1. Introduction

1.1 Ammonia Issues in the Chesapeake Bay

The Clean Water Act of 1977 requires the Environmental Protection Agency (EPA) to publish water quality criteria that reflect the latest scientific knowledge on the effects of pollutants in a body of water. Fulfilling this requirement, the EPA published the Ambient Aquatic Life Water Quality Criteria for Ammonia (Saltwater) – 1989 [1], herein referred to simply as the Criteria. This document proposes criteria to be used as a guide in assisting the states in setting discharge standards. Two standards are presented in the Criteria: 1) The acute criterion, which is the one-hour average concentration not to be exceeded more than once every three years. 2) The chronic criterion, which is a four-day average concentration not to be exceeded more than once every three years. The procedures for deriving these criteria are described in "Guidelines for Deriving Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses" [2], herein referred to as the Guidelines.

The acute criterion was established at 0.233 mg/L as unionized ammonia. It is based on 21 species from 18 genera (See Table 4.2). The Genus Mean Acute Value (GMAV) for the four most sensitive species was used to calculate the acute criterion. The chronic criterion was established at 0.035 mg/L as unionized ammonia. It is based on acute-chronic ratios for two marine and four saltwater organisms (See Table 4.3). At the time the Criteria was published, chronic data for saltwater species were only available for the Mysid Shrimp and Inland Silverside.

This research is intended to provide more data, in conjunction with research performed by Li [3] and Hudgins [4], so that the criteria may be reevaluated.

1.2 Ammonia in Recirculating Aquaculture

Aquaculture has gained importance in recent years as traditional fishing grounds have become depleted due to overfishing. In recirculating aquaculture, the water is constantly being recycled through some type of treatment process, often filtration, aeration, settling, or biological filtering [5]. Recirculating aquaculture offers many advantages over flow-through systems. First, it uses much less water, so sites that don't have an adequate supply of water for a flow-through system can be used. Second, energy savings could be significant if the water supply must be treated (e.g. heating) [5]. Even though the water is treated, ammonia can build up in a recirculating system and cause toxicity [5]. Ammonia is a by-product of the metabolic processes of the organisms being cultured and of the breakdown of organic nitrogen [5]. Toxicity data can be used to establish guidelines of acceptable ammonia concentrations in aquaculture.

2. Overview of Ammonia in the Aquatic Environment

2.1 The Nitrogen Cycle

The Nitrogen Cycle is one of the major biogeochemical cycles of the biosphere [6]. Earth's atmosphere is almost 80% nitrogen gas (N_2), however plants can't use it in this form. Therefore, the nitrogen cycle is critical to life on earth. The major processes are illustrated in Figure 2.1 and are described below. They are:

-Fixation

-Ammonification

-Nitrification

-Denitrification

-Assimilation

Fixation occurs by free-living bacteria, symbiotic bacteria in the root nodules of legumes (*Rhizobium*), blue-green algae in the aquatic environment, and by industry, especially fertilizer producers [6]. Nitrogen fixation is especially important since most plants and all animals can't use it in its molecular form (N_2). However, excess fixation and concentration of fixed nitrogen can have deleterious environmental effects such as accelerated ammonia toxicity, which is discussed in this report, and eutrophication of lakes [6]. Fixation requires energy. The reduction of one molecule of N_2 to NH_3 requires about the same amount of energy that is released by the reduction of an atom of organic carbon to CO_2 [7]. On a global scale, nitrogen fixation roughly balances production of N_2 by denitrification and fluxes amount to approximately 2% of the total cycling of nitrogen through the biosphere [7].

Ammonification is the process by which organic nitrogen is decomposed back to ammonia (NH_3). It is carried out by many species of bacteria and eukaryotes [6]. During ammonification the energy potential of the nitrogen atom doesn't change [7].

Nitrification is the process by which nitrogen is oxidized from ammonia to nitrite (NO_2) and nitrite is oxidized from nitrite to nitrate (NO_3) [7]. Both steps release much of the potential chemical energy stored in the nitrogen atom [7]. The transformation from ammonia to nitrite is carried out by *Nitrosomonas* in the soil and freshwater systems and

by *Nitrosococcus* in marine systems [7]. The transformation from nitrite to nitrate is carried out by *Nitrobacter* in the soil and freshwater systems and by *Nitrosococcus* in marine systems [7].

Denitrification occurs in anoxic environments. In these environments, where oxygen is not present to serve as an electron acceptor, nitrate and nitrite may become electron acceptors [7]. When nitrate is the electron acceptor, it forms nitrite. When nitrite is the electron acceptor, it forms nitric oxide (NO) which is then transformed to nitrogen gas by physical processes [7]. Bacteria such as *Pseudomonas denitrificans* accomplish the denitrification reactions

Assimilation is the process by which nitrate is reduced to organic nitrogen. Assimilation requires energy, however, it is required for many organic molecules [6]. Animals can not assimilate nitrate to organic nitrogen and must eat plants to acquire their organic nitrogen [6].

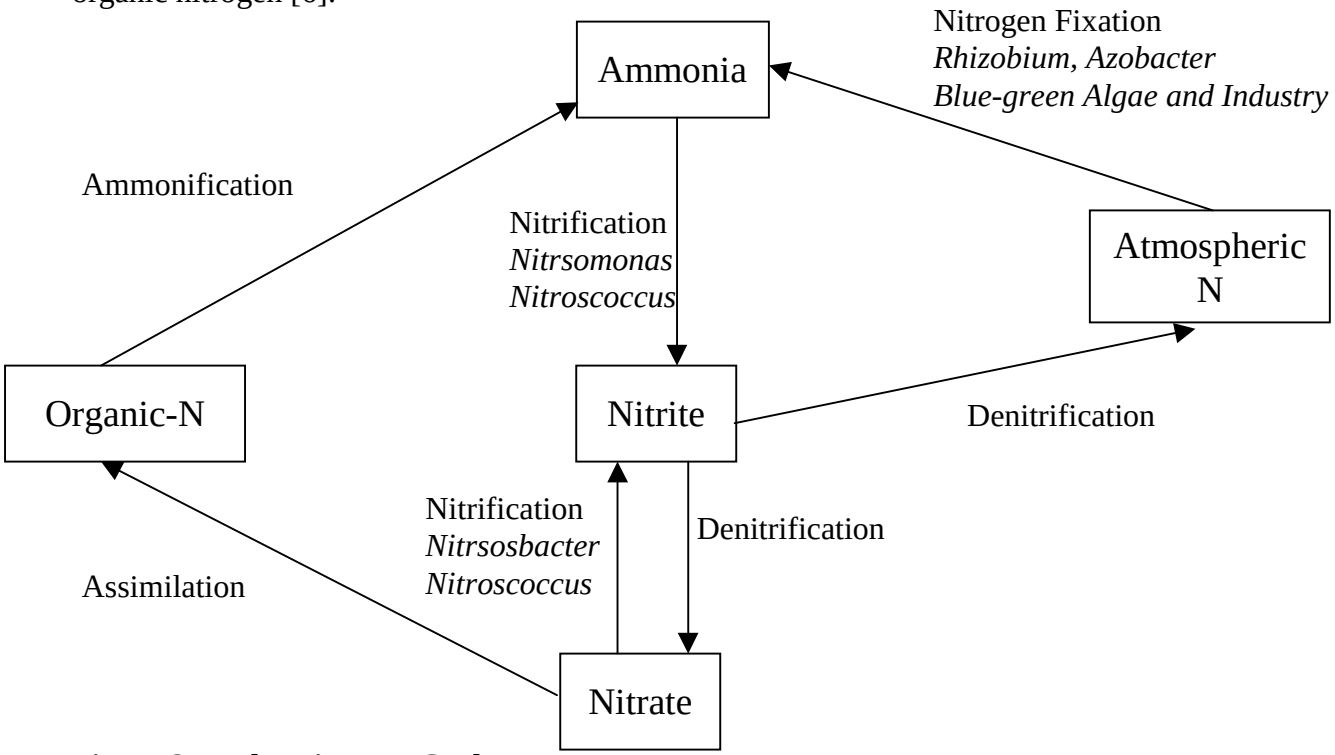

Figure 2.1- The Nitrogen Cycle

2.2 Ammonia Chemistry in Water

Ammonia is present in the aqueous environment in two forms, unionized ammonia (NH_3), and ionized ammonium (NH_4^+). They exist in equilibrium represented by the equation:

$$NH_4^+ + H_2O \leftrightarrow NH_3 + H_3O^+ \qquad \textbf{Eq. 2.1}$$

It is important to know the relative amounts of NH_3 and NH_4^+ because they exhibit different toxicities, with NH_3 being much more toxic [8]. In fact, current ammonia regulations are written in terms of NH_3 [1]. It is necessary to know the physical parameters that determine the relative proportions of NH_3 and NH_4^+ because current measurement techniques only measure total ammonia [9]. This section will discuss the major physical parameters that determine these proportions; pH, temperature, and salinity, and will briefly discuss other parameters that have minor effects; carbon dioxide, dissolved oxygen, and alkalinity.

2.2.1 pH

At 25^0C and no salinity, the ionization constant is given as:

$$K_a = \frac{[NH_3][H^+]}{[NH_4^+]} = 10^{-9.2} \qquad \textbf{Eq. 2.2 [1]}$$

As can be seen from equation 2.2, the hydrogen ion concentration and thus the pH determine the proportion of NH_3. As the pH increases, the hydrogen ion concentration decreases, and the proportion of unionized ammonia increases. From equation 2.2, the equation for the percent of total ammonia in the unionized form can be derived as;

$$\%\text{-unionized ammonia} = 100/(1+\text{antilog}\,(pK_a - pH)) \qquad \textbf{Eq. 2.3}$$

At the pHs acceptable to most aquatic life, 6.5-8.5, a small increase in pH can greatly affect the %-unionized ammonia as seen in Table 2.1.

Table 2.1 - %-unionized ammonia at 25^0C at various pHs

pH	**6.5**	**7.0**	**7.5**	**8.0**	**8.5**
%-unionized ammonia	0.180	0.566	1.77	5.38	15.3

From this data, it can be seen that an increase of pH by 1 unit roughly corresponds to a ten-fold increase in the %-unionized ammonia. The strong dependence of %-unionized ammonia on pH has two important consequences:

1. The pH of the receiving stream is extremely important when discussing ammonia effects in the environment.
2. Strict pH control of experimental bioassays must be exercised to obtain useful data.

2.2.2 Temperature

Emerson et al. [10] determined temperature effects the ionization constant for aqueous ammonia follows the equation:

$pK_a = 0.0918 + 2729.92/T$ **Eq. 2.4**

Where T is degrees Kelvin.

From this equation, it is apparent that an increase in temperature will increase the %-unionized ammonia. Table 2.2 lists the values for the ionization constant and the %-unionized ammonia at several different temperatures

Table 2.2 – Ionization constants and %-unionized ammonia for several temperatures.

Temperature (^oC)	pK_a	%-NH_3 at pH 7.5	%-NH_3 at pH 8.0
0	10.1	.256	.806
5	9.91	.388	1.22
10	9.74	.577	1.80
15	9.57	.846	2.63
20	9.41	1.22	3.77
25	9.25	1.74	5.31
30	9.10	2.45	7.36

Temperature effects have two major impacts:

1. The temperature characteristics of receiving waters are important when discussing ammonia toxicity in the environment.
2. Laboratory experiments must exercise control over temperature. This was relatively easy in the bioassays presented in this report by using constant temperature baths.

2.2.3 Salinity

As salinity or ionic strength increases, the %-unionized ammonia decreases due to the decrease in the ionization constant. Table 2.3 gives pK_as, ionic strengths and %-unionized ammonia values at various salinities.

Table 2.3 – Salinity Effects on %-unionized ammonia at 20^{o}C and pH 8

Salinity (ppt)	Ionic Strength	pKa	%NH_3
0	0	9.40	3.82
20	0.4	9.46	3.41
25	0.5	9.48	3.19
30	0.6	9.49	3.12
35	0.75	9.51	2.98

As can be seen from the table, an increase in salinity by about 10 parts per thousand (ppt) decreases the %-unionized ammonia by ~0.3%. Data on the salinity of receiving waters is important when discussing the toxicity of ammonia in the environment. In laboratory bioassay conditions, salinity is not a difficult to control, however good records of salinity are important so that the %-unionized ammonia can be accurately determined.

