Chapter 4 Test organisms, Equipment, and Reagents

Test organisms, equipment, and reagents used are important factors in ammonia toxicity experiments. The information about these factors is needed to develop reliable and accurate results. The topics are discussed in two major sections:

4.1 Test Organisms

4.1.1 Taxonomy, Morphology, and Distribution

4.1.2 Sources and Age of the Organisms

4.1.3 Acclimation of the Test Organisms

4.2 Equipment and Reagents

4.2.1 Equipment

4.2.2 Reagents

4.1 Test Organisms

The selection of the test organisms is important in characterizing the toxicity of a specific toxicant. The response to the same toxicant varies from one species to another. Also, the life stage of the species, and the origin and handling of the test species must be considered before toxicity tests are conducted. Two marine species were used in this research, the Atlantic silverside (*Menidia menidia*) and the ghost shrimp *(Palaemonetes pugio*). The subjects covered in this section include taxonomy, morphology, and distribution of the test organisms. Their source, original cultural conditions, and life stage will be covered, as well.

4.1.1 Taxonomy, Morphology, and Distribution

A brief life history, taxonomy, and morphology for each species are provided in the following:

• Atlantic Silverside (*Menidia menidia*)

The Atlantic silverside is a marine fish and belongs to the family Antherinidae. The adult Atlantic silverside has a length of up to 117 mm and females, in general, are slightly larger than males. They are characterized by the first dorsal fin usually having four or five spines, the second dorsal fin having one spine and eight or nine rays, and the anal fin having one spine with usually 21 to 26 rays [26].

The silverside genus (*Menidia*) has three species. All of three are routinely used in toxicity tests. The other two species are the inland silverside and tidewater silverside. The differences among these three species can be identified by means of the taxonomic key for marine fish. Figure 4.1 shows the morphology of a mature Atlantic silverside [26].

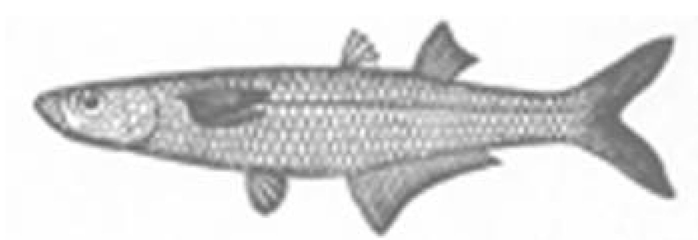

Figure 4.1 Morphology of an Adult Atlantic Silverside [26]

Atlantic silversides are distributed in estuaries along the Atlantic and Gulf coasts. They remain in the Atlantic estuaries throughout most of the year. This species is an important component of estuarine ecosystems, serving as forage fish for commercially and recreationally valued species such as striped bass and bluefish. They survive at intermediate to high salinity, typically 12-30 ppth [50]. These species can be cultured in-house, but require a lot of space. Culturing in-house also requires specific equipment such as a circulation pump which can generate a current velocity to help organisms spawn. Additionally, due to the large number of organisms required for toxicity testing, it was decided that the purchase of test organisms from an outside commercial source would be more appropriate.

- Ghost Shrimp *(Palaemonetes pugio*).

Ghost shrimp is in the family Palaemonidae (Figure 4.2). It is so named because its body is transparent. The major characters of this species are rostrum reaching slightly beyond end of antennal scale and abdomen smooth. Adults have an average total length of 40mm. Males generally are smaller and have a more slender rostrum than females [53]. Ghost shrimp is one of the commonly used marine species in toxicity tests. Other species in the genus, *Palaemonete*, similar to ghost shrimp, can be identified using the taxonomic key developed by Williams (1984).

Figure 4.2 Morphology of an Adult Female Ghost Shrimp [53]

Ghost shrimp is a resident of estuaries of the Atlantic Ocean and the Gulf of Mexico, particularly in beds of submerged vegetation. They can survive in a wide range of salinity from 0 to 40 ppth, and are abundant in salinity of 10 -20 ppth [53]. This species also can be cultured in-house. In this study the organisms were purchased from outside sources because of the low production rate of young generated by juvenile females and for the reasons given for the Atlantic silverside.

4.1.2 Sources and Age of the Organisms

A number of factors related to test organisms can alter the results of toxicity testing. For the continual use of organism in toxicity tests, the sources of test organism should be kept as consistent as possible in order to eliminate variations in species [50, 51]. Age and life stage of organism are crucial for toxicity tests. The following section will address the sources and age of the test organisms used in this research.

The Atlantic silverside and the ghost shrimp were supplied by Sea Plantations (SP), Inc., which is a company certified by the state of Massachusetts. Both species originally were collected off the shores of Massachusetts and identified by biologists of SP, Inc.. The both species were cultured in the laboratory with natural seawater. Water quality parameters of the culture solution for the Atlantic silverside were pH 7.8-8.0, salinity 30ppth, and temperature 23-25°C. For the ghost shrimp, the parameters were pH 7.8-8.0, salinity 25 ppth, and temperature 22-24°C. The fish and shrimp were contained in the cubitainers during their overnight shipping. The address for SP Inc. is provided in Appendix G.

The larval stage of both species were used in this study based on the believe that the younger organisms are, the more sensitive to ammonia they will be. As a result, if the sensitive stage of organisms is protected, the adults would be protected too. The Atlantic silversides were 13-15 days old, and the ghost shrimps were 9-11 days old. The average lengths of the Atlantic silverside and the ghost shrimp were approximately 11.15 ± 0.25 mm and 9.23 ± 0.16 mm, respectively.

4.1.3 Acclimation of the Test Organisms

Acclimation is a necessary step in preparing test organisms for test conditions. The same methods of acclimation suggested by Hudgins (1996) and recommended by other researchers [43, 46], were used for both species. Special attention was paid to Atlantic silverside since different levels of salinity were used in the test. The original salinity was gradually adjusted up or down to the designed test conditions. The acclimation steps were described as follows:

i. Upon arrival, the original solution was aerated gently, and then poured into holding tanks or trays. The pH, temperature and salinity of the original water were measured before addition of dilution water. Gradual adjustments were made to ensure that the original conditions were not drastically different from the test conditions.

ii. Temperature of the water was adjusted by placing the trays in a water bath. The water bath temperature was kept at 20 ± 1°C for acute tests and 25 ± 1°C for chronic test.

iii. Salinity and pH were changed by adding 20% of the dilution water into the original water each time. Every hour, dilution water was added to the solution of the original water and dilution water. Each time, when water was added, the pH and salinity of the solution were measured to ensure no drastic change in these two factors. After five cycles, the pH and salinity in the holding trays were essentially the same as those in the dilution water. A period of 48 hours was used for acclimation of both species and all types of bioassay, except that the acclimation of the Atlantic silverside on the acute tests took 3 days, and was achieved by replacing 80% solution with dilution water on the last two days.

iv. Dissolved oxygen was maintained by continuous aeration. The organisms were fed twice daily with newly hatched, brine shrimp (*Artemia*). The photoperiod was maintained at 16 hr light /8 hr darkness during acclimation.

v. At the initiation of tests, healthy organisms were transferred to test vessels with a plastic pipet having an opening large enough not to harm the organisms.

4.2 Equipment and Reagents

The equipment, reagents and their usage for measuring pH, temperature, salinity concentration, dissolved oxygen, and ammonia concentration are covered in this section.

4.2.1 Equipment and Manufacturing Companies

2095 Bath Circulator	Forma Scientific
Automatic Timer	Precision
pH Meter	Orion model 420
Dissolved Oxygen Meter	YSI model 51B
Salinity Meter	YSI model 30/25 FT
Balance	Metter AE240
Drying Oven	Blue M, single wall, gravity convection oven
Automatic Pipette	Wheaton 100-1000 μl
Transfer Pipette	Samco
Beakers and Cylinders	Kimax

All equipment was maintained in accordance with the Standard Operation Procedures provided by Olver Laboratory. Circulators, air lines, and timers were routinely checked twice a day. The pH meter was calibrated daily with National Bureau of Standard (NBS) buffers. The dissolved oxygen meter was calibrated immediately before the initiation of tests.

