174.64671
EC75	5.674	178.03767	167.01495	197.03528
EC80	5.842	184.62758	172.22677	207.09432
EC85	6.036	192.61742	178.36244	219.6477
EC90	6.282	203.16349	186.2346	236.73178
EC95	6.645	219.86578	198.32405	264.82349
EC99	7.326	254.98888	222.72424	327.45819



Test ID: 1N10
Sample ID: NO2STOCK
Sample Type: NANO2
Method: Dunnett's Test



Test ID: 1N10
Sample ID: NO2STOCK
Sample Type: NANO2
Method: Maximum Likelihood-Probit

Acute Shrimp Test-12 hr Survival

Start Date:	3/1/2008 13:00	Test ID:	1AvNs	Sample ID:	NH3NO2
End Date:	3/3/2008 13:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	NH3NO2
Sample Dat		Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei
Comments:					

Conc-mg/L	1	2	3
I8 D-Control	1.0000	1.0000	1.0000
29.56	0.8750	1.0000	1.0000
35.87	1.0000	1.0000	0.8750
40.1	0.8750	0.7500	0.8750
43.73	0.7500	0.7500	0.6250
54.98	0.5000	0.3750	0.3750

			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
I8 D-Control	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3				0	24
29.56	0.9583	0.9583	1.3319	1.2094	1.3931	7.961	3	0.889	2.500	0.1722	1	24
35.87	0.9583	0.9583	1.3319	1.2094	1.3931	7.961	3	0.889	2.500	0.1722	1	24
*40.1	0.8333	0.8333	1.1554	1.0472	1.2094	8.107	3	3.452	2.500	0.1722	4	24
*43.73	0.7083	0.7083	1.0020	0.9117	1.0472	7.805	3	5.677	2.500	0.1722	7	24
*54.98	0.4167	0.4167	0.7012	0.6591	0.7854	10.403	3	10.046	2.500	0.1722	14	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates non-normal distribution (p <= 0.01)	0.8542656	0.858	-0.707522	-0.984641
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	35.87	40.1	37.926073		0.0862667	0.0890495	0.2091414	0.0071161	2.5E-06	5, 12
Treatments vs .248 D-Control										

Maximum Likelihood-Probit

Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	8.3550615	1.8119857	4.8035696	11.906553	0	1.3179099	9.4877291	0.86	1.7161032	0.1196879	3
Intercept	-9.338148	2.9740109	-15.16721	-3.509087							
TSCR											

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	27.394759	19.152953	31.923232
EC05	3.355	33.054768	26.324053	36.736621
EC10	3.718	36.535663	31.019866	39.806732
EC15	3.964	39.089011	34.467622	42.24743
EC20	4.158	41.245003	37.262606	44.550835
EC25	4.326	43.18918	39.599694	46.910208
EC40	4.747	48.504346	44.8852	54.941377
EC50	5.000	52.011962	47.738822	61.256247
EC60	5.253	55.773235	50.519848	68.640343
EC75	5.674	62.637082	55.208253	83.383663
EC80	5.842	65.589624	57.129801	90.16799
EC85	6.036	69.207285	59.428525	98.818358
EC90	6.282	74.043932	62.423154	110.94353
EC95	6.645	81.841268	67.097449	131.79112
EC99	7.326	98.75044	76.734955	182.26716

Acute Shrimp Test-24 hr Survival

Start Date:	3/1/2008 13:00	Test ID:	1AvNs	Sample ID:	NH3NO2
End Date:	3/3/2008 13:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	NH3NO2
Sample Dat		Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei
Comments:					

Conc-mg/L	1	2	3
l8 D-Control	1.0000	1.0000	1.0000
29.56	0.6250	0.6250	0.7500
35.87	0.5000	0.6250	0.6250
40.1	0.0000	0.3750	0.2500
43.73	0.0000	0.2500	0.0000
54.98	0.0000	0.0000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
l8 D-Control	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3				0	24
*29.56	0.6667	0.6667	0.9569	0.9117	1.0472	8.173	3	3.555	2.470	0.3031	8	24
*35.87	0.5833	0.5833	0.8696	0.7854	0.9117	8.388	3	4.266	2.470	0.3031	10	24
*40.1	0.2083	0.2083	0.4535	0.1777	0.6591	54.740	3	7.657	2.470	0.3031	19	24
*43.73	0.0833	0.0833	0.2930	0.1777	0.5236	68.155	3	8.965	2.470	0.3031	22	24
54.98	0.0000	0.0000	0.1777	0.1777	0.1777	0.000	3				24	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.9640511	0.835	-0.105777	0.8349011
Equality of variance cannot be confirmed				

Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	<29.56	29.56			0.1826463	0.1885381	0.5685885	0.0225861	3.3E-05	4, 10
Treatments vs .248 D-Control										

					Maximum Likelihood-Probit						
Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	11.006073	2.0237516	7.0395199	14.972626	0	4.6723968	9.4877291	0.32	1.5339736	0.0908589	3
Intercept	-11.88303	3.1757052	-18.10741	-5.658643							
TSCR											

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	21.018615	15.172278	24.602877
EC05	3.355	24.239516	18.912636	27.39116
EC10	3.718	26.153702	21.253641	29.027023
EC15	3.964	27.529882	22.982322	30.201834
EC20	4.158	28.675089	24.444452	31.184026
EC25	4.326	29.695467	25.76056	32.067238
EC40	4.747	32.430581	29.303248	34.518672
EC50	5.000	34.195864	31.536102	36.230288
EC60	5.253	36.057236	33.754378	38.234871
EC75	5.674	39.378302	37.182087	42.501959
EC80	5.842	40.779545	38.440304	44.551288
EC85	6.036	42.475922	39.862451	47.181136
EC90	6.282	44.710957	41.622228	50.839164
EC95	6.645	48.241767	44.241531	56.959599
EC99	7.326	55.634358	49.383984	70.816885

Acute Shrimp Test-48 hr Survival					
Start Date:	3/1/2008 13:00	Test ID:	1AvNs	Sample ID:	NH3NO2
End Date:	3/3/2008 13:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	NH3NO2
Sample Dat		Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei
Comments:					

Conc-mg/L	1	2	3
I8 D-Control	1.0000	0.8750	1.0000
29.56	0.5000	0.3750	0.2500
35.87	0.1250	0.0000	0.2500
40.1	0.0000	0.0000	0.1250
43.73	0.0000	0.0000	0.0000
54.98	0.0000	0.0000	0.0000

			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
I8 D-Control	0.9583	1.0000	1.3319	1.2094	1.3931	7.961	3				1	24
*29.56	0.3750	0.3913	0.6560	0.5236	0.7854	19.958	3	6.276	2.420	0.2606	15	24
*35.87	0.1250	0.1304	0.3542	0.1777	0.5236	48.854	3	9.079	2.420	0.2606	21	24
*40.1	0.0417	0.0435	0.2389	0.1777	0.3614	44.379	3	10.149	2.420	0.2606	23	24
43.73	0.0000	0.0000	0.1777	0.1777	0.1777	0.000	3				24	24
54.98	0.0000	0.0000	0.1777	0.1777	0.1777	0.000	3				24	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.9559686	0.805	-0.061091	-1.190493
Bartlett's Test indicates equal variances (p = 0.90)	0.5693772	11.344867		

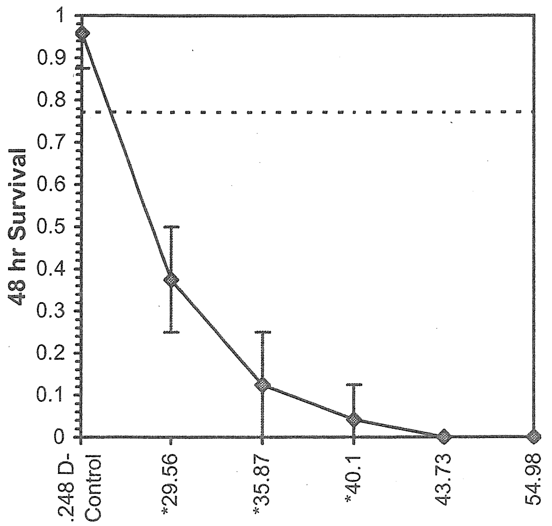
Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	<29.56	29.56			0.1734414	0.1837321	0.7213504	0.017394	3.2E-05	3, 8

Treatments vs .248 D-Control

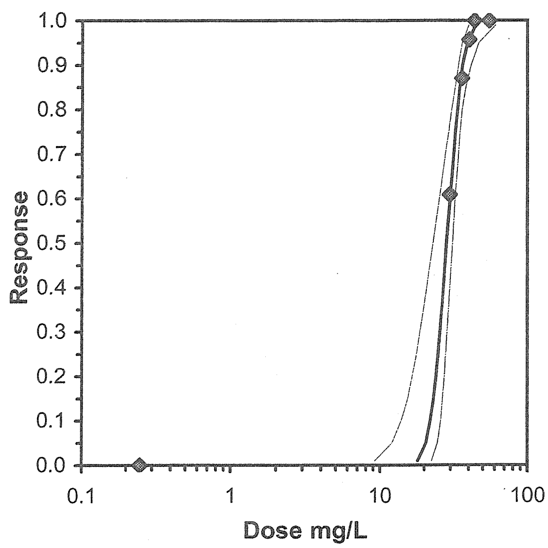
Maximum Likelihood-Probit							
Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
0.0416667	0.461398	9.4877291	0.98	1.4509388	0.0843786	4

