Hardness-Dependent Ammonia Toxicity and the Potential Use of the Water-Effect Ratio

Final Report for Arid West Water Quality Research Project

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FOREWORD

The Arid West Water Quality Research Project (AWWQRP or "Project") was established in 1995 as a result of a federal appropriation (Public Law 103-327) and the establishment of an Assistance Agreement between the U.S. Environmental Protection Agency (USEPA) and Pima County Wastewater Management (PCWWM), Tucson, Arizona. The establishment of this Agreement provided a significant opportunity for western water resource stakeholders to (1) work cooperatively to conduct scientific research to recommend appropriate water quality criteria, standards and uses for effluent-dependent and ephemeral waters in the arid and semi-arid regions of the West ("arid West"), and (2) improve the scientific basis for regulating wastewater and stormwater discharges in the arid West. Effluent-dependent waters are created by the discharge of treated effluent into ephemeral streambeds or streams that in the absence of effluent discharge would have only minimal flow.

With the establishment of the AWWQRP, a management infrastructure was created to support the development of peer-reviewed research products. From within the Capital Development Division of PCWWM, the AWWQRP Project Director, Program Manager and support staff administer the Project. A Regulatory Working Group (RWG), comprised of 15 stakeholders representing both public and private interests, works to ensure that Project research has a sound regulatory basis and that research activities focus on important regulatory concerns. The Scientific Advisory Group (SAG), comprised of scientists with experience in water quality research, makes certain that project research has a sound scientific basis and that studies are properly designed and technically sound.

This report represents the sixth in a series of research reports produced by the AWWQRP, and builds upon already completed work. The first report in the series, *Pre-Research Survey of Municipal NPDES Dischargers in the Arid and Semi-Arid West*, resulted from an RWG recommendation that the Project survey arid West wastewater facilities to compile information about their effluent discharges and associated water quality concerns.

The second report, the *Habitat Characterization Study*, utilized the findings of the Discharger Survey. Recognizing that an understanding of the attributes of effluent-dependent waters was critical to the development of appropriate water quality criteria and standards for these waters, the RWG recommended that the AWWQRP commission a major study to describe the physical, chemical, and biological characteristics of effluent-created habitats.

The *Habitat Characterization Study* evaluated the physical, chemical and biological characteristics of effluent-dependent habitats at ten case study sites in the arid West: Santa Cruz River below Nogales and below Tucson, Arizona; Salt River below Phoenix, Arizona; Santa Ana River below San Bernardino, California; Fountain Creek below Colorado Springs, Colorado; South Platte River below Denver, Colorado; Las Vegas Wash below Las Vegas, Nevada; Santa Fe River below Santa Fe, New Mexico; Carrizo Creek below Carrizo Springs, Texas; and Crow Creek below Cheyenne, Wyoming (Figure F-1). The primary objectives of this effort were to (1) review existing physical, chemical and biological data; (2) conduct a site reconnaissance to characterize habitats using established protocols and protocols adapted for arid West conditions; (3) identify similarities and differences among sites; (4) discuss potential approaches to protect these habitats in the context of existing regulatory programs; and (5) recommend areas for additional study. The final report may be downloaded from the AWWQRP website, www.co.pima.az.us/wwm/wqrp, or obtained from the AWWQRP Office in a CD hyperlinked format.

The AWWQRP's third report, *Extant Criteria Evaluation*, evaluated the applicability of national water quality criteria, as well as the methods to modify those criteria, to effluent-dependent and ephemeral waters in the arid West. This work built upon the findings presented in the *Habitat Characterization Study* using the expertise of national water quality criteria researchers. The AWWQRP used the findings and recommendations contained in the *Extant Criteria Evaluation* as the primary driver for the selection and execution of three subsequent research projects, including evaluations of 1) the Biotic Ligand Model of copper toxicity in arid west streams, 2) use of the EPA recalculation procedure in effluent-dependent streams, and 3) potential hardness-modifications to ammonia toxicity and their implications for use of the water-effect ratio.

Figure F-1. *Habitat Characterization Study* Case Study Sites.

The purpose of this sixth report, *Hardness-Dependent Ammonia Toxicity and the Potential Use of the Water Effect Ratio (WER)*, ("Ammonia WER Study") was to conduct a simple empirical study as a "proof of concept" to determine whether hardness exerts a significant enough effect on acute ammonia toxicity to be used as a basis for deriving site-specific ammonia standards in hard effluent-dependent waters.