4.2.2 Reagents

The major reagents used in toxicity tests were reagent grade ammonia chloride (NH_4Cl) and the salt Forty Fathoms. The preparation steps were as follows.

Ammonia chloride was used as the toxicant in the entire study. The stock ammonia solution was 10,000 mg/L of total ammonia and refrigerated at 4°C for later use.

Synthetic saltwater was used as dilution water and made up with deionized water and Forty Fathoms, as recommended by EPA. Newly prepared synthetic saltwater was stored in a 20L or 50L carboy. The dissolved oxygen of the synthetic saltwater was kept at saturation by means of aeration, and the synthetic saltwater was aged at least 24hr before use.

Chapter 5 Methods and Procedures

One marine fish and one marine shrimp were chosen for this research. Since ammonia toxicity is mainly affected by pH, temperature, and salinity, these factors were controlled throughout all the experimental steps. This section includes training in Olver Lab, the methods used in toxicity tests, the experimental design, and the control of environmental factors for the research. The methods of data analyses are included, as well.

5.1 Experimental Design and Methods

Both acute and chronic tests were conducted with two saltwater species. All tests were performed at Olver Laboratory, Inc. in Blacksburg.

5.1.1 Training

Olver Lab is a certified bioassay laboratory by the state of Virginia. The techniques routinely performed in the Olver Lab include the acute and chronic toxicity tests with fathead minnows (*Pimephales promelas*) and two cladocerans (*Ceriodaphnia dubia* and *Daphnia magna).* Both static-non-renewal and static-with-renewal tests are performed.

5.1.2 Test Methods and Conditions

48 hr and 96hr acute tests, and 7-day chronic tests were performed for the two species under study. The acute and chronic tests were conducted in accordance with the documents outlined by EPA's Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms (EPA 600/4-90/027) and Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms (EPA 600/4-89/001) [50, 51], respectively.

The endpoint in acute tests was survival and the endpoints in 7-day chronic tests were survival and growth. Five ammonia test concentrations plus a control were used in each test, shown in Table 5.1. The following section provides the test conditions for each species.

Table 5.1 Test Dilutions for Toxicity Experiments (mg/L total ammonia)

Concentration of total ammonia, mg/L					
		48 -hour		96-hour	7-day
Atlantic silverside	(14ppth*)	(22ppth)	(30ppth)		(30ppth)
(Menidia menidia)	control	control	control		control
	5	5	5		5
	10	10	10		10
	20	20	20		15
	30	30	30		20
	40	40	40		25
Ghost shrimp				(25ppth)	(25ppth)
(Palaemonetes pugio)				control	control
				10	5
				20	10
				30	15
				40	20
				50	30

* ppth: salinity unit as part per thousand in parentheses.

Atlantic Silverside. The Atlantic silverside was acutely assessed before [11, 49], but not chronically. The influence of different salinity levels on ammonia toxicity has not been studied with this species. In this research, the 48 hour acute tests at three levels of salinity and the 7-day chronic test were conducted with this organism. The test conditions were chosen on the basis of average summer conditions in the Chesapeake Bay [31, 34, 54]; and, the recommended temperature for warmwater species was provided by the EPA [50, 51]. The temperature was 20°C for acute tests and 25°C for the chronic test. The pH value was 8.2. The three salinity levels selected for the acute tests were 14, 22, 30 part per thousand (ppth), after the distribution range of salinity for Atlantic silverside was also taken into account. A salinity of 30 ppth was used for the chronic test [26].

The 48hr acute tests were conducted three times at three levels of salinity. Each of five test dilutions plus a control had two replicates, and each replicate was tested with 10 organisms. Sixty organisms were exposed at each dilution.

The 7-day chronic tests were conducted twice. Each time five test dilutions plus one control were set up. Each dilution had three replicates, and a total of 60 organisms were exposed at each dilution.

The test conditions of both acute and chronic tests for the Atlantic silverside are summarized in Tables 5.2 and 5.3.

Table 5.2 48hr Acute Bioassay Conditions for Atlantic Silverside (*Menidia menidia*)

Test type:	Static non-renewal
Test duration:	48-hour
Endpoint:	Survival
Temperature:	20°C
Photoperiod:	16 hr light/8 hr darkness
Replicates per test:	2
Test dilutions:	5
Test beaker size/test solutions:	1 L/500ml
Number of organisms per replicate:	10
Age of organisms:	14 days
Feeding regimen:	None
DO:	> 4.0 mg/L
pH:	8.2±0.1
Salinity:	14, 22, and 30ppth

Table 5.3 7-day Chronic Bioassay Conditions for Atlantic Silverside *(Menidia menidia)*

Test type:	Static renewal
Endpoint:	Survival and growth
Test duration:	7 days
Temperature:	25°C
Photoperiod:	16 hr light/8 hr darkness
Replicates per test:	3
Test dilutions:	5
Test beaker size/test solutions:	1 L/500ml
Number of organisms per replicate:	10
Age of organisms:	14 days
Renewal and feeding regimen:	Renewal daily, feeding twice daily with less than 24 hr brine shrimps
DO:	> 4.0 mg/L
pH:	8.2±0.1
Salinity:	30±1 ppth

Ghost Shrimp. The ghost shrimp was assayed in the first phase of this project but only a 48-hour acute test was conducted. In order to refine the national criteria, a 96-hour acute test and chronic test were needed. The 96-hour acute ammonia toxicity for the ghost shrimp was tested before, but under different test conditions, and no chronic data are available [8]. Thus, in this research, a 96-hour acute and a 7-day chronic test were performed with the ghost shrimp. The test temperature and pH were the same as with the Atlantic silverside and all tests were conducted at the same level of salinity (25 ppth).

The 96hr acute tests were performed three times and each time had five test dilutions and a control. Each dilution had four replicates and a total of 120 organisms were exposed at each dilution.

The 7-day chronic test were conducted twice. Each of five dilution and a control had four replicates and 80 organisms were exposed at each dilution.

The test conditions of both acute and chronic tests for the ghost shrimp are summarized in Tables 5.4 and 5.5.

Table 5.4 96hr Acute Bioassay Conditions for Ghost Shrimp *(Palaemonetes pugio*)

Test type:	Static renewal
Test duration:	96-hour
Endpoint:	Survival
Temperature:	20°C
Photoperiod:	16 hr light/8 hr darkness
Replicates per test:	4
Test dilutions:	5
Test beaker size/test solutions:	1 L/500ml
Number of organisms per replicate:	10
Age of organisms:	10 days
Renewal and feeding regimen:	Renewal and feeding once at the point of 48 hr, feeding with less than 24 hr brine shrimps.
DO:	> 4.0 mg/L
pH:	8.2±0.1
Salinity:	25 ±1 ppth

Table 5.5 7-day Chronic Bioassay Conditions for Ghost Shrimp *(Palaemonetes pugio*)

Test type:	Static renewal
Endpoint:	Survival and growth
Test duration:	7 days
Temperature:	25°C
Photoperiod:	16 hr light/8 hr darkness
Replicates per test:	4
Number of test dilutions:	5
Test beaker size/test solutions:	1 L/500ml
Number of organisms per replicate:	10
Age of organisms:	10 days
Renewal and feeding regimen:	Renewal daily, feeding twice daily with less than 24 hr brine shrimps
DO:	> 4.0 mg/L
pH:	8.2±0.1
Salinity:	25±1 ppth

5.1.3 Experimental Set-up Procedures

Detailed procedures for both acute and chronic tests are presented in the following:

Set-up procedures for the 48-hour and 96-hour acute tests

a. Clean glass beakers were marked to track different dilutions and replicates. The newly prepared solutions were buffered to pH 8.2 with Na_2CO_3 before being poured into marked beakers. DO and salinity were measured and recorded at initiation of tests. 250 ml samples were taken from the initial dilution in order to analyze the concentration of total ammonia.

b. The beakers then were placed in random order (a random number was labeled on each beaker). 10 acclimated organisms, fed one hour before tests, were transferred into beaker with a transfer pipette. The order of 4, 4, and 2 organisms were put into the beakers at three separate times in order to avoid differences in the health of organisms.

c. The beakers containing organisms were placed in a water bath. The temperature of the water bath was kept constant at 20°C. The beakers were covered with a plastic panel to avoid evaporation of the solution.

d. pH was checked and adjusted every four hours during the daytime, but was not checked for eight hours at night.

e. Every 24hr, pH, temperature, and salinity were measured and recorded. The number of living organisms was counted and the dead ones were removed.

f. Solutions were renewed and ghost shrimp were fed at 48hr in the 96hr static-with-renewal acute test, in accordance with the static renewal test's procedure [50]. The 48hr acute test with Atlantic silverside was non-renewal and the fish were not fed during the entire test.

g. At the termination of the test, final pH, temperature, and salinity were measured and recorded. Again, the number of living organisms was counted and the data were used to determine LC_{50} and NOEC. Final samples were taken from each dilution to verify the concentration of total ammonia.