2.2.4 Other Factors (Carbon Dioxide, Dissolved Oxygen, and Alkalinity)

Carbon dioxide, dissolved oxygen, and alkalinity are minor factors that influence the %-unionized ammonia. Alkalinity and carbon dioxide affect the %-unionized ammonia indirectly by affecting the pH of the water. Increasing the concentration of carbon dioxide in water increases the hydrogen concentration, which decreases the %-unionized ammonia. Alkalinity works in conjunction with carbon dioxide in determining pH. An increase in dissolved oxygen may increase the toxicity of ammonia. This is related to the fact that one of the acute effects of ammonia intoxication is hypoxia. Alabaster et al. [11] showed that at dissolved oxygen levels 50% below saturation, survival times were reduced for several fish species exposed to lethal doses of ammonia. However, no work has been done to study the affects of dissolved oxygen on sublethal concentrations of ammonia.

3 Ammonia Toxicity

3.1 National Criteria

Two different criteria are present in the criteria; the acute criterion and the chronic criterion. Methods for determining the data are presented in the Guidelines [2]. The method for determining each is discussed below followed by what each criterion means and then how effluent limits are determined based on the criterion. Figure 3.1 offers a schematic representation of how the endpoints are used to derive each criterion.

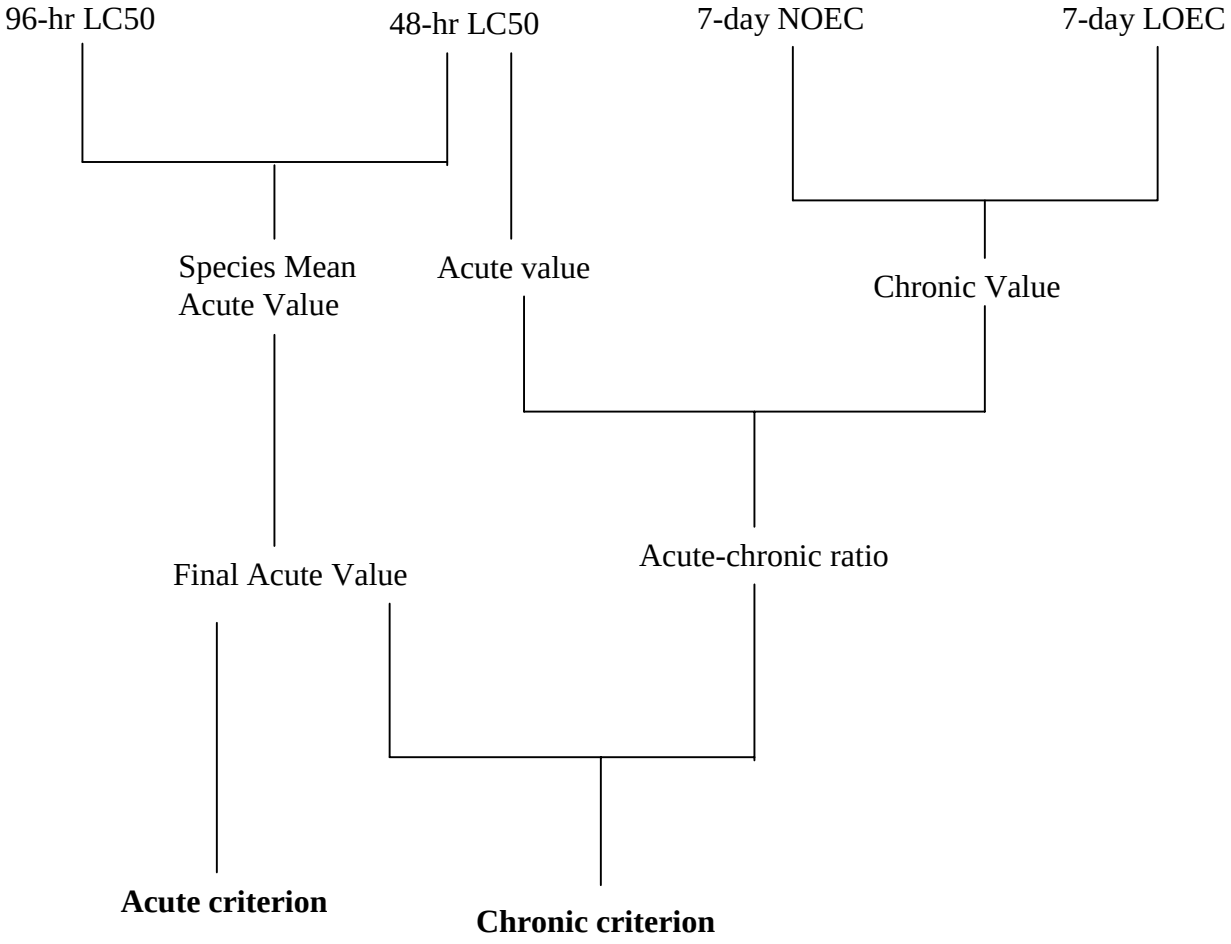

Figure 3.1 Schematic Representation of Derivation of the Acute and Chronic Criteria

3.1.1 Derivation of the Acute Criterion

Data for 21 marine species from 18 genera are available. The derivation of the acute criterion as described in the Guidelines is as follows:

1. For each species, the Species Mean Acute Value (SMAV) is determined. It is the geometric mean of the 48-hour and 96-hour LC_{50}s.
2. For each genus tested, the Genus Mean Acute Value (GMAV) is determined. It is the geometric mean of the SMAVs for each species tested of the given genus. Note that if only one species of a given genus is tested than GMAV = SMAV.
3. The GMAVs are then ranked for most sensitive (R=1) to least sensitive (R=N). Note that 18 genera were tested (N=18).
4. The data for the four most sensitive genera is then used in the five equations below to determine the Final Acute Value (FAV).

$$P = R/(N+1) \quad \textbf{Eq. 3.1}$$

$$S^2 = \frac{\sum((\ln(GMAV))^2) - (\sum(\ln(GMAV))\ /4)}{\sum P - (\sum(\sqrt{P}))\ /4} \quad \textbf{Eq. 3.2}$$

$$L = \frac{(\sum(\ln(GMAV)) - S(\sum(\sqrt{P})}{4} \quad \textbf{Eq. 3.3}$$

$$A = S(\sqrt{0.05}) + L \quad \textbf{Eq. 3.4}$$

$$FAV = e^A \quad \textbf{Eq. 3.5}$$

Table 3.1 Calculations for each of the terms to determine the acute criterion

Species	**GMAV**	**ln(GMAV)**	**ln(GMAV)^2**	**R**	**P**	$\sqrt{\mathbf{P}}$
Winter Flounder	0.492	-0.709	0.503	1	0.053	0.229
Red Drum	0.545	-0.607	0.368	2	0.105	0.324
Sargassum Shrimp	0.773	-0.257	0.066	3	0.158	0.397
Prawn	0.777	-0.252	0.064	4	0.211	0.459
Σ		-1.83	1.00		0.526	1.41

$$S^2 = \frac{\sum((\ln(GMAV))^2) - (\sum(\ln(GMAV))\ /4)}{\sum P - (\sum(\sqrt{P}))\ /4} = 5.74 \quad \textbf{Eq. 3.6}$$

$$L = \frac{(\sum(\ln(GMAV)) - S(\sum(\sqrt{P})}{4} = -1.30 \quad \textbf{Eq. 3.7}$$

$$A = S(\sqrt{0.05}) + L = -0.765 \quad \textbf{Eq. 3.8}$$

$$FAV = e^A = 0.465 \quad \textbf{Eq. 3.9}$$

5. Finally, the acute criterion is FAV/2, which yields a value of 0.233 mg/L (unionized ammonia).

3.1.2 Derivation of the Chronic Criterion

Acute-chronic ratios are used to define the chronic criterion. The acute-chronic ratio is determined in the following way [2]:

1. The geometric mean of the chronic no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) is calculated. This is the chronic value.
2. The acute-chronic ratio is the ratio of the 48-hour LC_{50}, also known as the acute value, and the chronic value.

At the time the criteria were published, acute-chronic ratios were available for two saltwater (Inland silverside *Menidia berrylina*, and the Mysid, *Mysidopsis bahia*.) and ten freshwater species [1]. The chronic criterion was developed based on the two saltwater species as well as four freshwater species due to the limited data on saltwater species. U.S.EPA developed the chronic criterion based on the following three conclusions that it made [1]:

1. Acute-chronic ratios of freshwater species appear to increase with decrease in pH.
2. Data on the effects of temperature on the ratios are lacking.
3. Acute-chronic ratios for the most acutely and chronically sensitive species are technically more applicable when trying to define concentrations chronically acceptable to acutely sensitive species.

Based on these conclusions, mean acute-chronic ratios were selected from freshwater tests whose chronic sensitivity was less than or equal to the median conducted at pH less than 7.7. Toxicity data for the species used to derive the chronic criterion is listed in Table 3.2.

Table 3.2 Toxicity data of species used to determine saltwater chronic criterion.

	Acute Value (mg/L)	Chronic Value (mg/L)	Acute-Chronic Ratio
Saltwater species			
Mysid *Mysidopsis bahia*	1.70	0.232	7.2
Inland Silverside *Menidia beryllina*	1.30	0.061	21.3
Freshwater Species			
Channel Catfish *Ictalurus punctatus*	-*	-*	10.0*
Fathead Minnow *Pimephales promelas*	2.55	0.13	20
Bluegill Sunfish *Lepomis marchirud*	1.08	0.926	12
Rainbow Trout *Salmo gairdneri*	0.422	0.0311	14

* Acute-chronic ratio is a composite of several studies.

To derive the chronic criterion, the final acute value, 0.465 mg/L is divided by the geometric mean of these six acute-chronic ratios, 13.1, which yields a chronic criterion of 0.035 mg/L.

3.1.3 Regulatory implications of the acute and chronic criterion

The acute criterion is defined as the one-hour average concentration that should not be exceeded more than once every three years and the chronic criterion is defined as the four-day average concentration that cannot be exceeded more than once every three years. For a specific site, the total ammonia discharged is related to the 90^{th} percentile pH and temperature and the average salinity. Site-specific numbers can then be determined for the purpose of NPDES permitting.

3.2 Routes of Exposure and Mechanisms of Ammonia Toxicity

Hudgins [4] previously presented detailed analysis of existing data concerning the routes of exposure and mechanisms of ammonia toxicity. This section will summarize his analysis and highlight some studies that have occurred since his theses.

3.2.1 Routes of Entry, Absorption, and Excretion

Unionized ammonia is the toxic form of ammonia to aquatic life because it can most readily gain entry to aquatic organisms. The predominant route of entry for ammonia is the gills for both vertebrates and invertebrates. This is because the un-ionized ammonia molecules can readily pass through the cell membranes at the gill surface. The neutral form is soluble in the lipid segments of the cell membrane and need only rely on diffusion. [12]. Diffusion of ionized ammonia is extremely slow through cell membranes due to its charge. It occurs as a large hydrated and charged entity, which can not pass through the charge-lined micropores of the hydrophobic membrane components. Several active transport mechanisms have been proposed to transport ammonium through the cell membrane. Ammonium is transported into the cell at the binding site of ionized potassium (K^+) because it has the same ionic radius. However, even with these active transport mechanisms, passive diffusion of unionized ammonia is the dominant route of entry [13].

In most fish, ammonia is the major form of nitrogen excretion. It's mainly excreted from the gills. Goldstein et al. [14] found that ammonia was excreted chiefly in the ionized form. They postulated that ammonium is excreted by NH_4^+-Na^+ exchange mechanisms.

3.2.2 Mechanisms of Action

Hudgins summarizes the data available about the mechanisms of action up to 1996 [4]. Generally, the mechanisms of action are as follows;

- Once ammonia enters the cell membrane at the gills, it diffuses through the organism in the blood stream.
- Damage to organisms has been found to occur in the gills, kidney, liver, and blood.
- On the cellular level, several modes of toxicity have been observed including;
  - Disturbance of the electrochemical gradients, which disrupts the transport of nutrients across the cell membrane.
  - Disruption of various enzymatic reactions such as the conversion of glutamate to α-ketoglutarate that is a pathway used to degrade amino acids.
  - An increased demand of maintenance energy on the cells.
- Mechanisms of acute toxicity are the reduction of red blood cells, which leads to an increase in permeability of water to the organism.
- The major mechanism of action for chronic toxicity is the necrosis of the gill tissue, which eventually leads to asphyxiation.