Parameter	Value	SE	95% Fiducial Limits	
Slope	11.851343	3.1111268	5.7535339	17.949151
Intercept	-12.19557	4.7538452	-21.51311	-2.878036
TSCR	0.0418119	0.0288892	-0.014811	0.0984347

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	17.974001	9.1705304	22.422353
EC05	3.355	20.518628	12.020933	24.521863
EC10	3.718	22.019287	13.879723	25.733309
EC15	3.964	23.093299	15.288726	26.592824
EC20	4.158	23.98413	16.505575	27.303492
EC25	4.326	24.77572	17.622077	27.935192
EC40	4.747	26.8882	20.749266	29.639189
EC50	5.000	28.24482	22.849658	30.770699
EC60	5.253	29.669887	25.096558	32.029557
EC75	5.674	32.199663	28.971386	34.662198
EC80	5.842	33.262405	30.435474	36.041694
EC85	6.036	34.545512	31.994388	38.003587
EC90	6.282	36.230502	33.708989	41.059337
EC95	6.645	38.880271	35.910881	46.699948
EC99	7.326	44.384657	39.70374	60.55181



Test ID: 1AvNs
Sample ID: NH3NO2
Sample Type: NH3NO2
Method: Dunnett's Test



Test ID: 1AvNs
Sample ID: NH3NO2
Sample Type: NH3NO2
Method: Maximum Likelihood-Probit

Acute Shrimp Test-48 hr Survival

Start Date:	3/3/2008 17:00	Test ID:	1AsNv	Sample ID:	NH3NO2
End Date:	3/5/2008 17:00	Lab ID:	CATML-Telonicher Marine	Sample Type:	NH3NO2
Sample Dat		Protocol:	ASTM94-ASTM (1994)	Test Species:	LV-Litopenaeus vannamei
Comments:					

Conc-mg/L	1	2	3
.0 D-Control	1.0000	1.0000	1.0000
127.4	1.0000	1.0000	1.0000
146.8	0.8750	1.0000	0.8750
155.2	0.8750	0.8750	0.7500
162.8	0.7500	0.6250	0.8750
183.2	0.5000	0.6250	0.6250

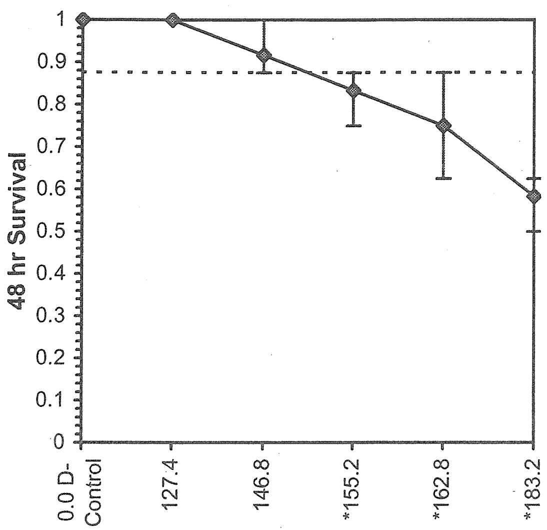
			Transform: Arcsin Square Root						1-Tailed		Number	Total
Conc-mg/L	Mean	N-Mean	Mean	Min	Max	CV%	N	t-Stat	Critical	MSD	Resp	Number
.0 D-Control	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3				0	24
127.4	1.0000	1.0000	1.3931	1.3931	1.3931	0.000	3	0.000	2.500	0.1817	0	24
146.8	0.9167	0.9167	1.2706	1.2094	1.3931	8.345	3	1.684	2.500	0.1817	2	24
*155.2	0.8333	0.8333	1.1554	1.0472	1.2094	8.107	3	3.271	2.500	0.1817	4	24
*162.8	0.7500	0.7500	1.0561	0.9117	1.2094	14.113	3	4.636	2.500	0.1817	6	24
*183.2	0.5833	0.5833	0.8696	0.7854	0.9117	8.388	3	7.201	2.500	0.1817	10	24