<u>Set-up procedures for the 7-day chronic test:</u>

a. Same as for the acute test.

b. Same as for the acute test.

c. The beakers containing organisms were placed in the water bath. The water bath temperature was kept constant at 25°C. The beakers were covered with a plastic panel to avoid evaporation of the solution.

d. pH was checked and adjusted every four hours during the daytime, but was not adjusted for eight hours at night. Organisms in each test beaker was fed twice daily with newly hatched *Artemia nauplii*.

e. Test solutions in each beaker were renewed on a daily basis. 80% of the solution was siphoned off and newly prepared solutions buffered to pH 8.2 were added. Before renewal, waste in beakers and dead organisms were removed and the number of living organisms was counted.

f. Daily, the final pH, temperature, and salinity were measured and recorded. Final samples were taken from each dilution to verify the concentration of total ammonia. The initial pH, temperature, and salinity were measured and recorded again.

g. A day before the end of the tests, pans were weighed, oven dried at 105°C for two hours, and then placed in a dessicator for one hour. The dry mass for each pan was weighed with a Metter AE240 balance and recorded.

h. At the termination of the tests, the final count of living organism was recorded and living organisms were transferred to the weighing pans. The pans then were oven dried, cooled and weighed, as described previously. The dry mass of each fish was determined and later was used to calculate the NOEC and LOEC.

5.1.4 Analytical Chemistry of Ammonia

The total ammonia concentration of each dilution for both species was measured according to Method-4500 B. and C. detailed in Standard Methods for the Examination of Water and Wastewater [2]. Samples, taken at initiation and the end of each test, were preserved in 6M H_2SO_4 and stored at 4°C for no longer than 2 weeks. The samples were analyzed with distillation followed by titration. The average of the initial and final measured ammonia concentrations were used in the calculation of LC_{50} and NOEC.

It was found that no significant loss of total ammonia was observed through a 48hr acute test. Thus, for the chronic tests with 24hr base renewal, it was decided that samples need not be taken each day. Total ammonia was measured to three significant figures as specified for the analytic method. The un-ionized ammonia was calculated based on pH, temperature, and salinity. The resulting values for un-ionized ammonia to three significant figures were used in this report.

5.2 pH Control

A small change in pH strongly affects the fraction of un-ionized ammonia. Fluctuations in pH have been considered a major source of variability in ammonia toxicity testing. Therefore, the causes of changes in pH and the procedures to control pH are presented in the following. This section will also address the methods for determination of average pH.

5.2.1 Causes of pH Fluctuation

There are several factors which will cause fluctuations in pH. Typically, pH tends to decline during the period of ammonia toxicity tests. This is due to the slow equilibrium of the dissociation of the ammonium salt in sea water and the production of wastes by tests organisms [49]. Other physical factors, such as dissolved oxygen and salinity, also affect the pH value [1, 37].

In addition, some researchers suggest that the inaccuracy in measuring pH is a factor related to repeated pH fluctuations [38, 54]. A major reason is that pH meters used to measure high ionic strength solutions after being calibrated with low ionic strength buffers (NBS). Millero (1986) found that the pH (NBS buffers) scale overestimates pH relative to the free hydrogen ion scale by 0.02 pH unit at 30 ppth, 0.045 unit at 20 ppth and 0.075 unit at 10 ppth. A pH variation of ± 0.1 has been considered to be acceptable by Thurston *et al.* (1983), Kohn (1994), and Hermanutz (1996). Therefore, pH was kept $\leq \pm 0.1$ throughout all the tests in this research and, in most cases, $\leq \pm 0.05$.

5.2.2 The pH Control Procedure

There are three pH control methods. They are: acid/base addition, the nitrogen headspace technique, and use of buffers. The Na_2CO_3 buffer system, suggested by Hudgins (1996) and other researchers [8, 28], was used in this work. It requires the initial buffering of each test dilution to the desired pH and subsequent buffering periodically during the test to maintain the pH. The study by Hudgins (1996) indicated that the pH of test solutions needed to be adjusted at intervals of eight hours or less for a variation of less than $\pm$ 0.1 unit. Therefore, it was decided that pH should be measured every four hours during the daylight hours with a lapse of 8 hour overnight. The procedures for pH control were as follows:

a. Initially, the pH of dilution water was adjusted to 8.2 with 1N Na_2CO_3 before the addition of the organisms.

b. pH was measured every four hours and adjusted to 8.2 with 1N Na_2CO_3, if needed.

c. Steps1 and 2 were used in both renewal tests and in non-renewal tests. In a daily renewal test, pH of the new dilution was adjusted to 8.2 before replacing the old solutions.

5.2.3 Methods for Determination of Average pH

The observed average pH value from the tests was used to calculate the concentration of un-ionized ammonia [8, 23]. The average pH was calculated as follows. The measured pH values for every four hours were converted to concentrations of hydrogen ions. The concentrations of hydrogen ion were averaged, and then converted back to pH units.

5.3 Methods of Data Analysis

Survival data from acute tests and survival and growth data from chronic tests were used in determining LC_{50} and NOEC values. Data were analyzed with Toxstat Version 3.4 software, which was developed based on the methods depicted in the documents: EPA/ 600/4-90/027 and EPA /600/4-89/001. In addition, a single factor analysis of variance (ANOVA) was also employed to determine the significant differences in LC_{50} values among three levels of salinity in acute tests with the Atlantic silverside. The LC_{50} and NOEC were determined using different statistical methods which are briefly presented in the following. The methods used in determining a final LC_{50} and NOEC are included in this section as well.

5.3.1 Methods for Determination of LC_{50}

Survival data were used in determining the LC_{50} values from multi-concentration tests. Four methods are outlined in the EPA document, 600/4-90/027. Those methods are the Graphical Method, the Spearman-Karber Method, the Trimmed Spearman-Karber Method, and the Probit Method. Statistical analysis steps are illustrated in Figure 5.1. Based on requirements by those analytical methods, the Probit Method was used to determine the LC_{50} values for Atlantic silverside and ghost shrimp. A 95% confidence interval (C.I.) was shown along with each LC_{50} value.