Recent work by Vedel et al. [15] with rainbow trout has discovered some other possible mechanisms. They found fish exposed to ammonia had a decrease of muscular potassium, which indicates that ammonium (NH_4^+) may substitute for potassium (K^+) in some Na^+/K^+-ATPase reactions, or that ammonia intoxicated nervous tissue continuously stimulated muscle contraction, causing a loss of potassium. They also found indicators of increased diuresis and tissue necrosis, which is consistent with existing data. Finally they

found an increase in glutamine concentration and a decrease of glutamate concentration in the brain. This was thought to be due to the detoxification reaction:

$$\text{Glutamate} + NH_4^+ + \text{ATP} \rightarrow \text{Glutamine} + \text{ADP} + H^+$$

The resulting decrease of brain glutamate may be a problem for fish because it is thought to be a neurotransmitter in the brain.

4 Previous Toxicity Tests

Li [3] and Hudgins [4] reviewed the literature on acute and chronic toxicity tests published up to 1996. This section will summarize their reviews, add their data into the review and add literature published since 1996 and up to the present. More freshwater than saltwater species have been tested in every category, acute vertebrate and invertebrate tests and chronic vertebrate and invertebrate tests. The current criteria for ammonia in marine environments are currently based on saltwater and freshwater organisms because of the lack of data for saltwater organisms. This summary will consist of the following sections:

Acute Toxicity Data in Freshwater and Saltwater Species

Chronic Toxicity Data in Freshwater and Saltwater Species

Comparisons with Freshwater and Saltwater Species

4.1 Acute Toxicity Data in Freshwater and Saltwater Species

Li's thesis summarizes the results of 60 freshwater and 21 saltwater species that had been tested up to her thesis. Since then, no other acute data is available except Li [3]

and Hudgins [4] on marine organisms. One study was done on the freshwater Pale Chub (*Zacco Platypus*) [16], however this organism is native only to waters of Japan, Korea and China and the endpoints they used were not standard 96-hour and 48-hour LC_{50}s, so they are excluded.

4.1.1 Freshwater Fish and Invertebrates

Table 4.1 lists 96-hour acute LC_{50}s for several vertebrates and invertebrates with references listed in brackets. The data are given in terms of mg/L of unionized ammonia. Several generalizations can be made about the acute toxicity of ammonia to freshwater organisms:

- Salmonids are the most sensitive (salmon and trout), while cyprinid fish (carp, minnow and dace are the least sensitive fish.
- Of the fresh water invertebrates, crayfish are the most sensitive and clams are the most tolerant.
- Invertebrates are more tolerant that vertebrates by a factor of about 2-3. For this reason, the freshwater regulations are based on fish.
- Sensitivity to ammonia is greater in egg and larval stages of fish than in saltwater organisms.

Table 4.1 96-hour LC_{50} for various freshwater organisms

Vertebrates	96 hr LC_{50} NH_3 mg/L	Invertebrates	96 hr LC_{50} NH_3 mg/L
Rainbow trout[17]	0.37-0.56	Fingernail clam[22]	1.10
Cutthroat trout[18]	0.52-0.80	Snail[21]	2.40
Atlantic salmon[19]	0.03-0.15	Cladeoceran[20]	2.94
Fathead minnow[20]	0.70-1.20	Amphipod[20]	3.12
Walleye[20]	0.51-1.10	Mayfly[20]	3.90
White sucker[20]	1.70-2.20	Isopod[20]	5.02
Channel catfish[20]	1.00-1.30	Caddisfly[20]	10.1
Carp[21]	1.00-1.50	Crayfish[20]	18.3
Bluegill sunfish[21]	0.95-1.18		
Smallmouth bass[21]	0.90-1.15		

[] - Reference

4.1.2 Saltwater Fish and Invertebrates

Saltwater organisms have been studied less than freshwater organsisms for acute toxicity to ammonia. Before Li [3] and Hudgins [4], 21 species were tested. These data have been collected by the EPA [1] and are presented in Table 4.2. The data are presented as the Species Mean Acute Value (SMAV), which is the geometric mean of the 48-hour LC_{50} and the 96-hr LC_{50}. A column noting whether an organism is a fish or an invertebrate has been added.

Table 4.2 Acute data for saltwater fish and invertebrates [1]

Rank	Fish (F)/ Invert. (I)	Species	SMAV (mg/L)	pH Range	Temp. Range(^{o}C)	Salinity (ppt)
1	F	Striped Bass (*Morone saxatilis*)	0.481	7.2-8.1	15-23	5-34
2	F	Winter Flounder (*Pseudo-pleuronectes americanus*)	0.492	7.9-8.1	7.5	31
3	F	Red Drum (*Sciaenops ocellatus)*	0.545	8.0-8.2	25-26	28-30
4	I	Sargassum Shrimp (*Latreutes fucorum*)	0.773	8.07	23.4	28
5	I	Prawn (*Macro-brachium rosenbergii*)	0.777	6.8-8.3	28	12
6	I	Copepod (*Eucalanus pileatus*)	0.793	8.2	20.5	34
7	F	Planehead filefish (*Monocanthus hispidus*)	0.826	8.07	23.4	28
8	I	Copepod (*Eucalanus elongatus*)	0.867	8.0	20.3	34
9	I	Mysid* (*Mysidopsis bahia*)	1.02	7.0-9.2	19-26	10-31
10	F	Spot (*Leiostomus xanthurus*)	1.04	7.92	20.4	9.3
11	F	Atlantic Silverside* (*Menidia menidia*)	1.05	7.0-9.0	11-25	10-30
12	F	Inland Silverside (*Menidia beryllina*)	1.32	7.1-9.1	18-26	11-33
13	F	Striped Mullet (*Mugil cephalus)*	1.54	7.99	21	10
14	I	Ghost Shrimp* (*Palaemonetes pugio*)	1.65	7.9-8.1	19-20	10-28
15	F	White Perch (*Morone americana*)	2.13	8.0	16	14
16	I	Lobster (*Homarus americanus*)	2.21	8.1	21.9	33.4
17	F	Sheepshead Minnow* (*Cyprinodon variegatus*)	2.73	7.6-8.1	10-33	10-33
18	F	Three-spined stickleback (*Gasterosteus aculeatus*)	2.93	7.6	15-23	11-34
19	I	Brackish water clam (*Rangia cuneata*)	3.08	7.95	20.2	9.2
20	I	Quahog Clam (*Mercenaria mercenaria*)	5.36	7.7-8.2	20	27
21	I	Eastern Oyster (*Crassostrea virginica*)	19.1	8.0	20	27

* Additional work performed by Hudgins and Li

Hudgins calculated a SMAVs for the Sheepshead Minnow and Mysid Shrimp (*M. bahia*) of 2.37 mg/L and 0.87 mg/L, respectively [4]. Li determined an SMAV of 2.40 mg/L for the Ghost Shrimp (*P. pugio*) using data on the 48-hr LC_{50} from Hudgins and data she collected on the 96-hr LC_{50} [3]. She also determined a SMAV of 1.07 mg/L for the Atlantic Silverside. The impact of this data is discussed in the discussion section of the manuscript.

4.2 Chronic Toxicity Data in Freshwater and Saltwater Species

Chronic ammonia toxicity to saltwater organisms has not been extensively studied. Before Hudgins and Li, only two marine species, the Mysid Shrimp and the Inland Silverside, had been tested, compared with 15 freshwater species [1]. As mentioned previously, the chronic criterion for marine environments is based on two marine organisms and four freshwater organisms. Li's and Hudgin's reports added chronic values for two more species, the Sheepshead Minnow and Ghost Shrimp. Their data will be used in conjunction with the data presented in this report to calculate a new chronic criterion based solely on marine organisms. Table 4.3 lists the current chronic data available for marine species as well as several freshwater species. Including the four freshwater species used to calculate the chronic criterion.

Table 4.3 – Chronic values for various saltwater and freshwater organisms

Freshwater species	Chronic value mg/L NH_3	Saltwater Species	Chronic value mg/L NH_3
Channel Catfish [a] (*Ictalurus punctatus*)	0.25	Mysid [a] (*Mysidopsis bahia*)	0.23
Fathead Minnow [a] (*Pimephales promelas*)	0.13	Inland Silverside [a] (*Meninidia berillina*)	0.06
Bluegill Sunfish [a] (*Lepomis macrchirud*)	0.09	Sheephead Minnow [b] (*Cyprinodon variegatus*)	0.39
Rainbow Trout [a] (*Salmo gairdneri*)	0.03	Ghost Shrimp [c] (*Paleomentes pugio*)	0.46

[a] Species used to determine chronic criterion by EPA [1]
[b] Additional work by Hudgins [4]
[c] Additional work performed by Li [3]

In addition to their data, Person-Le Ruyet et al. [23] studied the chronic toxicity of ammonia to juvenile turbot, *Scophthalmus maximus.* They did not, however, present their data in terms of standard endpoints so it is difficult to integrate it into the chronic criteria. Nevertheless, the data is useful to review. Juvenile turbots of three sizes were observed for 4-6 weeks to determine the effects of ammonia on survival, growth, feeding, and physiological status. 28-day LC_{50}s were observed to be 0.8 mg/L, 1 mg/L, and 1.1 mg/L for 13 g, 22 g, and 104 g fish, respectively. For growth, the larger turbot were more sensitive. The 14-day Lowest Observed Effect Concentration (LOEC) were 0.21 mg/L and 0.40 mg/L for 23 g and 14 g fish, respectively. The 34-day LOEC for 104 g fish was 0.09 mg/L. Food uptake experiments indicated that consumption decreased with increased ammonia concentration, however food usage efficiency was not affected. Physiologically, large turbot were more sensitive that small ones.

4.3 Comparisons with Freshwater and Saltwater Species

Several general trends can be deduced when comparing saltwater and freshwater species:

- For acute toxicity, freshwater fish are generally more sensitive than freshwater invertebrates.
- No general trend is evident with saltwater organisms. Marine fish and invertebrates seem to have a mixture of tolerances for ammonia. For this reason, both marine fish and invertebrates need to be studied
- For both freshwater and saltwater species, the life stage of the organism can affect toxicity. Previous studies have shown that the earlier life stages are more susceptible, however work by Person-Le Ruyet et al. [23] seems to contradict this trend.

5 Materials and Methods

Most of the materials and methods follow standard procedures described in U.S. EPA 600/4-90/027 [24] and U.S. EPA 600/4-89/001 [25] and are described in the manuscript section of this document. This section describes variations from these procedures and describes certain parts in more detail than the manuscript.

5.1 Morphology, taxonomy and life history of test organisms

5.1.1 Quahog Clam

The Quahog Clam, *Mercinaria mercenaria*, ranges from the Gulf of Mexico to Cape Cod. It inhabits intertidal to sub-tidal zones down to 60 feet. Its habitat varies from sandy to muddy sand bottoms. It is usually found at salinities above 15 ppt.

The shell of *M. mercenaria* is thick, strong, and broadly oval and its beaks are shifted forward. It is distinguished by a purple stain on the inside of its shell, which is mostly white and by external sculpting that changes with age. The young have sharply raised concentric ridges, which are mostly gone in older clams. *M. mercenaria* grows quickly, but may live 20- 25 years. Adults range from 4 to 6 inches [26]. Quahogs are an important commercial clam. They are still harvested in the wild, although they are increasingly being aquacultured. The most common method of aquaculturing them is to fertilize the eggs by mixing with sperm in seawater and the raise them in tanks on phytoplankton until they finish their free swimming stage and settle. They are then transported to leased areas where they feed in the wild until they harvesting [27].

M. mercenaria has several common names. In New England it is called the Quahog Clam. Farther south it is called the Round Clam or Hardshell Clam. Commercial names are based on it size. Below 1.5 inches in shell length it is called the Littleneck Clam. Between 1.5 and 2 inches it is referred to as the Cherrystone Clam. Above 2 inches it is called the Chowder Clam. Its scientific name, *mercenaria,* comes from the shells use by Native Americans to make currency or wampum [26].