The SAG provided a technical review of the findings from the Ammonia WER Study. After the SAG comments were addressed, the report was submitted to the RWG and USEPA for additional technical and regulatory review. Comments of a technical nature were covered in a response matrix, with major comments addressed in the report, as necessary. In particular, even though our studies confirm previous work suggesting that some elements of water quality in very hard waters can affect acute ammonia toxicity, these effects are highly species-specific. It is also as yet difficult to generalize the effects of specific water quality factors (e.g., sodium) on ammonia toxicity in natural waters for regulatory purposes (i.e., development of site-specific water quality standards). Because additional research is needed to better resolve these scientific questions, it is strongly recommended that local state and regional USEPA staff should be consulted prior to using these preliminary findings to support or propose regulatory change.

The AWWQRP has made a significant effort to share Project results and their implications in a variety of technical, regulatory, industry and public interest forums, including publication in the primary scientific literature. This outreach effort is designed to create a broader understanding of water quality issues unique to the arid West and provide scientific and regulatory data in support of a regional approach to the development of water quality criteria, standards and uses. Heightened interest in arid West water quality issues has been fueled by the recognition that treated effluent can have a valuable role in the support and enhancement of riparian ecosystems, particularly in light of increasingly limited water resources. The AWWQRP looks forward to continuing its support of research that not only provides critical data to address unique western water quality issues, but also supports the development of innovative solutions.

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ACRONYMS

µg/L	microgram(s) per liter
AWQC	ambient water quality criteria
AWWQRP	Arid West Water Quality Research Project
CEC	Chadwick Ecological Consultants
EDW	effluent-dependent waters
LC50	median lethal concentration – point estimate for 50% mortality
mg/L	milligram(s) per liter
NPDES	National Pollutant Discharge Elimination System
NH_3	ammonia, or "unionized" ammonia
NH^{4+}	ammonium ion, or "ionized" ammonia
PCWMD	Pima County Wastewater Management Department
SMAV	species mean acute value
TA-N	total ammonia as nitrogen
UA-N	unionized ammonia as nitrogen
USEPA	U.S. Environmental Protection Agency
WER	Water Effect Ratio

EXECUTIVE SUMMARY

Ammonia is unique among regulated toxicants, as it is an endogenously produced compound that organisms must either excrete or detoxify for survival. In aqueous solution, total ammonia nitrogen (TA-N) exists in two forms, the ammonium ion (NH_4^+) and un-ionized ammonia (NH_3), and their relative chemical abundance is primarily dependent upon the pH and temperature of the solution. Accordingly, ammonia toxicity to aquatic organisms is also largely a function of pH and temperature, with toxicity increasing with increasing pH. This is because ammonia toxicity is primarily dependent on the relative concentration of un-ionized ammonia which becomes more abundant at higher pH. As a result, the U.S. Environmental Protection Agency's (USEPA) most recent (1999) national recommended acute Ambient Water Quality Criteria (AWQC) for ammonia depend directly on pH (in addition to the presence or absence of salmonid fish). The 1999 AWQC also mentions that ions other than pH (e.g., hardness cations such as calcium or magnesium) may also affect acute ammonia toxicity, but this was not considered significant enough to base criteria calculation on hardness.

Although the 1999 AWQC is not expressed as a function of hardness, some toxicity studies have suggested that ammonia toxicity may vary with hardness for both invertebrates and fish. This clearly could be a significant issue for ephemeral and effluent-dependent waters in the arid West with elevated hardness, because if ammonia/hardness relationships can be confirmed, it may be possible to consider water-effect ratio (WER)-based studies to derive site-specific water quality standards for these waters. However, additional scientific study is needed to further evaluate empirical relationships between hardness and acute ammonia toxicity. Therefore, a simple empirical study was conducted as a "proof of concept" to determine whether hardness exerts a significant enough effect on acute ammonia toxicity to be used as a basis for deriving site-specific ammonia standards in hard, effluent-dependent waters. This study consisted of three general components:

- A literature review for scientific studies conducted since publication of the 1999 AWQC to evaluate whether any new studies support or reject the hardness-ammonia toxicity relationships mentioned above.
- A series of acute toxicity tests that independently varied hardness and pH to further evaluate the significance of hardness-ammonia toxicity relationships for both freshwater fish and invertebrates.
- A limited set of confirmatory WER studies in effluent dependent waters of varying hardness to determine whether WER magnitudes were a function of hardness.