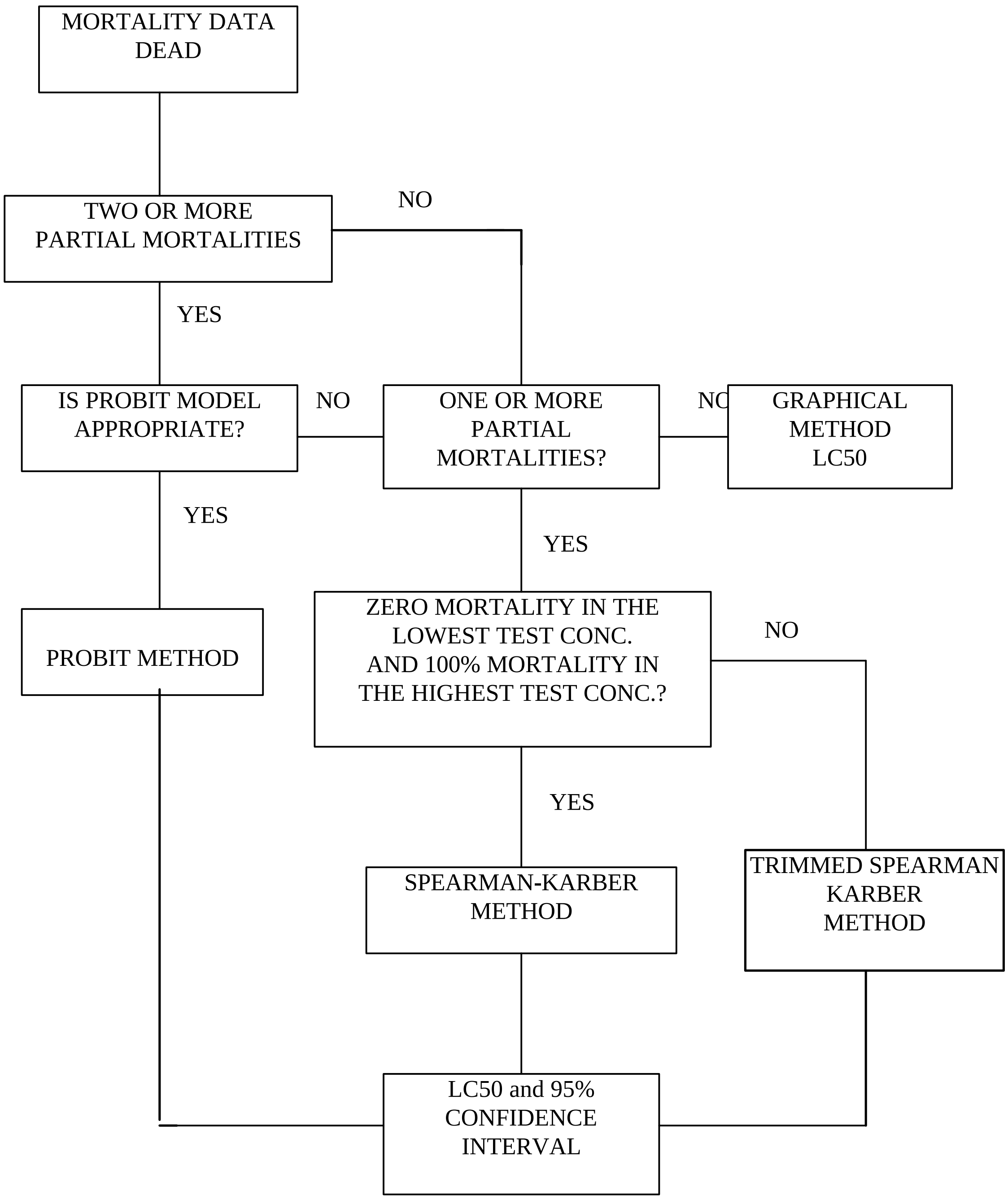

Figure 5.1 Flowchart for Statistical Analysis of the LC_{50} Based on Survival Data [50]

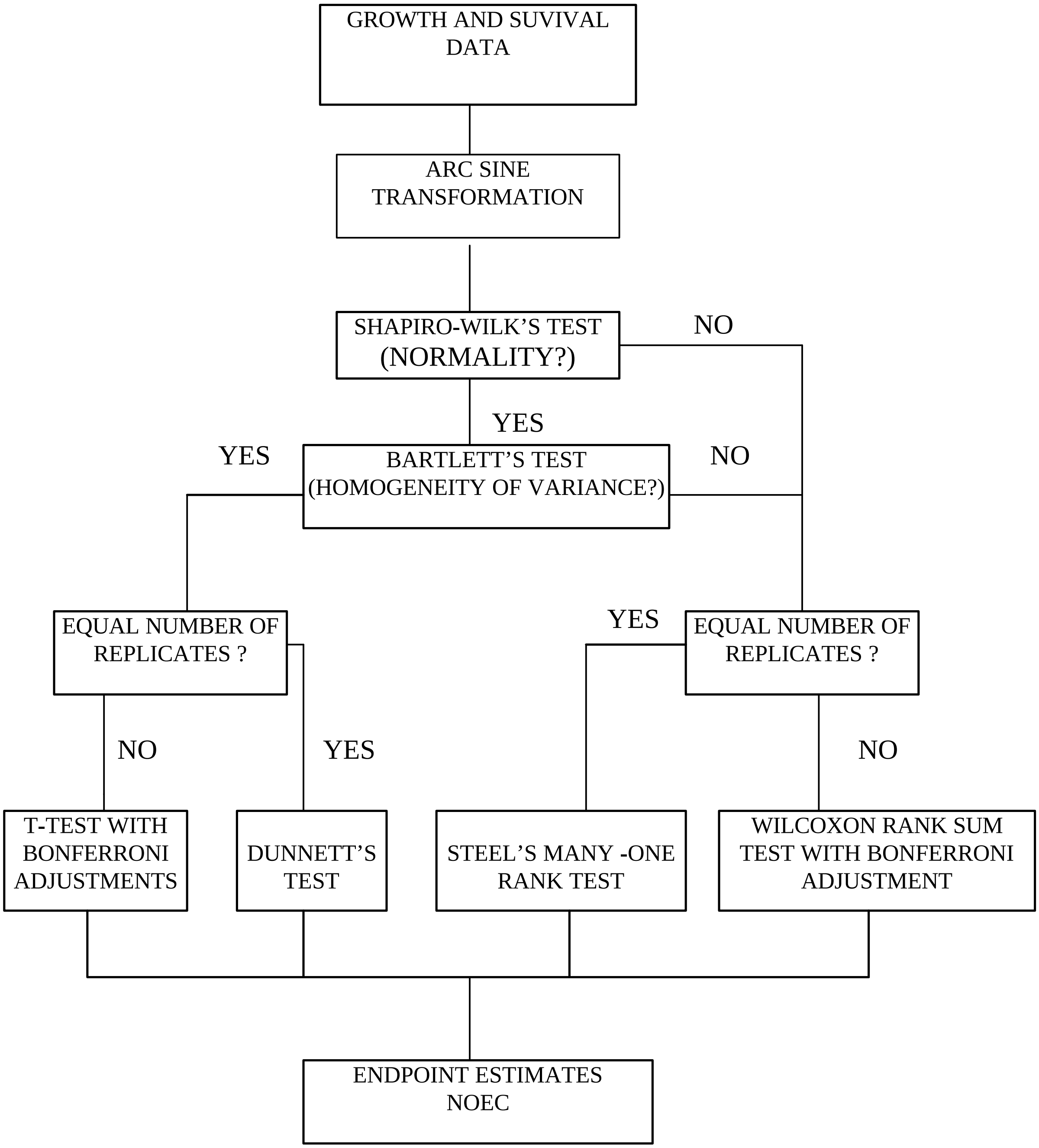

Figure 5.2 Flowchart for Statistical Analysis of the NOEC Based on Growth and Survival Data [51]

5.3.2 Methods for Determination of NOEC and LOEC

The NOEC for acute and chronic tests was determined using hypothesis testing and both survival and growth data were used [50, 51]. In these analyses, growth and survival data, which were expressed as the proportion surviving, were transformed by the arc-sine-square-root transformation. This transformation is commonly used for proportionality data to stabilize the variance and satisfy normality requirement. The Shapiro Wilk's Test was then used to test normality. Bartlett's Test was used to determine the homogeneity of variance. Failure of either of these tests leads to the use of modified T tests. In the T-test, if equal numbers of replicates across all dilutions and the control are met, a non-parametric Steel's Many-one Rank Test was used to determine the NOEC and LOEC. If not, then Wilcoxon Rank Sum Test was used to estimate the endpoints.

However, if the data met the assumptions of normality and homogeneity of variance, a parametric Dunnett's Procedure analysis was used if equal numbers of replicates were considered. A Bonferroni adjustment was used if there were unequal numbers of replicates.