5.1.2 The morphology of the Summer Flounder (*Paralichthys dentatus*)

The Summer Flounder (*Paralichthys dentatus*) ranges from the southern Gulf of Maine to South Carolina [27]. It is an important commercial and recreational fish in the Mid-Atlantic (Cape Cod to Cape Hatteras). It spawns during an offshore migration to the outer continental shelf during autumn and winter. The larvae are transported by water currents to coastal areas. Development of the post-larvae and juveniles occurs in bays and estuarine areas, including the Chesapeake Bay [27].

The Summer Flounder is a member of the family, Bothidae. Bothidae are known as Lefteye Flounders, because both eyes migrate to the left side of the head. Other distinguishing characteristics include a round or oblong body, an asymmetrical mouth with a slightly prominent protractile lower jaw, the ability to change color to match surroundings and the ability to raise and move both eyes independently. All Flounder are carnivorous [28]. Usually, they lay camouflaged and partially buried on the bottom and wait for unsuspecting prey to wander by. Their typical prey includes shrimp and small fish. This large family has 37 genera and 212 species [28]. Data indicate that females may live up to 20 years and grow up to 11.8 kg (26 lbs.). However males rarely live more than 7 years [27].

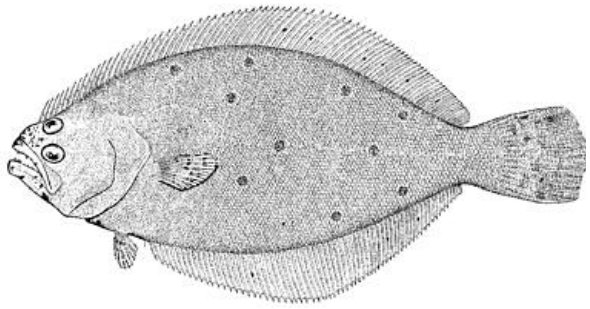

Figure 5.1 – Adult Summer Flounder [27]

5.1.3 Morphology of Atlantic Silverside (*Menidia menidia)*

The Atlantic Silverside, *Menidia menidia*, is found in estuaries from Maine to Northern Florida. It occurs at salinities from 12 to 30 ppt. This species is an important forage fish for many commercial and recreational species including striped bass, bluefish and spotted sea trout [24].

Adults usually grow to about 117 mm. Females are usually slightly larger than males. They can be distinguished by the number of rays and spines on their fins. The first dorsal fin usually has four to five spines. The second dorsal fin has one fin and eight or nine rays. The anal fin has one spine and 19 to 29 rays. The pectoral fin has 12 to 16 rays [29].

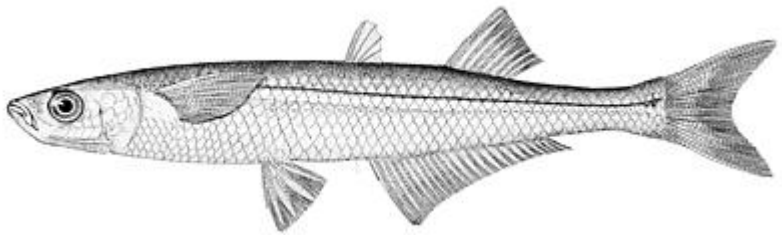

Figure 5.2 – Adult Atlantic Silverside [27]

5.2 Tests Performed

The manuscript section of this document does not differentiate which tests were performed as part of the research for this thesis and which tests were performed by Hudgins and Li. The following section describes which tests were performed as part of this research. The intent of this section is to make it clear which work was performed by the author and which work was performed by Hudgins and Li.

5.2.1 Acute Tests

Table 5.1 lists the acute tests performed for this research. This research used performed acute tests on two organisms that were not studied by Hudgins or Li, the Summer Flounder and the Quahog Clam and performed natural seawater tests, while Hudgins and Li used only synthetic seawater.

Table 5.1 Acute Tests

Organism	Test Type	Number Performed	Natural / Synthetic	Salinity (ppt)
Summer	48-hour	2	Synthetic	30.0
Flounder	48-hour	1	Natural	27.0*
	96-hour	1	Synthetic	30.0
	96-hour	1	Natural	27.0*
Quahog Clam	48-hour	3	Synthetic	27.0
	96-hour	3	Synthetic	27.0
Atlantic	48-hour	3	Natural	8.3- 26.0*
Silverside	96-hour	3	Natural	8.3- 26.0*

* Salinity of the natural seawater was dependent on water in Hampton, VA at the time of sampling.

5.2.2 Chronic Tests

Two chronic tests were performed on the Summer Flounder. Survival data was tabulated from these tests, however growth was insignificant over the 7 days of the tests.

5.3 Data Analysis

Data analysis is briefly explained in the manuscript section of this document, however not in the detail required of a thesis. This section goes into more detail on the data analysis.

After the tests were completed, the numbers of mortalities for the acute and chronic tests were used to determine the LC_{50}**,** NOEC, and LOEC for each test species. The data were analyzed in two ways; each test's data were analyzed individually and then their endpoints were averaged together (multi-test method) and all the data for a specific test on each organism were pooled together to come up with one set of endpoints (multi-replicate method). Methods for analyzing mortality data to determine the LC_{50}**,** NOEC, and LOEC are presented in EPA 600/4-90/027 [24] for acute tests and EPA 600/4-89/001 [25] for a chronic test these methods are detailed below. These methods include manual computation as well as computer programs such as Toxstat Version 3.3 and 3.4. However, manual computational techniques were not necessary based upon the EPA guidelines.

5.3.1 Acute Tests

In acute tests, the most useful data are LC_{50}s and NOECs. EPA 600/4-90/027 [24] describes four well-tested methods for determining the LC_{50} for multi-concentration acute toxicity tests. These methods are the Graphical Method, the Spearman-Karber Method, the Trimmed Spearman-Karber Method and the Probit Method. The analysis scheme is suggested in EPA 600/4-90/027 and is presented in Figure5.3. Based on this scheme, the Probit method was used for all of the acute tests to determine the LC_{50}. For details of these methods as well as the other ones outlined, refer to EPA 600/4-90/027.

Determination of the NOEC is accomplished using hypothesis testing and is outlined in EPA report 600/4-90/027 [24]. The first step in determining the NOEC is to transform the data to the arc-sine-square-root transformation. For this, the mortality data must be stated as a proportion surviving. This transformation is used to stabilize the variance and satisfy the normality requirement. The normality assumption is tested by the Shapiro-Wilk's test. If the normality assumption is verified, the Bartlett's test for equality of variances was used to test the homogeneity of the variance assumption. If the data satisfies both the normality and homogeneity of variance test, the Dunnett's test was used. If the data does not satisfy both of the assumptions, the Steel's Many-One Rank Test was used. Figure 5.4 shows a flowchart of this procedure to determine the NOEC. Based on this scheme, the Steel's Many-One Rank was used to determine the NOEC for all of the acute tests.

5.3.2 Chronic Tests

The survival data were used to determine 7-day LC_{50}, LOEC and NOEC values based on 6 replicates of the Summer Flounder. The analysis scheme is outlined in EPA 600/4-89/001 [25]. The data were analyzed using Toxstat Version 3.4. To determine the LC_{50} and NOEC, the same steps were used as described in section 5.3.1, except 7-day mortality data were used. The LOEC is simply the next highest concentration after the NOEC. Figures 5.3 and 5.4 can again be followed to determine which test is appropriate. Based on this scheme, the probit method was used for all of the chronic tests to determine LC_{50} and Steel's Many One Rank was used for all chronic tests to determine the NOEC.

5.3.3 Natural Seawater Tests

To determine whether the 48-hour and 96-hour LC_{50}s for tests involving natural seawater were significantly different from tests using synthetic seawater, the values were tested with a single-factor ANOVA analysis at $\alpha \leq 0.05$. Differences between LC_{50} values were considered significant if $\alpha \leq 0.05$. For the Summer Flounder tests, the natural seawater tests were compared with synthetic seawater contained in this research. For the 48-hour acute tests involving Atlantic Silverside, the natural seawater tests were compared with 48-hour acute tests performed by Li [3]. For 96-hour acute tests involving Atlantic Silverside, data were compared with data presented in the Criteria [1].

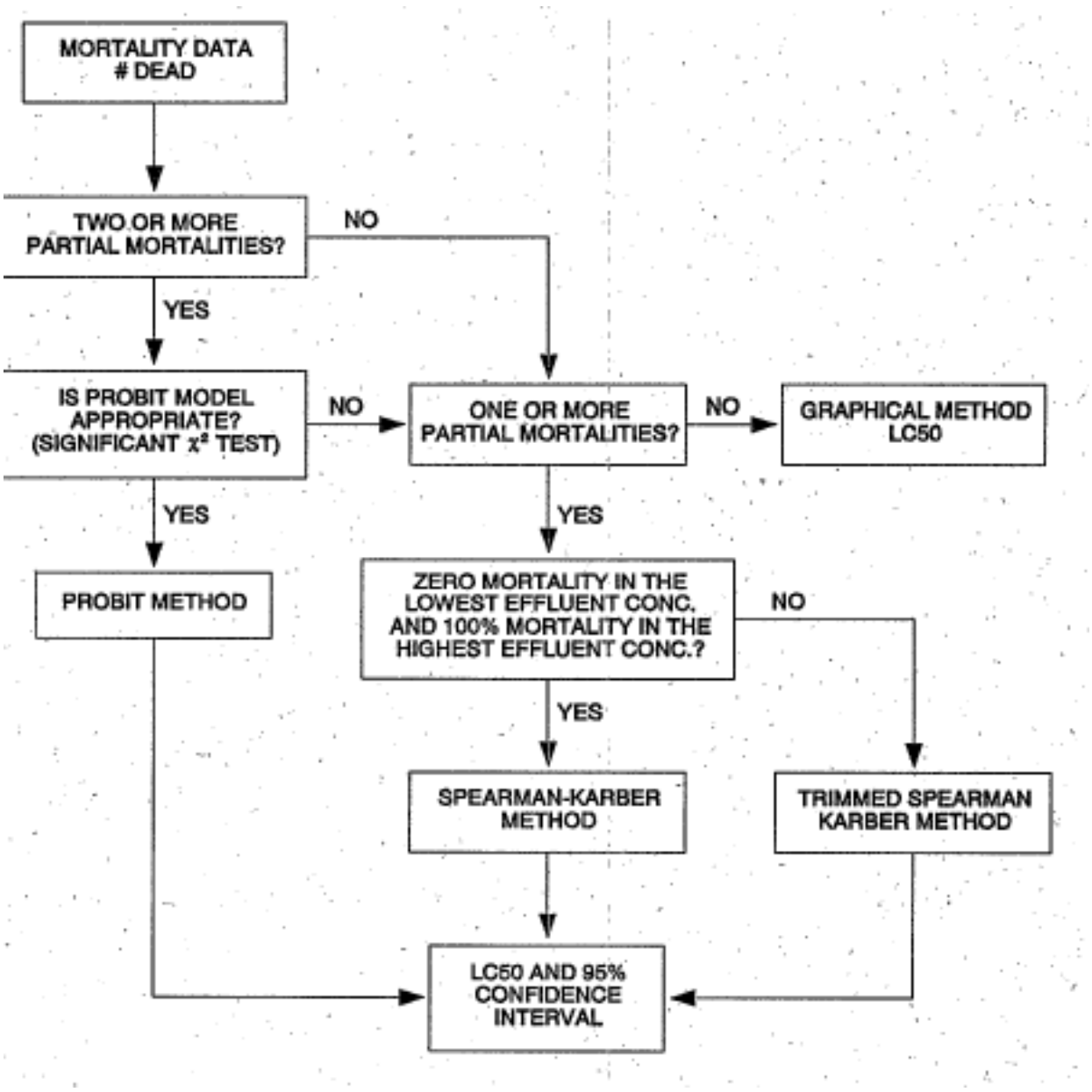

Figure 5.3 Flowchart for determination of the LC_{50} for multi-concentration acute toxicity tests [24]