Auxiliary Tests	Statistic	Critical	Skew	Kurt
Shapiro-Wilk's Test indicates normal distribution (p > 0.01)	0.9567849	0.858	0.0905005	0.2721209
Equality of variance cannot be confirmed				

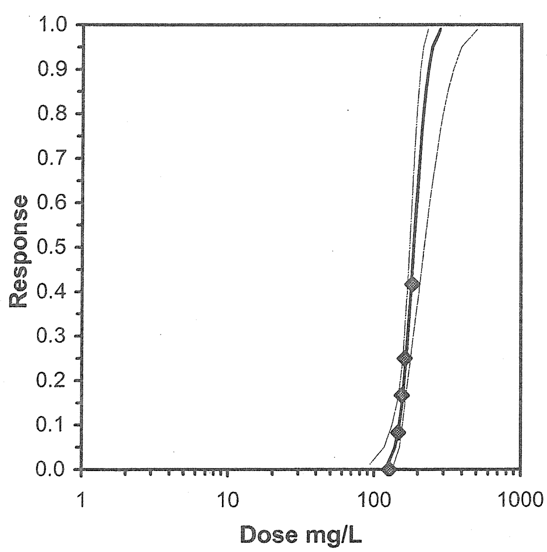
Hypothesis Test (1-tail, 0.05)	NOEC	LOEC	ChV	TU	MSDu	MSDp	MSB	MSE	F-Prob	df
Dunnett's Test	146.8	155.2	150.94158		0.0924715	0.0954545	0.1264531	0.0079253	6.1E-05	5, 12
Treatments vs 0.0 D-Control										

					Maximum Likelihood-Probit						
Parameter	Value	SE	95% Fiducial Limits		Control	Chi-Sq	Critical	P-value	Mu	Sigma	Iter
Slope	13.155528	3.4329235	6.4269978	19.884058	0	0.7556097	7.8147278	0.86	2.2714749	0.0760137	4
Intercept	-24.88245	7.5936706	-39.76605	-9.998858							
TSCR											

Point	Probits	mg/L	95% Fiducial Limits	
EC01	2.674	124.34854	92.969318	137.42601
EC05	3.355	140.10176	117.93244	149.65282
EC10	3.718	149.29989	133.1983	157.40482
EC15	3.964	155.84463	143.72418	163.85249
EC20	4.158	161.25019	151.57203	170.40125
EC25	4.326	166.03686	157.50378	177.50507
EC40	4.747	178.73808	169.35291	201.56545
EC50	5.000	186.84219	175.43826	219.40417
EC60	5.253	195.31374	181.30637	239.39581
EC75	5.674	210.25454	191.02766	277.42166
EC80	5.842	216.49589	194.9388	294.27153
EC85	6.036	224.00518	199.55902	315.27107
EC90	6.282	233.82469	205.48269	343.91126
EC95	6.645	249.17605	214.52075	391.33694
EC99	7.326	280.74318	232.42126	498.94649



Test ID: 1AsNv
Sample ID: NH3NO2
Sample Type: NH3NO2
Method: Dunnett's Test



Test ID: 1AsNv
Sample ID: NH3NO2
Sample Type: NH3NO2
Method: Maximum Likelihood-Probit

Appendix C:

Instant Ocean Composition

Ion	Instant Ocean (ppm)	Seawater (ppm)
Chloride	19,290	19,353
Sodium	10,780	10,781
Sulfate	2,660	2,712
Magnesium	1,320	1,284
Potassium	420	399
Calcium	400	412
Carbonate/bicarbonate	200	126
Bromide	56	67
Strontium	8.8	7.9
Boron	5.6	4.5
Fluoride	1	1.28
Lithium	0.3	0.173
Iodide	0.24	0.06
Barium	less than 0.04	0.014
Iron	less than 0.04	less than 0.001
Manganese	less than 0.025	less than 0.001
Chromium	less than 0.015	less than 0.001
Cobalt	less than 0.015	less than 0.001
Copper	less than 0.015	less than 0.001
Nickel	less than 0.015	less than 0.001
Selenium	less than 0.015	less than 0.001
Vanadium	less than 0.015	less than 0.002
Zinc	less than 0.015	less than 0.001
Molybdenum	less than 0.01	0.01
Aluminum	less than 0.006	less than 0.001
Lead	less than 0.005	less than 0.001
Arsenic	less than 0.004	0.002
Cadmium	less than 0.002	less than 0.001
Nitrate	None	1.8
Phosphate	None	0.2

Provided by Instant Ocean