Figure 5.2 provides a flowchart of these statistical analysis procedure for the NOEC and LOEC. In accordance with this procedure, the NOEC and LOEC of the Atlantic silverside were determined with Steel's Many-one Rank Test; and, the NOEC and LOEC of the ghost shrimp were estimated with Bonferroni adjustment analysis.

5.3.3 Methods for Determination of Differences.

To determine whether the 48hr LC_{50} values at the three levels of salinity were significantly different from each other, a group of three LC_{50} values at each level of salinity was tested with a single-factor ANOVA analysis at $\alpha \leq 0.05$. Differences between LC_{50} values were considered significant if $\alpha \leq 0.05$, but not at $\alpha > 0.05$.

Initially, in determining whether a group of three LC_{50} values was sufficient enough to be used in the final results, a method of trial and error was employed. This method involved the hypothesized numbers selected among the original average LC_{50}

values at each salinity level to form a group of four LC_{50} values. And then, the result from the hypothesized group of four was compared with that from the actual group of three. If the hypothesized result showed the same pattern as actual result, no more tests were needed. If not, at a minimum, an additional test would be conducted at each level of salinity. It was determined that a group of three LC_{50} values could represent the final results so no additional tests were performed.

5.3.4 Methods for Determination of Final LC_{50} and NOEC

In order to obtain a final LC_{50} and NOEC, two methods were used in this research. In one of the methods each of the tests was analyzed individually and then, LC_{50} , NOEC values, and the upper and lower limit of 95% C.I from each test were averaged. This method is known as the averaging method. The other method is known as the total-replicate method. Through this method, all of the replicates were analyzed together and then a final value of LC_{50}, NOEC, and 95% C.I were generated. In this research, both methods were used and the two resulting sets of data were presented on a total ammonia basis. Then, the unionized ammonia LC_{50} and NOEC were determined based upon the conditions of average pH, temperature, and salinity.

Chapter 6 Results and Discussions

Both the total-replicate method and the averaging method were used to determine the final LC_{50}, NOEC, and LOEC values. The lower one of these values generated from the total-replicate and the average methods was selected. The values were then used to refine the national criteria. The species mean acute values (SMAV) and acute-chronic ratio were also determined for both test organisms based on the LC_{50}, NOEC, and LOEC. This section includes the acute and chronic results for the Atlantic silverside and the ghost shrimp, the determination of SMAV, the acute-chronic ratio results, discussions of the results, and the refined national criteria.

6.1 Acute Toxicity Results

The LC_{50} and NOEC values from acute tests were determined using the Probit method, as described in Section 5.3. The 48hr LC_{50} values for the Atlantic silverside were determined at each of three levels of salinity. The 96hr LC_{50} for the ghost shrimp was estimated, as well. This section lists the LC_{50} for both species separately. For acute tests, an LC_{50} value is more useful than a NOEC to refine the national water criteria, so NOEC values were simply listed, and are not discussed.

6.1.1 The 48hr LC_{50} and NOEC values for Atlantic Silverside

The 48hr LC_{50} values for the Atlantic silverside at three levels of salinity are listed in Tables 6.1 and 6.2. The two tables give the LC_{50} and NOEC values as total ammonia and un-ionized ammonia. For each experiment, date of test initiation, 95% C.I. in parentheses, the LC_{50} values found in the literature for each species, and average pH are listed for comparison. Bench sheets for individual tests are given in Appendix B.

Table 6.1 shows the results from the averaging method. The 48hr LC_{50} values were calculated individually at each salinity level, and three 48hr LC_{50} values were obtained. The 48hr LC_{50} values at each salinity were 1.50 NH_3 mg/L at 14 ppth, 1.17 NH_3

mg/L at 22 ppth, and 1.08 NH_3 mg/L at 30 ppth. Among these three values, two fall in the range of 1.03 to 1.47 NH_3 mg/L listed in literature [11]. One of them, however, exceeds the range by 2%. The LC_{50} of 1.47 NH_3 mg/L in the literature was obtained under the conditions of pH 8.5 and salinity of 10ppth, which were different from those used in this work [11].

Table 6.1 48hr Individual Test and Average LC_{50} and NOEC for Atlantic Silverside (*Menidia Menidia*) at Three Levels of Salinity

Date	Total ammonia LC_{50}(mg/L)	Un-ionized ammonia LC_{50}(mg/L)	Avg. pH	Total ammonia NOEC (mg/L)	Un-ionized ammonia NOEC (mg/L)
		Salinity = 14 ppth			
5/24/96	28.09 (24.97-31.21)	1.52 (1.35-1.68)	8.17	20	1.09
6/28/96	28.17 (24.54-31.81)	1.52 (1.32-1.72)	8.16	20	1.08
6/29/96	27.14 (23.38-30.92)	1.47 (1.26-1.67)	8.16	20	1.08
Average	27.8 (24.18-31.36)	1.50 (1.30-1.69)	8.16	20	1.08
		Salinity = 22 ppth			
5/24/96	24.27 (21.51-27.05)	1.14 (1.01-1.27)	8.13	20	0.89
6/28/96	26.49 (23.53-29.45)	1.27 (1.10-1.38)	8.14	20	0.91
7/13/96	24.03 (20.74-27.31)	1.13 (0.97-1.28)	8.16	20	0.90
Average	24.93 (22.14-28.25)	1.17 (1.04-1.33)	8.14	20	0.90
		Salinity = 30 ppth			
5/24/96	24.68 (21.66-27.70)	1.15 (0.99-1.27)	8.17	20	0.93
6/28/96	22.75 (18.25-24.36)	1.05 (0.84-1.12)	8.16	20	0.92
7/13/96	22.56 (18.80-24.70)	1.03 (0.86-1.14)	8.15	20	0.91
Average	23.43 (19.96-26.03)	1.08 (0.92-1.19)	8.16	20	0.92

In addition, these results indicated that the 48hr LC_{50} values decreased as salinity increased. The results of the ANOVA analysis are shown in Appendix C. This results indicated that the LC_{50} at salinity of 14ppth was statistically greater than these at salinity of 22ppth and 30ppth. The LC_{50} at salinity of 22ppth was higher than that at salinity of 30ppth, but not statistically different. Discussions of the influence of different salinity strengths on the acute ammonia toxicity will be presented in section 6.4.1.

Table 6.2 48hr LC_{50} and NOEC Based on Six Replicates for Atlantic Silverside (*Menidia menidia*) at Three Levels of Salinity

Salinity (ppth)	Total ammonia LC_{50}(mg/L)	Un-ionized ammonia LC_{50}(mg/L)	Total ammonia NOEC(mg/L)	Un-ionized ammonia NOEC(mg/L)	Literature Un-ionized LC_{50}(mg/L)
14	28.02 (25.51-29.53)	1.51 (1.38-1.59)	20	1.08	
22	24.98 (22.99-26.50)	1.17 (1.08-1.25)	20	0.90	1.03-1.47
30	23.58 (20.83-24.33)	1.09 (0.95-1.19)	20	0.92	

The 48hr LC_{50} values based upon the 6-replicate method are 1.51, 1.17, and 1.09 NH_3 mg/L at salinity of 14, 22 and 30ppth, respectively. These values are slightly greater than those obtained from the averaging method.

More strict value should be selected in order to protect aquatic species. Thus, based on the averaging method, the reported 48hr LC_{50} values for the Atlantic silverside were 1.50, 1.17, and 1.08 at salinity of 14, 22 and 30ppth, respectively. The 48hr NOEC values for the Atlantic silverside were 1.08, 0.90, and 0.92 NH_3 mg/L at salinity of 14, 22, and 30ppth, respectively.

6.1.2 The 96hr LC_{50} and NOEC for Ghost Shrimp

The 96hr LC_{50} values for the ghost shrimp are listed in Tables 6.3 and 6.4. These tables provide the LC_{50} values with both total ammonia and un-ionized ammonia. The average pH, date of test initiation, 95% C.I. in parentheses, and the 96hr LC_{50} for this species presented in the literature are included in the tables, as well. Bench sheets for individual tests are given in Appendix D.