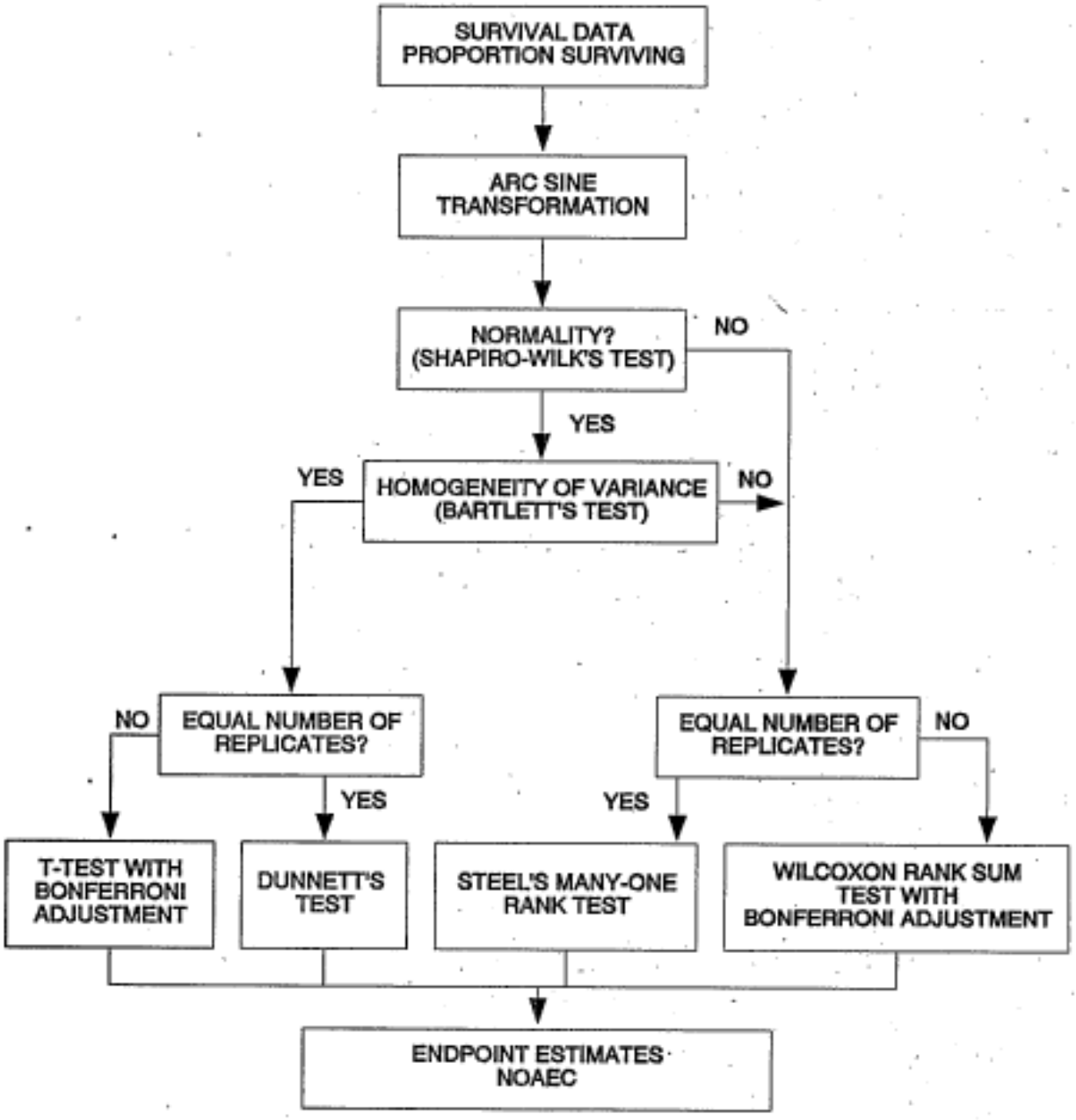

Figure 5.4 – Flowchart for Analysis of multi-concentration test data [24].

6 Manuscript

THE TOXICITY OF AMMONIA TO THREE MARINE FISH AND THREE MARINE INVERTEBRATES

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Abstract - Laboratory toxicity tests were performed to obtain more data pertaining to the toxicity of ammonia to saltwater organisms. The standards for instream ammonia limits in marine environments are presently based on toxicity tests involving both freshwater and saltwater organisms. Acute tests (48-h and 96-hour) were performed at 20 ^{0}C, and chronic tests (7-day) were performed at 25 ^{0}C. Synthetic seawater and natural seawater taken from the Chesapeake Bay were used. The results obtained are given below in terms of mg/L of unionized ammonia. For the Sheepshead Minnow (Age-1-14 days), the 48-h LC_{50} and the 96-hour LC_{50} were 2.68 mg/L and 2.09 mg/L, respectively, and the 7-day NOEC (growth) was 0.34 mg/L. For the Summer Flounder (2 months), the 48 and 96-hour LC_{50}s in synthetic seawater were 1.22 mg/L and 1.08 mg/L, respectively, and the 7-day NOEC (mortality) was 0.34 mg/L. In Chesapeake Bay water, the 48 and 96-hour LC_{50}s were 1.09 mg/L and 0.889 mg/L, respectively. For the Atlantic Silverside (14 days), the 48-h LC_{50} in synthetic seawater at salinities 14, 22, and 30 ppt were 1.50 mg/L, 1.17 mg/L and 1.08 mg/L, respectively. The 7-day NOEC (growth) was 0.48 mg/L. In Chesapeake Bay water, the 48 and 96-h LC_{50}s were 1.45 mg/L and 1.08 mg/L, respectively. For the Mysid Shrimp (<2 days), the 48 and 96-h LC_{50}s were 1.00 mg/L and 0.76 mg/L, respectively. For the Ghost Shrimp (10 days), the 48 and 96-h LC_{50}s were 3.48 mg/L and 1.97 mg/L, respectively, and the 7-day NOEC (growth) was 1.08 mg/L. For the Quahog Clam (5mm shell height), the 48 and 96-h LC_{50}s were 216 mg/L and 36.6 mg/L. Based on these results, it appears the chronic criterion for ammonia in marine environments can be increased from 0.035 mg/L (unionized ammonia) to 0.081 mg/L.

Keywords: ammonia, acute toxicity, chronic toxicity, salinity effects, Chesapeake Bay, bioassays

6.1 Introduction

In order to reevaluate the in-stream ammonia limit in the Chesapeake Bay watershed, toxicity tests were performed on three fish, the Sheepshead Minnow (*Cyprindon variegatus*), Summer Flounder (*Paralichthys dentatus*), and Atlantic Silverside (*Menidia menidia*) and three invertebrates, the Ghost Shrimp (*Palaemonetes pugio*), Mysid Shrimp (*Mysidopsis bahia*), and Quahog Clam (*Mercenaria mercenaria*).

Effluent standards for specific chemicals are based primarily on toxicity tests. One such effluent standard is the national criteria set for the discharge of ammonia into saltwater. However, due to a lack of data on the toxicity of ammonia to saltwater organisms, the current saltwater criteria are based on freshwater and saltwater organisms. These criteria can be found in "Ambient Aquatic Life Water Quality Criteria for Ammonia (Saltwater)-1989" [1]. Two criteria are presented in this document: the acute criterion, a one-hour average concentration not to be exceeded more than once every three years and the chronic criterion, a four-day average concentration not to be exceeded more than once every three years. The acute criterion is calculated using species mean acute values (SMAVs), which are the geometric means of the 48-h LC_{50} and the 96-h LC_{50}. The chronic criterion is derived from acute-chronic ratios. Procedures for developing these criteria are described in "Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses" and "Ambient Water Quality Criteria for Ammonia" (1984) [2,3]. The acute criterion was developed based on 21 species from 18 genera and the chronic criterion was developed

based on two saltwater and four freshwater species [1]. Currently, the acute criterion for ammonia in saltwater is 0.233 mg/L as unionized ammonia, whereas the chronic criterion is 0.035 mg/L as unionized ammonia [1].

Another motivation for this research was to provide toxicity data to aquaculturists involved in the recirculating aquaculture of Summer Flounder, *Paralichthys dentatus*. Although relatively new, aquaculture of Summer Flounder has been undertaken by several private companies and universities in the past several years [4-6]. For example, the Virginia Tech Seafood Research and Extension Unit has begun researching recirculating aquaculture of Summer Flounder. Ammonia toxicity data is very important in recirculating aquaculture systems because ammonia is a natural by-product of the cultured organism's metabolism and can build up in a recirculating system [7].

Unionized ammonia is the toxic form of ammonia to aquatic life because it can most readily gain entry to aquatic organisms. The predominant route of entry for both vertebrates and invertebrates is the gills. This is because the unionized ammonia molecules can readily pass through cell membranes at the gill surface [8]. In most organisms, ammonia is the predominant form of nitrogen excreted. The gills of the organisms rapidly eliminate ammonia [9]. Several modes of action have been suggested for ammonia toxicity. These include disturbance of electrochemical gradients [10-12], cerebral effects caused by a depletion of the cerebral energy in the form of ATP [12-14], acid-base disturbance in the body of the organism [15] and a decrease in glutamate, a potential neurotransmitter, in the brains of fish [16].

Because unionized ammonia is the toxic form of ammonia, it is important to understand the physical parameters that affect the proportion of total ammonia that is in

the unionized state. Ammonia is present in water in two forms, unionized ammonia (NH_3) and ionized ammonium (NH_4^+). They exist in equilibrium represented by the equation;

$$NH_4^+ + H_2O \leftrightarrow NH_3 + H_3O^+ \qquad \textbf{Eq. 6.1}$$

The ionization constant for this reaction at 25^0 C and no salinity is;

$$K_a = \frac{[NH_3][H^+]}{[NH_4^+]} = 10^{-9.2} \qquad \textbf{Eq. 6.2}$$

The three major parameters that affect the proportion of unionized ammonia are pH, temperature, and salinity. These factors affect the proportion of unionized ammonia in the following ways; increase in pH increases the proportion of unionized ammonia, increasing temperature increases the proportion of unionized ammonia, and increasing salinity decreases the proportion of unionized ammonia. Greater discussion of these effects can be found in USEPA 1989 [1], Emerson et al. [17], and Whitfield et al. [18].

Salinity is an important factor that should be considered during ammonia toxicity tests with marine species. Atlantic salmon and Chinook salmon were found to be less sensitive to ammonia in saltwater that in freshwater [19,20]. However, the effect of different salinity levels on ammonia toxicity is not well known. Therefore, tests were performed with the Atlantic Silverside to further investigate the relationship of ammonia toxicity and salinity.

Tests were also performed on two fish, the Atlantic Silverside and Summer Flounder, to determine if interactions of ammonia with natural seawater taken from the Chesapeake Bay were important (e.g. synergistic or antagonistic).

6.2 Materials and Methods

6.2.1 Organism source and care

The Summer Flounder were purchased from GreatBay Aquafarms of Portsmouth, New Hampshire. The flounder were approximately two months old and post metamorphosis. This age group was chosen because this is approximately the age when they are switched from a flow-through system to a recirculating system and from live food to chow. They were fed both *Artemia* cultured in the lab and chow provided by GreatBay twice daily.

The Sheepshead Minnow were purchased from Cosper Environmental Services Inc. of Bohemia, New York. The minnows arrived less than five days old and were fed both *Artemia* and Tetrimin® flakes twice daily.

The Atlantic Silverside and Ghost Shrimp were supplied by Sea Plantations (SP), Inc., of Salem, Massachusetts. The larval stages of both species were used in this study based on the belief that the younger organisms are, the more sensitive to ammonia they will be. The Atlantic Silversides were 13-15 days old, and the Ghost Shrimp 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.

The Mysid shrimp were purchased from Cosper Environmental Services of Bohemia, New York. They were cultured in 10-gallon tanks at salinities between 25 and

28 ppt. Before each test, young less than two days old were acquired by collecting adult females bearing brood pouches and isolating them in a separate 4-liter tank. These females were placed in a large (10 cm by 15 cm) standard fish transfer net for two days that allowed the juveniles to pass through while keeping the females inside to avoid predation. The young were then collected with a fine mesh net for use in testing. These procedures follow EPA document no. 600/4-90-027 [21]. In order to verify the health of the culture, standard 48-h acute reference tests were conducted each month. The standard toxicant was an EPA-certified solution of cadmium ($CdCl_2$). The LC_{50} was determined to be appropriate based on work performed by Burton and Fisher [22].

The quahog clams (*Mercenaria mercenaria*) were purchased from Cherrystone Aquafarms in Cheriton, VA. They were approximately 5 mm in shell height and 9 months old at test initiation.