LITERATURE REVIEW

The literature review revealed few studies that have specifically examined the role of hardness on ammonia toxicity to aquatic organisms, and that most of these studies were conducted with invertebrates. The majority of research investigating the relationship between hardness and ammonia in fish consists of physiological studies that examine the link between hardness and ammonia excretion rather than a toxic response to ammonia. Several of the studies reviewed suggested that the Na^+ - NH_4^+ exchange mechanism plays a key role in the ammonia/hardness relationship. In general, the literature review indicated that changes in the ion composition of freshwaters can indeed decrease ammonia toxicity for some (but not all) species, but this is not likely to be a consistent function of hardness *per se*. Varying responses to elevated hardness may instead be more of a function of changes in sodium ion concentrations rather than calcium or magnesium ions.

ACUTE TOXICITY TESTS

Acute toxicity tests were conducted with two freshwater fish species: *Pimephales promelas* (fathead minnow) and *Oncorhynchus mykiss* (rainbow trout), and two freshwater invertebrate species: *Hyalella azteca* and *Ceriodaphnia dubia*. For each species, six toxicity tests were conducted at three nominal hardness levels (100, 300, and 600 mg/L as $CaCO_3$) and two nominal pH levels (7, 8). Tests were conducted in synthetic freshwaters in which alkalinity was held constant to control for the potential confounding effects of sodium.

For both fish species examined, ammonia toxicity was relatively constant with increasing pH when expressed on an un-ionized basis, while ammonia toxicity significantly increased with pH when expressed on the basis of total ammonia-N. These results were consistent with those from previous studies that suggest the effect of pH on ammonia toxicity in fish is best explained by the pH-dependent speciation of un-ionized ammonia. In contrast, for both invertebrate species tested, ammonia toxicity, expressed on an un-ionized basis, decreased with increasing pH. As suggested by previous researchers, these results indicate that the toxicity of ammonia to invertebrates may be best explained by a joint toxicity model wherein both the ionized and un-ionized fractions play an important role in ammonia toxicity. This is important because USEPA uses a form of this joint toxicity model in the derivation of the acute ammonia AWQC.

No significant relationships were observed between hardness and the toxicity of ammonia to either of the fish species examined. These findings contradict the conclusions of several physiological studies that suggested an ammonia/hardness relationship might exist owing to an increase in ammonia excretion with increasing hardness. However, these physiological studies were conducted at ambient ammonia concentrations much lower than those tested in the acute toxicity tests, and in natural waters where the ionic composition was likely very different from that of the acute toxicity test waters, wherein calcium and magnesium where the only ionic constituents manipulated. Furthermore, even though a physiological relationship between hardness and ammonia excretion may exist under ambient conditions in natural waters, this condition may not necessarily elicit a toxicological response.

For the invertebrate species tested, the only significant hardness/ammonia toxicity relationships observed were that at pH 8, ammonia toxicity increased with increasing hardness for *H. azteca* and decreased with increasing hardness for *C. dubia* when expressed on the basis of total ammonia-N. These results were not in agreement with previous studies that found the toxicity of total ammonia to *H. azteca* decreased with increasing hardness and the toxicity of total ammonia to *C. dubia* increased with hardness. However, the previous *H. azteca* studies were confounded by the fact that alkalinity (and, likely, sodium) co-varied with hardness, while alkalinity was held constant in the acute toxicity tests conducted in the present study. To determine whether or not this discrepancy in experimental design could explain the inconsistency in study results, a series of additional acute *H. azteca* studies were conducted wherein sodium was independently manipulated in conjunction with hardness and alkalinity. The results of these studies confirmed that allowing alkalinity to fluctuate with hardness likely had a significant effect on the results previously observed, and that elevated sodium levels offer considerable protection to *H. azteca* against ammonia toxicity, especially when coupled with elevated hardness. Differences in test water ionic composition related to hardness (e.g., sodium) may also help explain why our results with *C. dubia* did not agree with previous studies. But because chemical data were not reported, we can not suggest specific explanations for the differences we observed between these two studies.

WER STUDIES

Acute ammonia toxicity tests using paired site-water and reconstituted laboratory water as dilution water were also conducted. Four effluent-dependent waters (Las Vegas Wash, Nevada; Salt River, Arizona; Santa Ana River, California; and the South Platte River, Colorado) were chosen due to the wide range of water hardness present at these sites. Acute toxicity tests were conducted with *C. dubia*, *P. promelas*, and *Chironomus tentans*. Differences in ammonia toxicity between sites and laboratory water were evaluated by calculating water-effect ratios (WERs) for each of the acute tests.