Table 6.3 shows the results from the averaging method. The final 96hr LC_{50} was obtained by averaging the LC_{50} values from three individual tests (4 replicates per test). The 96hr LC_{50} for the ghost shrimp was 1.66 NH_3 mg/L. This value shown in Table 6.4 is in the range of 1.06-2.57 NH_3 mg/L reported in the literature [8, 49].

Table 6.3 96hr Individual Test and Average LC_{50} and NOEC for Ghost Shrimp (*Palaemonetes Pugio*)

Total ammonia NOEC(mg/L)	Total ammonia LC_{50}(mg/L)	Un-ionized ammonia LC_{50}(mg/L)	Avg. pH	Total ammonia NOEC(mg/L)	Un-ionized ammonia NOEC(mg/L)
11/9/96	36.84 (34.59-38.81)	1.66 (1.56-1.82)	8.15	30	1.35
1/13/97	34.60 (31.09-37.43)	1.52 (1.37-1.65)	8.14	30	1.32
2/6/97	38.50 (35.35-41.56)	1.81 (1.16-1.95)	8.17	30	1.41
Average	36.64 (33.22-39.50)	1.66 (1.52-1.82)	8.16	30	1.38

Table 6.4 lists the 96hr LC_{50} and NOEC from the 12-replicate method. The 96hr LC_{50} of 1.67 NH_3 mg/L was slightly higher than that from the averaging method.

Table 6.4 96hr LC_{50} and NOEC Based on 12 Replicates for Ghost Shrimp (*Palaemonetes Pugio*)

Total ammonia LC_{50} (mg/L)	Un-ionized ammonia LC_{50}(mg/L)	Total ammonia NOEC (mg/L)	Un-ionized ammonia NOEC(mg/L)	Lit. Un-ionized LC_{50}(mg/L)
36.20 (34.86-38.04)	1.67 (1.60-1.75)	30	1.38	1.06-2.57

For the ghost shrimp, the 96hr LC_{50} values calculated with both methods differed slightly. Therefore, the 96hr LC_{50} for the ghost shrimp was reported to be 1.66 NH_3 mg/L, based on the averaging method. The 96hr NOEC was 1.38 NH_3 mg/L.

6.2 Chronic Toxicity Results

The NOEC, LOEC, and 7-day LC_{50} for the Atlantic silverside and the ghost shrimp were determined based upon both survival and growth data. For the chronic tests, the NOEC and LOEC values are more useful than the LC_{50} values to refine the national water criteria so the NOEC and LOEC values are discussed in detail and the 7-day LC_{50} value was simply listed.

6.2.1 Atlantic Silverside

The averaging method and the total-replicate method were used to determine the final NOEC, LOEC, and 7-day LC_{50} for the Atlantic silverside. Bench sheets for individual test are provided in Appendix E.

Table 6.5 lists the NOEC, LOEC, and 7-day LC_{50} values for the Atlantic silverside based on survival data. This table also gives the NOEC, LOEC, and the 7-day LC_{50} on total ammonia and un-ionized ammonia basis, date of test initiation, 95% C.I in parentheses for the 7-day LC_{50}, and average pH. The 7-day LC_{50} for the Atlantic silverside was 1.16 NH_3 mg/L based on the 6-replicate method. According to the 6-replicate method, the NOEC and LOEC for the Atlantic silverside based on survival were 0.63 and 0.95 NH_3 mg/L, respectively.

Table 6.5 7-Day Individual Test and 7-Day LC_{50}, NOEC, and LOEC for Atlantic Silverside (*Menidia Menidia*) Based on Survival

Date	Total ammonia LC_{50} (mg/L)	Un-ionized ammonia LC_{50}(mg/L)	Avg. pH	Total NOEC (mg/L)	Un-ion NOEC (mg/L)	Total LOEC (mg/L)	Un-ion LOEC (mg/L)
6/6/96	18.16 (16.60-19.48)	1.18 (1.08-1.27)	8.16	10	0.65	15	0.98
7/12/96	18.82 (17.65-19.90)	1.22 (1.11-1.25)	8.14	15	0.93	20	1.24
Average	18.49 (17.13-19.69)	1.20 (1.08-1.24)	8.15	12.5	0.78	17.5	1.10
6-replicate method	18.45 (17.56-19.28)	1.16 (1.10-1.21)	8.15	10	0.63	15	0.95

Table 6.6 provides the NOEC and LOEC values for the Atlantic silverside based on growth data. This table includes the NOEC and LOEC at both total ammonia and un-ionized ammonia, and the dates of test initiation. The NOEC and LOEC values for the averaging method and the 6-replicate method differed by 24%. The value for the averaging method was lower. According to the averaging method, it was determined that the NOEC and LOEC based on growth were 0.48 and 0.79 NH_3 mg/L, respectively.

Table 6.6 7-Day Individual Test and Average NOEC and LOEC for Atlantic Silverside (*Menidia menidia*) Based on Growth

Date	Total ammonia NOEC (mg/L)	Un-ionized ammonia NOEC(mg/L)	Total ammonia LOEC(mg/L)	Un-ionized ammonia LOEC(mg/L)
6/6/96	5	0.33	10	0.65
7/12/96	10	0.62	15	0.93
Average	7.5	0.48	12.5	0.79
6-replicate method	10	0.63	15	0.95

In comparing the chronic results, it was found that growth of the Atlantic silverside was slightly more sensitive than survival. Therefore, the NOEC and LOEC of un-ionized ammonia for the Atlantic silverside based on growth were 0.48 and 0.79 mg/L, respectively.

6.2.2 Ghost Shrimp

The NOEC, LOEC, and 7-day LC_{50} for the ghost shrimp were determined by the averaging method and the total-replicate method. The results for survival and growth are provided in Table 6.7 and Table 6.8, respectively. Bench sheets for individual tests are given in Appendix F.

Table 6.7 lists the NOEC and 7-day LC_{50} values for the ghost shrimp based on survival. This table gives the NOEC and 7-day LC_{50} in terms of total ammonia and unionized ammonia, average pH, date of test initiation, and 95% C.I in parentheses for the 7-day LC_{50}. The 7-day LC_{50} for the ghost shrimp based on the 8-replicate method was 1.43 NH_3 mg/L. According to the 8-replicate method, it was determined that the NOEC and LOEC were 0.99 and 1.31 NH_3 mg/L, respectively.

Table 6.7 7-Day Individual Test and Average LC_{50}, NOEC, and LOEC for Ghost Shrimp (*Palaemonetes Pugio*) Based on Survival

Date	Total ammonia LC_{50} (mg/L)	Un-ionized ammonia LC_{50}(mg/L)	Avg. pH	Total NOEC (mg/L)	Un-ion NOEC (mg/L)	Total LOEC (mg/L)	Un-ion LOEC (mg/L)
1/1/97	20.04 (18.18-22.31)	1.30 (1.18-1.45)	8.18	15	0.98	20	1.30
1/12/97	23.86 (21.68-26.04)	1.57 (1.43-1.71)	8.16	15	0.99	20	1.32
Average	21.95 (19.93-24.18)	1.45 (1.32-1.60)	8.17	15	0.99	20	1.31
8-replicate method	21.66 (20.20-23.31)	1.43 (1.33-1.54)	8.17	15	0.99	20	1.31

Table 6.8 lists the NOEC and LOEC values for the ghost shrimp based on growth. This table includes the NOEC and LC_{50} in terms of total ammonia and un-ionized ammonia, and date of test initiation. According to the 8-replicate method, the NOEC and LOEC for the ghost shrimp was 0.33, and 0.66 NH_3 mg/L, respectively.