6.2.2 Dilution water

Synthetic seawater used as dilution water was made up with deionized water and Forty Fathoms® as recommended by the EPA [21]. Newly prepared synthetic seawater was kept at saturation by means of aeration, and the water was aged at least 24 hours to assure complete dissolution of the salts before use.

Natural seawater was collected from the Chesapeake Bay at Hampton, Virginia. It was kept refrigerated at 4 ^{0}C until 24 hours prior to use when it was brought to room temperature and aerated to bring dissolved oxygen to saturation. All water used in testing was less than two weeks old , as suggested by EPA [24].

6.2.3 Experimental verification of ammonia dose

The total ammonia concentration of each dilution for all tests were measured according to Method-4500 B. and C., as detailed in Standard Methods for the Examination of Water and Wastewater [23]. Samples taken at initiation and the end of each test were preserved in 6M H_2SO_4 and stored at 4^0C for no longer that two 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 the LC_{50}, LOEC, and NOEC.

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

6.2.4 Test Conditions

All tests were conducted according to EPA guidelines; specifically the acute methods are presented “Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms” [21] and the chronic methods are presented in “Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms” [24]. These experiments were performed at Olver Laboratories, Incorporated. All tests were conducted as static experiments with 10 organisms per replicate, and a light regime of 16 hours light/ 8 hours darkness. The pH was kept at 8.2 ± 0.1 using 0.1 N $NaHCO_3$ and Na_2CO_3. The

temperatures for acute and chronic tests were 20 ^{0}C and 25 ^{0}C, respectively. The dissolved oxygen for all tests remained above 4.0 mg/L. All 48-h acute tests were non-renewal; all 96-h acute tests were renewed once after 48 hours; and, all 7-day chronic tests were renewed once a day during the test duration. Other test conditions are summarized in Table 1.

6.2.5 pH Control

The pH had to be carefully controlled because small changes in pH can greatly affect the proportion of ammonia that is unionized. Previous research [25-27] and the ammonia criteria published by EPA [1] indicated that a pH change would be significant at >± 0.1 units. Therefore, these tests were kept at pH variation <± 0.1 and in most cases <± 0.05 pH units. The ammonium salt (NH_4Cl) used has a slow dissociation and is slightly acidic [1], so pH tended to drift down. Sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) were used to adjust pH. In all tests, the pH was checked and adjusted when appropriate every four hours during the daytime, with, at most, an eight hour gap in the evening. All new solutions were adjusted to pH 8.2 prior to use.

6.2.6 Statistical Analysis

After tests were completed, survival data and, where appropriate, growth data, were analyzed to determine the LC_{50}, NOEC, and LOEC for each species. The data were analyzed in two ways: each test's data were analyzed individually and then their endpoints were averaged together (multi-test method); and, all the data for a specific test on each organism were pooled together to come up with one set of endpoints (multi-replicate method). The data were then analyzed using Toxstat ® versions 3.3 and 3.4, following guidelines provided in EPA reports 600/4-90/027 [21] and 600/4-89/001 [24]

for the determination of the LC_{50}, NOEC, and LOEC. The average pH was determined by averaging the upper and lower hydrogen ion concentrations for a final average pH, which was used in determining the unionized ammonia LC_{50}, 95% confidence intervals and NOEC for both the acute and chronic toxicity tests. The endpoints determined from the "multi-replicate method" were then used to calculate the species mean acute value (SMAV), chronic values, and acute-chronic ratios, as described in "Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses" [2]. From these data, proposed, revised acute and chronic criterion were calculated again following procedures described in "Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses".

To determine whether the 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 $\alpha > 0.05$.

To determine if the LC_{50}s determined in the natural seawater testing were significantly different, single-factor ANOVA analysis at $\alpha \leq 0.05$ was again employed. The natural seawater tests involving the Summer Flounder were compared with synthetic seawater tests presented in this research. The 48-h acute tests involving the Atlantic Silverside were compared with each of the three different salinity levels of 48-h tests presented in this report. The 96-h tests involving the Atlantic Silverside were compared with data presented in the Criteria [1].

6.3 Results

6.3.1 Acute Results

Table 2 summarizes the acute results based on the "multi-test method" "multi-replicate method" for synthetic seawater testing at normal test conditions.

Eight 48-h and four 96-h acute tests were performed with Sheepshead Minnow. The LC_{50}s for these tests are similar to those presented in the literature for unionized ammonia. Miller et. al. [28] also worked with Sheepshead Minnow, but they used a flow-through system and their temperature and salinity were 25^0C and 30 ppt, respectively, as compared to 20^0C and 25-ppt in these tests. They also conducted their tests with four replicates as compared to the sixteen replicates in this research. The data in this research falls below their value of 2.79 mg/L as unionized ammonia [28]. The SMAV for the Sheepshead Minnow was determined to be 2.37 mg/L, which is reasonable considering that EPA indicates that the SMAV for Sheepshead Minnow is 2.74 mg/L [1].

Two 48-h and two 96-h acute tests (two replicates each) were performed on the Summer Flounder. No previous research with ammonia has been performed with the Summer Flounder. Research was performed by Cardin [29] on the Winter Flounder (*Pseudopleuronectes americanus*), which is in a different genus. Cardin reported 48-h LC_{50}s from 0.44-0.53 mg/L (unionized ammonia), about 50% of the 48-h LC_{50} determined in this research. When compared to organisms reported in the Criteria [1], its sensitivity ranks near the middle. It is less sensitive than 10 of the 21 organisms reported.

Eight 48-h (2 replicates each) and four 96-h static renewal tests (4 replicates each) were analyzed to determine the LC_{50} and NOEC for the Mysid Shrimp. The final LC_{50}

and NOEC for both the 48-h and 96-h tests were determined by the multi-test and multi-replicate methods. There was some mortality in the controls, although it wasn't significant. This mortality appeared to be from the cannibalistic behavior of the juvenile Mysids, since the carcasses in the controls had completely disappeared. Therefore, for future testing of the Mysid, additional surface area (approximately 20 $in.^2$ of mesh netting) should be added to the test beakers to reduce mortality in the controls. The 48-h LC_{50} value tabulated in this research is lower than the 96-h LC_{50} values determined by Miller et al. [28] of 1.3-1.9 mg/L. One would expect that the 96-h LC_{50} would be lower than the 48-h LC_{50}. Experiments conducted by Miller et al. were at 25^0C and 30-ppt salinity, as compared to 20^0C and 25-ppt for this research. However, these variations in the conditions do not explain the large discrepancies in the unionized ammonia LC_{50}. The 96-h LC_{50} derived from this research is also below the 96-h range presented by Miller et al. [28] for unionized ammonia. Again, experiments conducted by Miller et al. were at 25^0C and 30-ppt salinity, as compared to 20^0C and 25-ppt for this research. This variation in temperature and salinity should affect only the total ammonia LC_{50}. However, some data presented by the EPA [1] suggest that the 96-h LC_{50} is similar to the data reported in this document. The EPA lists 96-h LC_{50}s ranging from 0.7-1.2 mg/L.

A total of eight 48-h static nonrenewal (2 replicates per test) and three 96-h static renewal (4 replicates per test) were analyzed to determine the LC_{50} and NOEC for the Ghost Shrimp. The 48-h LC_{50} for this research is above the value presented by the EPA of 2.57 mg/L as unionized ammonia [1]. The conditions for the 48-h ghost shrimp tests listed in EPA [1] were similar to the conditions in this research except for the size (age) of the shrimp. The size of the shrimp used in EPA testing was 10-20 mm, but the size of

the shrimp used in this research was 15-30 mm. This difference in size (age) of the test organisms was possibly the reason for the discrepancies in the test data. As the aquatic organisms mature, they may become more tolerant to ammonia. The 96-h LC_{50} calculated in this research falls within the range reported by EPA of 1.06-2.57 mg/L [1].

A total of three 48-h static nonrenewal (2 replicates per test) and three 96-h static renewal (2 replicates per test) were analyzed to determine the LC_{50} and NOEC for the Quahog Clam. The SMAV of 88.9 mg/L as unionized ammonia is about 4.5 times greater than the least sensitive organism reported in the Criteria [1] (Eastern Oyster SMAV of 19.1 mg/L). It was apparent from observations of the test organisms that they were able to securely close their shells to avoid the high levels of ammonia. A more appropriate method would be to measure food uptake, such as performed by Epifano and Srna [30]. They determined an SMAV of 5.36 mg/L as unionized ammonia based on Effective Concentration- 50% (EC_{50}) measurements.

6.3.2 Salinity Results

The 48-h LC_{50} values for the Atlantic Silverside at three salinity levels are listed in Table 3. Table 3 gives the LC_{50} and NOEC values as total and unionized ammonia. For each experiment, 95% confidence intervals (C.I.) are given in parentheses, the LC_{50} values found in the literature for each species are listed for comparison. The 48-h LC_{50} values at each salinity (in terms of unionized ammonia concentrations) were 1.50 mg/L at 14 ppt, 1.17 mg/L at 22 ppt, and 1.08 mg/L at 30 ppt. Among these three values, two fall in the range of 1.03 to 1.47 mg/L listed in the literature [31]. One of these, the LC_{50} at 14 ppt, exceeds the range by 2%. The LC_{50} of 1.47 mg/L in the literature was obtained under the conditions of pH 8.5 and salinity 10 ppt [31], which were different from the

conditions in this work. In addition, these results indicated that the 48-h LC_{50} values decreased as salinity increased. The results of the ANOVA analysis are shown in Table 4. These results indicated that the LC_{50} at salinity of 14 ppt was significantly greater than at salinity 22 and 30 ppt. While the LC_{50} at 22 ppt was higher than at 30 ppt, it was not statistically significant.

6.3.3 Natural seawater testing results

One 48-h acute and one 96-h acute test were analyzed to determine if ammonia exhibits different toxicity in natural seawater than it does in synthetic seawater for Summer Flounder. The 48-h LC_{50} of 1.09 mg/L, in the natural seawater was significantly less than the 48-h LC_{50} in the synthetic seawater of 1.22 mg/L (See table 6.5 for α values.). The 96-h LC_{50} of 0.889 mg/L was less than in the synthetic seawater (1.08 mg/L), but not statistically significant.

Three 48-h acute and three 96-h acute test were analyzed to determine if ammonia exhibits different toxicity in natural seawater than it does in synthetic seawater for Atlantic Silverside. The 48-h LC_{50} of the natural seawater (1.52 mg/L) was significantly less than the LC_{50} determined for salinities of 22 ppt and 30 ppt, 1.17 mg/L and 1.09 mg/L, respectively. However, it wasn't significantly different than the 48-h LC_{50} for 14 ppt of 1.51 mg/L. The 96-h LC_{50} in natural seawater determined in this research of 1.18 mg/L was greater than the 96-h LC_{50} presented in the Criteria [1] of 0.97 mg/L, but not significantly different.

6.3.4 Chronic Results

Prior to this research, the chronic toxicity of ammonia had only been tested with two marine organisms, the Mysid Shrimp and Inland Silverside [1]. The 7-day chronic

tests were performed with the Sheepshead Minnow, Atlantic Silverside, Summer Flounder and Ghost Shrimp in order to expand the data base for the chronic effects of ammonia to common saltwater organisms. Of the organisms tested, the Sheepshead Minnow was the most sensitive, followed by the Summer Flounder, Atlantic Silverside, and Ghost Shrimp. The acute-chronic ratios for the Atlantic Silverside, Summer Flounder, and Sheepshead Minnow of 1.77, 2.27 and 6.95 are all less than the acute-chronic ratios presented in the literature (21.3 for the Inland Silverside and 7.2 for the Mysid). However, the chronic test for the Inland Silverside was conducted over a 28-day period, so it was expected that the acute-chronic ratio would be greater than for the other tests which were performed over seven days. The acute-chronic ratio of 7.57 for the Ghost Shrimp falls in between the values for the Mysid and Inland Silverside.