WERs, expressed as total ammonia, were fairly consistent among species. In particular, fathead minnow WERs generally ranged from 0.5 – 2 among all sites, WERs were consistently highest for *C. tentans* among all sites (0.5 – 3), and WERs for *C. dubia* were ≤ 1 for all sites. WERs, expressed as total ammonia, were also fairly consistent among sites. The highest WERs were generally found in the South Platte River, the lowest WERs were generally found in the Santa Ana River, and the Salt River and Las Vegas Wash WERs were intermediate. WER magnitudes at these sites were not a function of hardness given that the South Platte River had the lowest hardness (198-214 mg/L $CaCO_3$), that the Santa Ana River had the second lowest hardness (258 mg/L $CaCO_3$), and that the Salt River and Las Vegas Wash had the two highest hardness values measured at any of the sites (374 and 480 mg/L $CaCO_3$, respectively). However, as previously discussed, other water quality parameters (i.e., alkalinity and sodium) may affect the toxicity of ammonia in natural waters; thus, the lack of a clear relationship between hardness and the WERs measured at these sites may be due to the fact that other factor(s) was contributing more heavily to the toxicity of ammonia to the species tested. Unfortunately, we were unable to evaluate the major ion composition of the lab and site waters tested in the present study, and so these hypotheses can not be experimentally confirmed.

CONCLUSIONS

This study has supported the limited toxicity literature available which suggests that hardness (and/or related cations) may influence acute ammonia toxicity. However, these effects have been shown to be species-specific, (i.e., no one ion composition will exert the same influence) and only valid for invertebrates, not fish. To further elucidate the mechanisms governing these effects, however, major ion composition other than hardness (sodium is of particular interest) needs additional independent experimental manipulation. This study has also shown that WERs >1 can be observed in effluent-dependent waters for both fish and invertebrates. The WERs found to be >1 may have been the result of a difference in ionic composition between the site and laboratory waters, but it is clear that the protective effect associated with these significant WERs was not due to hardness alone. Therefore, until these potential ion effects and/or mechanisms are better understood, it is difficult to predict whether a positive WER could be achieved for a given site without first conducting empirical tests.

1. INTRODUCTION

Ammonia toxicity to aquatic organisms is largely a function of pH- and temperature-dependent chemical speciation, because ammonia toxicity is primarily dependent on the relative concentration of un-ionized ammonia (USEPA 1999). Although the 1999 AWQC is not expressed as a function of hardness, some studies suggest that ammonia toxicity may vary as a function of hardness for both invertebrates and fish. Ankley *et al*. (1995) evaluated acute ammonia toxicity to the amphipod *Hyalella azteca* across a pH range from 6.5 – 8.5, and across a hardness range from 42 – 270 mg/L (as $CaCO_3$). As hardness increased, acute toxicity (as a function of total ammonia-N) decreased significantly, and became more pH-dependent. These results agreed with those of Borgmann (1994) who evaluated chronic ammonia toxicity in both Lake Ontario water (hardness = 130 mg/L), and Lake Ontario water that was diluted 1:10 with double-distilled water. Chronic ammonia toxicity (as a function of total ammonia-N) was significantly less toxic in the hard water relative to the diluted soft water. Both studies further suggested that ammonia toxicity decreased at elevated hardness in response to cationic interactions with Na^+-NH_4^+ membrane exchange mechanisms. Enhanced ammonia excretion via a similar Na-related mechanism at elevated hardness has also been observed in rainbow trout (Yesaki and Iwama 1992) and Lahontan cutthroat trout (Iwama *et al.* 1997).

The mechanistic similarity of hardness-enhanced ammonia excretion in both amphipods and trout suggest that ammonia toxicity may indeed be hardness-dependent for a wider range of taxa. This clearly could be a significant issue for ephemeral and effluent-dependent waters in the arid West with hardness greater than 270 mg/L (URS 2002, Parametrix 2003), and thus warrants additional study.

1.1 PROJECT APPROACH AND OBJECTIVES

If these ammonia/hardness relationships can be confirmed, it may be possible to consider water-effect ratio (WER)-based studies to derive site-specific water quality standards in waters with elevated hardness (i.e., > 200 mg/L). However, additional scientific study is needed to further evaluate empirical relationships between hardness and acute ammonia toxicity. Therefore, a simple empirical study was conducted as a "proof of concept" to determine whether hardness exerts a significant enough effect on acute ammonia toxicity to be used as a basis for deriving site-specific ammonia standards in hard, effluent-dependent waters. This study consisted of three general components:

- A brief literature review for scientific studies conducted since publication of the 1999 AWQC to evaluate whether any new studies support or reject the hardness-toxicity relationships already described above (See Section 2).
- A series of acute toxicity tests that independently varied hardness and pH to further evaluate the significance of hardness-ammonia toxicity relationships for both freshwater fish and invertebrates. This was important to help interpret the results of the WER studies by further elucidating whether ammonia toxicity is hardness dependent for species other than *Hyalella azteca*.
- A limited set of confirmatory WER studies in effluent dependent waters of varying hardness to determine whether WER magnitudes were a function of hardness in very hard waters (i.e., 200 mg/L or greater). These tests were conducted with two standard laboratory test species, in addition to an aquatic insect species.