Table 6.8 7-Day Individual Test and Average NOEC and LOEC for Ghost Shrimp (*Palaemonetes Pugio*) Based on Growth

Date	Total ammonia NOEC (mg/L)	Un-ionized ammonia NOEC(mg/L)	Total ammonia LOEC (mg/L)	Un-ionized ammonia LOEC(mg/L)
1/1/97	10	0.65	15	0.98
1/12/97	5	0.33	10	0.66
Average	7.5	0.49	12.5	0.83
8-replicate method	5	0.33	10	0.66

When the chronic results based on survival and growth were compared, the results indicated that the growth data was more sensitive. Therefore, for the ghost shrimp, the NOEC and LOEC of un-ionized ammonia based on growth were 0.33 and 0.66 mg/L, respectively.

6.3 SMAV and Acute-Chronic Ratio Results

The species mean acute values (SMAV) were determined using the 48hr LC_{50} and 96hr LC_{50} with the geometric mean method. The chronic values were determined by calculating the geometric mean of the NOEC and LOEC. Hence, the acute-chronic ratio was calculated through dividing the 48hr LC_{50} by the chronic value. The chronic value and the acute-chronic ratio for the Atlantic silverside were not calculated; and the reason for this will be given later. The SMAVs and acute-chronic ratio are important in reevaluating the national criteria. A summary of these results for both species along with their reported LC_{50}, NOEC, and LOEC are presented in Table 6.9.

Table 6.9 Summary of Acute and Chronic Results at Un-ionized Ammonia

Atlantic silverside (*M.menidia*) (mg/L)			Ghost shrimp (*P. pugio*) (mg/L)	
48hr LC_{50}	14 (ppth)	1.50		
	22 (ppth)	1.17	96hr LC_{50}	1.66
	30 (ppth)	1.08		
SMAV		1.07	SMAV	2.40
48hr NOEC	14 (ppth)	1.08		
	22 (ppth)	0.90	96hr NOEC	1.38
	30 (ppth)	0.92		
7-day LC_{50}		1.16	7-day LC_{50}	1.43
7-day NOEC(survival)		0.63	7-day NOEC(survival)	0.99
7-day LOEC(survival)		0.95	7-day LOEC(survival)	1.31
7-day NOEC(growth)		0.48	7-day NOEC(growth)	0.33
7-day LOEC(growth)		0.79	7-day LOEC(growth)	0.66
			Chronic value	0.46
			Acute-Chronic Ratio	7. 57

***** ppth: salinity unit as part per thousand in parentheses.

In determining the SMAV for the Atlantic silverside, the 48hr LC_{50} at a salinity of 22ppth was used because this salinity is closet to the average value in the Chesapeake Bay. Since 96hr tests were not performed in this research, the 96hr LC_{50} of 0.97 NH_3 mg/L reported by the EPA [49] was used. Therefore, the SMAV for the Atlantic silverside was calculated to be 1.07 NH_3 mg/L, as compared to 1.05 NH_3 mg/L proposed by the EPA [49]. The difference between these two values is 2%.

The SMAV for the ghost shrimp was determined to be 2.40 NH_3 mg/L at salinity levels of 25ppth. This value was calculated using the 48hr LC_{50} of 3.48 NH_3 mg/L reported by Hudgins's research [22] and the 96hr LC_{50} of 1.66 NH_3 mg/L determined in this study. The resulting SMAV for the ghost shrimp is 31% higher than the 1.65 NH_3 mg/L reported by the EPA [49].

The chronic value for the ghost shrimp was determined from the NOEC and LOEC provided by this research. The chronic value for the ghost shrimp was calculated to be 0.46 NH_3 mg/L. The acute-chronic ratio for the ghost shrimp was 7.56 and was obtained by dividing the 48hr LC_{50} of 3.48 NH_3 mg/L from Hudgins (1996) by the chronic value of 0.46 NH_3 mg/L. The ghost shrimp therefore was as chronically sensitive as the other marine invertebrate mysid (7.2), and less sensitive than inland silverside (21.3).

6.4 Discussion

6.4.1 Relationship of Salinity and Ammonia Toxicity

The relationships between ammonia toxicity and environmental factors, particularly salinity, are poorly understood with marine species. To date, the toxicity of ammonia at different salinity levels has only been studied with four species: striped bass (*Morone saxatilis*), three-spined stickleback (*Gasterosteus aculeatus*), mysid (*Mysidopsis bahia*), and inland silverside (*Menidia beryllina*) [19, 30]. In this study, the 48hr acute tests with Atlantic silverside were conducted at three levels of salinity. The purpose was to derive a better understanding of the relationship between ammonia toxicity and salinity, and to verify the previous results and the hypothesis that un-ionized ammonia is the cause of ammonia toxicity.

The results of this research showed that the 48hr LC_{50} values of un-ionized ammonia for *M. menidia* were 1.50 mg/L at 14ppth salinity, 1.17mg/L at 22ppth, and 1.08 mg/L at 30ppth. As illustrated in Figure 6.1, the 48hr LC_{50} decreased with increases in salinity, although the 48hr LC_{50} values between 22ppth and 30ppth were not statistically significantly different. As discussed in Section 2.1, if un-ionized ammonia is a major form of toxicity, the 48hr LC_{50} values should increase as salinity increases. In other words, the tolerance of species should increase as salinity increases. The result of this research do not support this hypothesis. As indicated in Section 2.1.3, salinity is not as important as pH and temperature in affecting ammonia equilibrium. Changes in 8 units of salinity causes only a 7% of change in un-ionized ammonia. This may explain why there was no statistical difference in LC_{50} values between 22 ppth and 30 ppth.

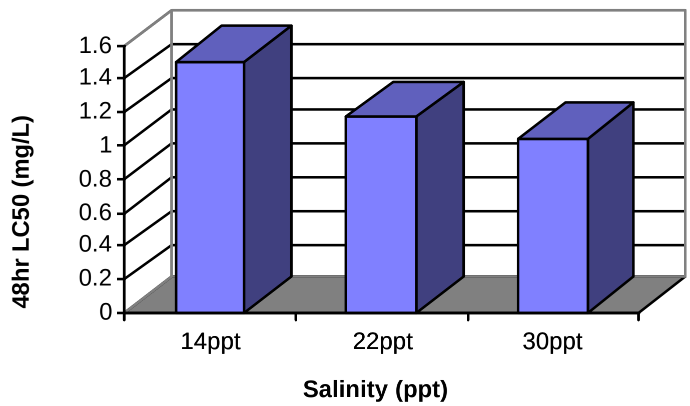

Figure 6.1 The Relationship between Salinity Strengths and 48hr LC_{50} as Un-ionized Ammonia for Atlantic silverside (*Menidia menidia*)

However, the 48hr LC_{50} values at 14ppth and 22ppth, as well as at 14ppth and 30ppth were statistically significantly different. The results of Mill *et al*.(1990) also revealed a similar pattern. In their study, the 96hr acute tests with mysid were performed at salinities of 11ppth, 30ppth, and the 96hr LC_{50} values were 1.71 at 11ppth and 1.23 at 30ppth. One of the interpretations for this results is that un-ionized ammonia might not be the only toxic form of ammonia. Both un-ionized ammonia and ionized-ammonia may play an important role in ammonia toxicity. In addition, the activity of ammonia and biological behavior of test organism may affect the toxicity of ammonia, as well.

6.4.2 The 7-day Chronic Toxicity Results

EPA has developed an approach to estimate a long-term chronic toxicity with 7-day or 9-day tests for marine species [48], because the 7-day chronic test can reflect long-term toxicity in more time-efficient way. The species used in the approach included inland silverside, sheephead minnow, and mysid. The 7-day chronic tests with the Atlantic silverside and the ghost shrimp were included in this study in order to determine whether the long-term chronic effect for these two species could be effectively estimated through this approach. The results are discussed for each species.