6.4 Discussion

6.4.1 Acute Testing and the Acute Criterion

While the results of the acute tests are useful in other areas, they have little impact on refining the acute criterion. The acute criterion is based on the four most acutely sensitive species tested, the Winter Flounder (*Pseudopleuronectes americanus*), Red Drum (*Sciaenops ocellatus*), Sargassum Shrimp (*Latreutes fucorum*) and Prawn (*Macro-brachium rosenbergii*). Their SMAVs range from 0.492 mg/L to 0.777 mg/L. The most sensitive species tested was the Mysid, with a SMAV of 0.87 mg/L. The Criteria note that while freshwater fish are generally more sensitive than freshwater invertebrates, no trend is apparent when comparing marine fish with marine invertebrates. This research agrees with that trend. Of the species tested, an invertebrate (Mysid) was the most sensitive followed by a three fish (Atlantic Silverside, Sheepshead Minnow and Summer

Flounder), and two invertebrates (Ghost Shrimp and Quahog Clam). The Criteria also note that mollusks are the most tolerant. The acute data on the Quahog Clam agree with that.

The results of the salinity testing with the Atlantic Silverside yielded some interesting results. Previous studies by Alabaster et al. [19], Herbert and Shurben [32], and Miller et al. [28] indicated that ammonia toxicity decreased as salinity increased. They all hypothesized that the decrease in toxicity was due to a decrease in osmotic stress on the organisms. The results of this research showed that the 48-h LC_{50} values of unionized ammonia for *M. menidia* decreased with an increasing pH, although values between 22 ppt and 30 ppt were not significantly different. One of the interpretations for this result is that unionized ammonia might not be the only toxic form of ammonia. Both unionized ammonia and ionized ammonia may play an important role in ammonia toxicity. In addition, the activity of ammonia and biological behavior of test organisms may affect the toxicity of ammonia.

The results of the natural seawater testing also yield interesting results. Ammonia toxicity appeared to be greater in the natural seawater for the Summer Flounder. This could be due to interactions with substances in the water, although no mortalities were observed in the controls. The Atlantic Silverside were more tolerant in the natural seawater than at 22 or 30 ppt, but were almost exactly as sensitive as the 14 ppt water. Interestingly, the average salinity of the natural seawater was 14.2 ppt. The results of the natural seawater testing may have an important impact on the recirculating aquaculture of marine organisms. If the aquaculture systems water supply is a nearby natural source, than the toxicity of ammonia may depend somewhat on water characteristics other than

pH, temperature and salinity. The results of the salinity and natural seawater tests also suggest that at different salinities, ammonia may exhibit very different toxicities. This implies that site-specific guidelines may be necessary.

6.4.2 Chronic Testing and the Chronic Criterion

The chronic results provide important additional data. The Criteria use two saltwater and four freshwater organisms to determine the chronic criterion. The freshwater organisms were used because no other data were available on the chronic toxicity of ammonia to saltwater organisms. However, this document presents chronic data for four more saltwater organisms. The chronic criterion is determined by dividing the final acute value (FAV) by the geometric mean of the acute-chronic ratios of the appropriate organisms for which chronic data are available [2]. The FAV for ammonia in saltwater is 0.465 mg/L. The six organisms used to calculate the current chronic criterion are; the Mysid, Inland Silverside, Channel Catfish, Fathead Minnow, Bluegill Sunfish, and Rainbow Trout, with acute-chronic ratios of 7.2, 21.3, 10.0, 20, 12, and 14, respectively [1]. The geometric mean of these six acute-chronic ratios is 13.1. The four organisms considered in this research were: the Atlantic Silverside, Sheepshead Minnow, Summer Flounder and Ghost Shrimp, with acute-chronic ratios of 1.77, 6.95, 2.54, and 7.57, respectively. The geometric mean of these four acute-chronic ratios and the two saltwater organisms (Mysid and Inland Silverside) is 5.75. The chronic criterion based on this result is 0.081 mg/L, significantly greater than the current chronic criterion of 0.035 mg/L.

6.5 Conclusions

In this research the acute toxicity of ammonia to three marine fish and three marine invertebrates and the chronic toxicity of ammonia to three marine fish and one marine invertebrate were studied. The acute criterion for ammonia in saltwater remains unchanged based on these results. The revised chronic criterion based on the Mysid Shrimp, Inland Silverside, Summer Flounder, Atlantic Silverside, Ghost Shrimp, and Sheepshead Minnow is 0.081 mg/L (unionized ammonia), significantly greater than the current chronic criterion of 0.035 mg/L. The toxicity of ammonia to Atlantic Silverside significantly increases with an increase in salinity (14ppt-22ppt). The sensitivity of Atlantic Silverside to ammonia in natural seawater and in synthetic seawater of the same salinity was similar. However, Summer Flounder were more sensitive to ammonia in natural seawater from the Chesapeake Bay than in the synthetic seawater.

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505 **Table 6.1 – Summary of Test Conditions**

	Sheepshead Minnow			Atlantic Silverside			Summer Flounder		
	48 hour	**96 hour**	**7day**	**48 hour**	**96 hour**	**7day**	**48 hour**	**96 hour**	**7day**
Endpoint	Surv.	Surv.	Surv./ Growth	Surv.	Surv.	Surv./ Growth	Surv.	Surv.	Surv.
Replicates	2	4	4	2	2	3	2	2	3
Beaker Size (mL)	600	600	600	1000	1000	1000	2000	2000	2000
Test Sol. (mL)	250	250	250	500	500	500	2000	2000	2000
Age	< 10days, 24 hr range	< 10days, 24 hr range	1-14 days, 24 hr range	14 days	14 days	14 days	2 months	2 months	2 months
Feeding Regime	none	*Artemia* 2x/ day	*Artemia* 2x/ day	none	*Artemia* 2x/ day	*Artemia* 2x/ day	None	Flakes 2x/ day	Flakes 2x/ day
Salinity (ppt)	25	25	25	14,22,30	30	30	30	30	30
Dilution water	Syn.	Syn.	Syn.	Syn./Nat.	Syn./Nat.	Syn.	Syn./Nat.	Syn./Nat.	Syn.

506 **Table 6.1 Continued**

	Mysid Shrimp		Ghost Shrimp			Quahog Clam	
	48 hour	**96 hour**	**48 hour**	**96 hour**	**7day**	**48 hour**	**96 hour**
Endpoint	Surv.	Surv.	Surv.	Surv.	Surv./Growth	Surv.	Surv.
Replicates	2	4	2	4	4	2	2
Beaker Size (mL)	600	600	1000	1000	1000	500	500
Test Sol. (mL)	250	250	1000	500	500	500	500
Age	< 2 days, 24 hr range	< 2 days, 24 hr range	1-2 months	10 days	10 days	1-2 months	1-2 months
Feeding Regime	none	*Artemia* 2x/ day	none	*Artemia* 2x/ day	*Artemia* 2x/ day	none	None
Salinity	25	25	25	25	25	30	30
Dilution water	Syn.	Syn.	Syn.	Syn.	Syn.	Syn.	Syn.

507 **Table 6.2 – Acute Results of Synthetic Seawater Tests[a]**

		Sheepshead Minnow	Atlantic Silverside	Summer Flounder	Ghost Shrimp	Mysid Shrimp	Quahog Clam
"multi-test method"	**48-h LC_{50}[b]**	2.69 (2.52-2.89)	1.08[c] (0.92-1.19)	1.22 (1.09-1.35)	3.49 (3.22-3.80)	1.03 (0.85-1.24)	218 (165-271)
	48-h NOEC	2.3	0.92[c]	0.93	2.7	0.69	38
	96-h LC_{50}[b]	2.07 (1.84-2.32)	-	1.08 (0.94-1.22)	1.66 (1.52-1.82)	0.76 (0.62-0.92)	37.9 (26.7-49.1)
	96-h NOEC	1.3	-	0.73	1.38	0.22	9.6
"multi-replicate method"	**48-h LC_{50}[b]**	2.68 (2.61-2.77)	1.09[c] (0.95-1.19)	1.22 (1.13-1.31)	3.48 (3.37-3.59)	1.00 (0.94-1.08)	216 (186-246)
	48-h NOEC	1.9	0.92[c]	0.93	2.34	0.45	37
	96-h LC_{50}[b]	2.09 (1.97-2.23)	-	1.07(0.968-1.17)	1.67 (1.60-1.75)	0.76 (0.69-0.83)	36.6 (30.5-42.8)
	96-h NOEC	1.3	-	0.47	1.38	0.22	9.3
	SMAV	2.37	0.92[c]	1.14	2.41	0.87	88.9

508 [a]All data in terms of mg/L of unionized ammonia.
509 [b] 95% Confidence Intervals in Parentheses
510 [c]Data for Atlantic Silverside tests performed at salinity 30 ppt.

Table 6.3 - 48-h LC_{50}s and NOECs based on 6 replicates for the Atlantic Silverside (*Menidia menidia*) and three salinity levels

Salinity (ppt)	Total ammonia LC_{50}(mg/L)[a]	Unionized ammonia LC_{50} (mg/L)[s]	Total ammonia NOEC(mg/L)	Unionized ammonia NOEC(mg/L)	Literature unionized LC_{50}(mg/L)
14	28.0 (25.5-29.5)	1.51 (1.38-1.59)	20.0	1.08	
22	25.0 (23.0-26.5)	1.17 (1.08-1.25)	20.0	0.900	1.03-1.47[b]
30	23.6 (20.8-24.3)	1.09 (0.950-1.19)	20.0	0.920	

[a] 95% confidence intervals in parentheses

[b] From EPA [1].

Table 6.4 - α values determined by Anova single factor (significance $\alpha \leq 0.05$)

Salinity (ppt)	**14**	**22**
30	0.000468	0.152
22	0.00255	**-**

516 **Table 6.5 - Natural seawater results compared with synthetic results[a]**

	Summer Flounder		Atlantic Silverside	
	48-h LC_{50}	**96-h LC_{50}**	**48-h LC_{50}**	**96-h LC_{50}**
Synthetic seawater	1.22 (1.13-1.31)	1.07 (0.968-1.17)	1.09 (0.950-1.19)	0.97[b]
Natural seawater	1.09 (0.954-1.23)	0.889 (0.776-1.00)	1.52 (1.41-1.63)	1.18 (1.08-1.28)
α value	0.0408	0.114	See below	0.208

517 [a] 95% confidence intervals in parentheses

518 [b]From EPA [1].

519 **Table 6.6 – Anova values of salinity tests compared to natural seawater tests for**

520 **Atlantic Silverside**

Salinity (ppt)	LC_{50}	α value
14	1.51 (1.38-1.59)	0.646
22	1.17 (1.08-1.25)	0.0133
30	1.09 (0.950-1.19)	0.00464

Table 6.7 – Chronic Results

		Sheepshead Minnow	Atlantic Silverside	Summer Flounder	Ghost Shrimp
"multi-test method"	**7-day LC_{50}[a]**	1.74 (1.61-1.89)	1.20 (1.08-1.24)	1.37 (1.20-1.54)	1.45 (1.32-1.60)
	NOEC (surv)	1.3	0.63	0.25	0.99
	NOEC (growth)	0.45	0.63	-	0.49
	LOEC (growth)		0.95	0.51[b]	0.83
"multi-replicate method"	**7-day LC_{50}[a]**	1.74 (1.66-1.83)	1.16 (1.10-1.21)	1.37 (1.25-1.49)	1.43 (1.33-1.54)
	NOEC (surv.)	1.3	0.63	0.34	0.99
	NOEC (growth)	0.34	0.48	-	0.33
	LOEC (growth)	0.45	0.79	0.68[b]	0.66
	Chronic Value	0.39	0.62	0.48	0.46
	Acute-chronic ratio	6.95	1.77	2.27	7.57

[b] 95% confidence intervals in parentheses

[a]LOEC for survival.

7 Engineering Significance

The ammonia limit is especially important because it will determine how much ammonia is allowed in a National Pollutant Discharge Elimination System (NPDES) permit. This limit will then be an important factor in determining the treatment system that is used. This limit is especially important to companies in Virginia that have previously been unregulated. These include seafood-processing companies whose wastewater discharges are often characterized as low flow, but contain high organic loads. Total ammonia concentrations in crab processing water, for example, average about 200 mg/L, but can reach as high as 1000 mg/L, if allowed to anaerobically digest [30]. Research on the treatment of these by McVeigh [30], Diz [31], and Wolfe [32]. The high concentration of ammonia in these wastewaters makes the possibility of an ammonia limit especially important.

Another significant implication of ammonia toxicity is in recirculating aquaculture. This is especially true for the relatively recent aquaculture of Summer Flounder. GreatBay Aquafarms in Portsmouth, New Hampshire recently became the first company in the country to commercially spawn Summer Flounder [33,34,35]. The Virginia Tech Seafood Research and Extension Center in Hampton, Virginia recently began researching the recirculating aquaculture of Summer Flounder. Because it is a new species to be aquacultured, the ammonia limits that are acceptable for the Summer Flounder are not known. The addition of this data will help set guidelines for this organism.