- **Atlantic Silverside**

As indicated in Tables 6.5 and 6.6, the NOEC for the Atlantic silverside was 0.48 NH_3 mg/L based on growth, and 0.63 NH_3 mg/L based on survival, respectively. The growth of the Atlantic silverside was inhibited before significant mortality occurred. These results showed that growth for the Atlantic silverside was more sensitive to ammonia than mortality. As compared to the NOEC of 0. 061 NH_3 mg/L for inland silverside, which has the same genus as Atlantic silverside, the NOEC of the Atlantic silverside is relatively high. The difference could primarily be contributed to variations in species and test duration. The chronic test for inland silverside was conducted by Mill *et al.* (1990) for a period of 28 days. Thus, the NOEC of the Atlantic silverside was assumed to be about four times greater than that of inland silverside. However, it was found that the growth NOEC for the Atlantic silverside was eight times greater than that of inland silverside. Hence, to estimate the chronic toxicity for the Atlantic silverside from the short term chronic test, this research suggested that 10 or 14-day chronic toxicity tests need to be performed. And for this same reason, it was also decided that the 7-day NOEC and LOEC for the Atlantic silverside in this research should not be used to refine the national water criteria for ammonia in saltwater.

- **Ghost Shrimp**

As described in Section 6.2.2, the NOEC for the ghost shrimp was 0.99 NH_3 mg/L based upon survival, and 0.33 NH_3 mg/L, based upon growth. These results suggest that the growth of the ghost shrimp in a 7-day chronic test was significantly inhibited but its mortality was not, at the same concentration. Significant mortality did not occur until the concentration of un-ionized ammonia was as high as 0.99 mg/L. It appeared that growth was more sensitive to ammonia than mortality. Hence, the 7-day chronic test for ghost shrimp can potentially be used to estimate long-term chronic toxicity. As a result, the NOEC and LOEC based on growth for the ghost shrimp were used to refine the national criteria for ammonia.

6.5 Refining the National Criteria

The acute LC_{50} values and the chronic NOEC values and LOEC values for the two species were used to refine the national criteria for ammonia. The procedures on deriving the national criteria for ammonia were outlined in the Guidelines [42]. The current acute criterion of un-ionized ammonia concentration is 0.233 mg/L, and the chronic criterion 0.035 mg/L. These criteria were determined based upon toxicity tests with both freshwater and marine species.

The SMAV, the chronic value, and the acute-chronic ratio were used to determine the final acute value and the final chronic value. Table 6.10 summarized the results from this research, which were used to determine the acute and chronic criteria. This section will present the refined results for the acute criterion and the chronic criterion.

6.5.1 The Acute Criterion

The SMAVs was used to determine the GMAV. The final acute value (FAV) was calculated based upon the GMAV. However, only the GMAVs from the top four most sensitive species were used to calculate the FAV. With the SMAVs generated for Atlantic silverside (*Menidia menidia*) and the ghost shrimp (*Palaemonetes pugio*) in this research, the previous rank of the top four species still remained unchanged. Therefore, the FAV was not calculated and still remains as 0.465 un-ionized ammonia mg/L. In other words, the current acute criterion of FAV/2 was not refined given the results of this research, and remains at 0.233 un-ionized ammonia mg/L.

Table 6.10 Test Results Used to Refine the National Criteria

Parameters	Atlantic silverside (*M. menidia*) (mg/L)	Ghost shrimp (*P. pugio*) (mg/L)
48-hour LC_{50}	1.17	3.48 *[2]
96-hour LC_{50}	0.97 *[1]	1.66
SMAV	1.07	2.40
7-day NOEC (growth)		0.33
7-day LOEC (growth)		0.66
Chronic Value		0.46
Acute-chronic ratio		7.57

*[1] : taken form references 49.
*[2] : taken from references 22.

6.5.2 The Chronic Criterion

The chronic criterion or the final chronic value (FCV) for ammonia was refined based on the results from this report. The current chronic criterion was calculated using the acute-chronic ratios from two saltwater and four freshwater species. The geometric mean of these six acute-chronic ratios was calculated and then divided into the FAV to yield the current chronic criterion of 0.035 un-ionized ammonia mg/L.

To refine the chronic criterion solely based on saltwater species, the four freshwater species were excluded. A total of four saltwater species with their acute-chronic ratios was used to refine the chronic criterion. They were inland silverside (21.3), mysid (4.35, Hudgins, 1996), sheephead minnow (6.95, Hudgins, 1996), and the ghost shrimp (7.56). The geometric mean of those four values is 8.35. Dividing the 8.35 into the FAV yields the refined chronic criterion of 0.056 un-ionized ammonia mg/L. The site-specific instream limits can be calculated based upon this value along with pH, temperature, and salinity.

The refined chronic criterion of 0.056 NH_3 mg/L differs from the current chronic criterion of 0.035 NH_3 mg/L by 29%. However, when compared to the 0.054 NH_3 mg/L proposed by Hudgins (1996), there is only a 4% difference. These results suggest that the current chronic criterion needs to be updated with test data derived solely from saltwater species.

Chapter 7 Conclusions and Recommendations

In this research, acute and the 7-day chronic ammonia toxicity tests were performed with two marine species: the Atlantic silverside (*Menidia menidia*) and the ghost shrimp (*Palaemonetes pugio*). The 48hr acute tests for the Atlantic silverside were performed at three salinity levels to better understand the influence of different salinity on ammonia toxicity. The acute LC_{50} values and the 7-day chronic NOEC values and LOEC values were determined in order to refine the national criteria for ammonia. The refined criteria based on more saltwater species data can be used to reevaluate the instream limits for the Chesapeake Bay. Based on the test results in this study, the following conclusions and recommendations are made.

7.1 Conclusions

1). For the Atlantic silverside, the 48hr LC_{50} values were 1.50, 1.17, and 1.08 NH_3 mg/L at salinities of 14, 22 and 30 ppth, respectively. Two of these values fall in the range of 0.97 to 1.47 NH_3 mg/L provided in the literature.

2). The 48hr LC_{50} values for the Atlantic silverside decreased with increases in salinity, although the 48hr LC_{50} values for 22ppth and 30ppth were not statistically different.

3). The 96hr LC_{50} for the ghost shrimp was determined to be 1.66 NH_3 mg/L. This value falls in the range of 1.06-2.57 NH_3 mg/L found in the literature.

4). The SMAV for the Atlantic silverside was determined to be 1.07 NH_3 mg/L, as compared to 1.05 NH_3 mg/L reported by EPA. The SMAV for the ghost shrimp was determined to be 2.40 NH_3 mg/L, as opposed to 1.65 NH_3 mg/L reported by the EPA.

5). The 7-day NOEC for the Atlantic silverside based on growth was 0.48 NH_3 mg/L, while the NOEC based on survival was 0.63 NH_3 mg/L. The results suggest that 10 or 14-day chronic tests should probably be used for long-term chronic data. Consequently, the 7-day NOEC and LOEC for the Atlantic silverside were not used to refine the national criteria.

6). The NOEC for the ghost shrimp based on growth was 0.33 NH_3 mg/L, as compared to a NOEC of 0.99 NH_3 mg/L based on survival. Growth was a more sensitive indicator than survival. Therefore, the NOEC based on growth for the ghost shrimp was used to refine the national criteria.

7). The acute-chronic ratio for the ghost shrimp was determined to be 7.56. It appeared that ghost shrimp was as chronically sensitive to ammonia as the marine invertebrate mysid (7.2), but less sensitive than inland silverside (21.3).

8). The current acute criterion of FAV/2 was not refined based upon the results provided by this research and remains at 0.233 un-ionized ammonia mg/L.

9). The refined chronic criterion for un-ionized ammonia was 0.056 mg/L, in contrast to the currently proposed chronic criterion of 0.035 NH_3 mg/L.

7.2 Recommendations

Future work needs to be performed in order to better understand the impact that ammonia has on marine vertebrates and invertebrates:

1). More invertebrates should be tested, both acutely and chronically. Possible candidates are given below:

- Blue crab (*Callinectes sapidus*)
- Sargassum shrimp (*Latreutes fucorum*)
- Sand shrimp (*Penaeus aztecus*)

2). The following vertebrates should be tested, particularly in chronic tests [49]:

- White perch (*Morone americana)*
- Killfish (*Fundulus simillis*)
- Spot (*Leostomus xanthurus*)

3). Further study of the relationship between toxicity of ammonia levels, pH, and temperature is needed.

4). The influence of salinity on ammonia toxicity should be evaluated in other species, especially invertebrates.

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