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Appendix A

% Unionized Ammonia as a Function of pH, Temperature, and Salinity

Salinity = 18-22 ppt

Temp		pH									
(^{0}C)	pK_a	7.5	7.6	7.7	7.8	7.9	8.0	8.1	8.2	8.3	8.4
0	10.1	0.251	0.315	0.397	0.499	0.627	0.788	0.990	1.24	1.56	1.96
5	9.94	0.362	0.455	0.572	0.719	0.904	1.14	1.42	1.79	2.24	2.80
10	9.78	0.522	0.656	0.825	1.04	1.30	1.63	2.05	2.56	3.21	4.00
15	9.61	0.770	0.968	1.22	1.53	1.91	2.40	3.00	3.74	4.67	5.81
20	9.45	1.11	1.39	1.75	2.19	2.74	3.43	4.28	5.32	6.61	8.18
25	9.29	1.60	2.00	2.51	3.13	3.91	4.88	6.06	7.52	9.28	11.4

Salinity = 23-27 ppt

Temp		pH									
(^{0}C)	pK_a	7.5	7.6	7.7	7.8	7.9	8.0	8.1	8.2	8.3	8.4
0	10.13	0.234	0.294	0.370	0.466	0.585	0.736	0.925	1.16	1.46	1.83
5	9.97	0.338	0.425	0.534	0.672	0.844	1.06	1.33	1.67	2.09	2.62
10	9.81	0.487	0.613	0.770	0.968	1.22	1.53	1.91	2.40	3.00	3.74
15	9.64	0.719	0.904	1.14	1.42	1.79	2.24	2.80	3.50	4.37	5.44
20	9.48	1.04	1.30	1.63	2.05	2.56	3.21	4.00	4.99	6.20	7.68
25	9.32	1.49	1.87	2.34	2.93	3.66	4.57	5.68	7.05	8.72	10.7

Salinity = 28-31 ppt

Temp		pH									
(^{0}C)	pK_a	7.5	7.6	7.7	7.8	7.9	8.0	8.1	8.2	8.3	8.4
0	10.14	0.229	0.288	0.362	0.455	0.572	0.719	0.904	1.14	1.42	1.79
5	9.98	0.330	0.415	0.522	0.656	0.825	1.04	1.30	1.63	2.05	2.56
10	9.82	0.476	0.599	0.753	0.946	1.19	1.49	1.87	2.34	2.93	3.66
15	9.65	0.703	0.883	1.11	1.39	1.75	2.19	2.74	3.43	4.28	5.32
20	9.49	1.01	1.27	1.60	2.00	2.51	3.13	3.91	4.88	6.06	7.52
25	9.33	1.46	1.83	2.29	2.87	3.58	4.47	5.56	6.90	8.54	10.5

Salinity = 32-40 ppt.

Temp (^{0}C)	pK_a	pH									
		7.5	**7.6**	**7.7**	**7.8**	**7.9**	**8.0**	**8.1**	**8.2**	**8.3**	**8.4**
0	10.2	0.218	0.275	0.346	0.435	0.547	0.687	0.863	1.08	1.36	1.71
5	10.0	0.315	0.397	0.499	0.627	0.788	0.990	1.24	1.56	1.96	2.45
10	9.84	0.455	0.572	0.719	0.904	1.14	1.42	1.79	2.24	2.80	3.50
15	9.67	0.672	0.844	1.06	1.33	1.67	2.09	2.62	3.28	4.09	5.10
20	9.51	0.968	1.22	1.53	1.91	2.40	3.00	3.74	4.67	5.81	7.20
25	9.35	1.39	1.75	2.19	2.74	3.43	4.28	5.32	6.61	8.18	10.1

Appendix B

Results of Individual Tests

Individual 48-Hour Acute Test Results for Summer Flounder

Date	Water	Total LC_{50} (mg/L)	Total NOEC (mg/L)	Avg. pH	Sal.	Un-ion. LC_{50} (mg/L)	Un-ion. NOEC (mg/L)
9/12/96	Syn.	26.7 (24.0-29.4)	20.0	8.17	30	1.22 (1.10-1.34)	0.914
2/14/97	Syn.	25.7 (22.7-28.6)	20.0	8.19	30	1.23 (1.09-1.37)	0.955
Avg.	-	26.2 (23.4-29.0	20.0	8.19	30	1.22 (1.09-1.35)	0.934
4-rep.	-	26.2 (24.2-28.2)	20.0	8.18	30	1.22 (1.13-1.31)	0.934
10/8/97	Nat.	22.9 (20.0-25.9)	10.0	8.18	27	1.09 (0.954-1.23)	0.477

Individual 96-Hour Acute Test Results for Summer Flounder

Date	Water	Total LC_{50} (mg/L)	Total NOEC (mg/L)	Avg. pH	Sal.	Un-ion. LC_{50} (mg/L)	Un-ion. NOEC (mg/L)
10/10/97	Syn.	22.3 (19.2-25.3)	10	8.18	27	1.06 (0.916-1.21)	0.477
10/14/97	Syn.	22.6 (19.9-25.3)	20	8.19	27	1.10 (0.973-1.23)	0.976
Avg.	-	22.4 (19.6-25.3)	15.0	8.19	27	1.08 (0.944-1.22)	0.726
4 Rep.	-	22.4 (20.3-24.6)	10	8.19	27	1.07 (0.968-1.17)	0.477
10/11/97	Nat.	18.6 (16.2-21.0)	10	8.18	27.3	0.889 (0.776-1.00)	0.477

Individual 48-Hour Acute Test Results for Atlantic Silverside.

Date	Total LC_{50} (mg/L)	Total NOEC (mg/L)	Avg. pH	Sal.	Un-ion. LC_{50} (mg/L)	Un-ion. NOEC (mg/L)
12/11/97	28.8 (26.3-31.3)	20	8.20	26	1.44 (1.31-1.56)	0.997
2/19/98	28.9 (25.6-32.2)	10	8.20	8.3	1.68 (1.48-1.87)	0.581
2/25/98	26.4 (22.0-30.9)	10	8.19	8.3	1.50 (1.25-1.75)	0.568
Avg.	28.0 (24.6-31.4)	13.3	8.20	14.2	1.54 (1.35-1.73)	0.715
6-rep.	27.9 (25.8-29.9)	10	8.20	14.2	1.52 (1.41-1.63)	0.544

Individual 96-Hour Acute Test Results for Atlantic Silverside.

Date	Total LC_{50} (mg/L)	Total NOEC (mg/L)	Avg. pH	Sal.	Un-ion. LC_{50} (mg/L)	Un-ion. NOEC (mg/L)
12/13/97	21.5 (18.2-24.8)	10	8.20	26	1.07 (0.908-1.24)	0.499
2/19/98	21.2 (18.3-24.2)	20	8.20	8.3	1.23 (1.06-1.40)	1.16
2/25/98	22.2 (19.0-25.4)	10	8.19	8.3	1.26 (1.08-1.44)	0.568
Avg.	21.7 (18.5-24.8)	13.3	8.20	14.2	1.19 (1.02-1.36)	0.743
6-rep.	21.6 (19.8-23.5)	20	8.20	14.2	1.18 (1.08-1.28)	1.09

Individual 48-Hour Acute Test Results for Quahog Clam (Salinity=30 ppt)

Date	Total LC_{50} (mg/L)	Total NOEC (mg/L)	Avg. pH	Un-ion. LC_{50} (mg/L)	Un-ion. NOEC (mg/L)
8/18/97	4895 (3552-6237)	800	8.18	229 (166-291)	37.4
8/19/97	4532 (3586-5479)	800	8.18	212 (167-256)	37.4
8/23/97	4473 (3362-5584)	800	8.19	213 (160-267)	38.2
Avg.	4633 (3500-5767)	800	8.18	218 (165-271)	37.6
6-rep.	4619 (3975-5262)	800	8.18	216 (186-246)	37.4

Individual 96-Hour Acute Test Results for Quahog Clam

Date	Total LC_{50} (mg/L)	Total NOEC (mg/L)	Avg. pH	Un-ion. LC_{50} (mg/L)	Un-ion. NOEC (mg/L)
8/11/97	802 (529-1075)	200	8.2	39.1 (25.8-52.4)	9.76
8/13/97	905 (670-1140)	200	8.2	44.1 (32.7-55.6)	9.76
8/23/97	652 (465-838)	200	8.18	30.4 (21.7-39.1)	9.34
Avg.	786 (555-1017)	200	8.19	37.9 (26.7-49.1)	9.62
6-rep.	784 (652-917)	200	8.18	36.6 (30.5-42.8)	9.34

Individual 7-day Chronic Test Results for Summer Flounder (Salinity=30ppt)

Date	Total LC_{50} (mg/L)	Total NOEC (mg/L)	Total LOEC (mg/L)	Avg. pH	Un-ion. LC_{50} (mg/L)	Un-ion. NOEC (mg/L)	Un-ion. LOEC (mg/L)
9/14/96	20.3 (17.5-23.2)	2.5	5	8.19	1.37 (1.18-1.57)	0.169	0.338
2/17/97	20.2 (18.0-22.4)	5	10	8.19	1.37 (1.22-1.52)	0.338	0.676
Avg.	20.3 (17.7-22.8)	3.75	7.5	8.19	1.37 (1.20-1.54)	0.253	0.507
6-rep.	20.3 (18.4-22.1)	5	10	8.19	1.37 (1.25-1.49)	0.338	0.676

Appendix C
Results of Single Factor ANOVA Analysis

Flounder 48-hour Acute Tests

48 syn	48 nat
1.22	1.09
1.23	

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
48 syn	2	2.45	1.225	5E-05
48 nat	1	1.09	1.09	#DIV/0!

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.01215	1	0.01215	243	0.040783	161.4462
Within Groups	5E-05	1	5E-05			
Total	0.0122	2				

Flounder 96-hour Acute Tests

96 syn	96 nat
1.06	0.889
1.10	

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
96 syn	2	2.16	1.08	0.0008
96 nat	1	0.889	0.889	#DIV/0!

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.024321	1	0.024321	30.40083	0.11422	161.4462
Within Groups	0.0008	1	0.0008			
Total	0.025121	2				

Atlantic Silverside 48-hour Acute Test v. 14 ppt

Natural	14 ppt
1.44	1.52
1.68	1.52
1.5	1.47

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Natural	3	4.62	1.54	0.0156
14 ppt	3	4.51	1.503333	0.000833

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.002017	1	0.002017	0.245436	0.646289	7.70865
Within Groups	0.032867	4	0.008217			
Total	0.034883	5				

Atlantic Silverside 48-hour Acute Test v. 22 ppt

Natural	22 ppt
1.44	1.14
1.68	1.27
1.5	1.13

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Natural	3	4.62	1.54	0.0156
22 ppt	3	3.54	1.18	0.0061

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.1944	1	0.1944	17.91705	0.01334	7.70865
Within Groups	0.0434	4	0.01085			
Total	0.2378	5				

Atlantic Silverside 48-hour Acute Test v. 30 ppt

Natural	30 ppt
1.44	1.15
1.68	1.03
1.5	1.05

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Natural	3	4.62	1.54	0.0156
30 ppt	3	3.23	1.076667	0.004133

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.322017	1	0.322017	32.63682	0.004644	7.70865
Within Groups	0.039467	4	0.009867			
Total	0.361483	5				

Atlantic Silverside 96-hour Acute Test v. Literature Values

nat water	Lit.
1.07	0.97
1.23	
1.26	

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
nat water	3	3.56	1.186667	0.010433
Other	1	0.97	0.97	#DIV/0!

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.035208	1	0.035208	3.374601	0.207612	18.51276
Within Groups	0.020867	2	0.010433			
Total	0.056075	3				

Appendix D
Organism Suppliers

GreatGay Aquafarms
153 Gosling Road
Portsmouth, NH 03801
(603) 430-8057

Sea Plantations, Incorporated
PO Box 848
Salem, Massachusetts 01970
(978) 745-4560

Cherrystone Aquafarms
PO Box 347
Cheriton, VA 23316
(804) 331-1208