This project approach was selected for the following reasons. First, we contend that combining WER studies with controlled hardness manipulation studies is preferable over simply conducting WERs using waters spanning the desired range of hardness. This is because for the WER approach to be effective and mechanistically meaningful, a water quality characteristic must be confirmed as a plausible causal agent in reducing ammonia bioavailability and toxicity in site waters relative to laboratory waters (USEPA 1994, Parametrix 2003). Therefore, to enhance confidence in the use of ammonia WERs in effluent dependent waters, we need to first empirically confirm that hardness-dependent ammonia toxicity does occur for several species (other than *Hyalella*) in reconstituted waters. Second, we contend that this initial study is best done only using acute tests. It would still be of scientific and regulatory significance to resolve acute toxicity relationships alone, particularly since WERs are rarely conducted using chronic toxicity tests and the vast majority of comparative toxicity data are acute. If hardness-toxicity relationships are confirmed with these acute tests, only then would it be prudent to proceed with chronic tests.

2. LITERATURE REVIEW: AMMONIA TOXICITY – HARDNESS RELATIONSHIPS

Ammonia is unique among regulated toxicants, as it is an endogenously produced toxicant that organisms must either excrete or detoxify for survival (Evans and Cameron 1986, Randall *et al.* 1989). In aqueous solution, total ammonia nitrogen (TA-N) exists in two forms, ammonium ion (NH_4^+) and un-ionized ammonia (NH_3), and the chemical speciation is primarily dependent upon the pH and temperature of the solution. In marine systems, the ionic strength of the water also affects the ionization potential of ammonia, thereby reducing NH_3 to NH_4^+ (Soderberg and Meade 1991). In freshwater, the ionic effect on chemical speciation is much less than the effects of pH or temperature and it is rarely accounted for in toxicological studies. Nonetheless, ionic strength may affect ammonia toxicity in aquatic organisms by disrupting the mechanisms of ion exchange across epithelial membranes (Evans and Cameron 1986, Soderberg and Meade 1991, Borgmann 1994, Ankley *et al.* 1995).

To best understand the following discussion, it is necessary to clarify a few terms that are frequently used herein: *ionic strength* specifically refers to a weighted concentration of all ions in solution, which include, but are not limited to, Na^+, K^+, $Ca^{+\ 2}$, Mg^+, HCO_3^-, SO_4^{-2}, Cl^- CO_3^-, whereas *hardness* is solely dependant upon the sum of calcium and magnesium in solution.

Seminal toxicology studies revealed that aquatic organisms became more sensitive to ammonia as pH increased (Chapman 1934, Wurham and Woker 1984). At ambient temperature (20°C) and pH 7 (freshwater), the percentage of TA-N that is NH_3 is 0.4%. This percentage rises slightly at pH 8 (3.8%), and at pH 9 the percentage begins to precipitously increase (28%). By pH 9.4, over half of total ammonia is in the un-ionized form. Based on this relationship, un-ionized ammonia was logically determined to be the more toxic form. Un-ionized ammonia is a neutral molecule (gaseous phase) that readily diffuses across the epithelial membranes of aquatic organisms.

With specific reference to fish, the presence of high ambient un-ionized ammonia concentrations either reduce or reverse the diffusive gradients across epithelial cells and cause ammonia concentrations to increase in gill tissue and the blood (Evans and Cameron 1986). Similar principles apply to the ammonium ion, although being a charged molecule (NH_4^+), the diffusive characteristics are much less than un-ionized ammonia (NH_3). Thus, the ammonium ion requires an active transport mechanism to cross the epithelial membrane. Consequently, the toxicity of ammonium ion has been considered to be less than un-ionized ammonia for most aquatic organisms. However, Borgmann (1994) and Ankley *et al.* (1995) have suggested that ammonium ion toxicity is a greater concern for *Hyalella azteca* and, quite possibly, for other aquatic invertebrates.

There has been considerable research and debate concerning the mechanisms that facilitate ammonium ion exchange in freshwater organisms (Evans and Cameron 1986, McDonald *et al.* 1989, Yesaki and Iwama 1992, Wright *et al.* 1993, Wilson *et al.* 1994), with a variety of evidence supporting different modes of excretion. The more commonly accepted mechanisms involve the direct link between Na^+ uptake and $NH4^+$ excretion in a 1:1 ratio, or an active exchange of $Na^+ - H^+ + NH_3$ (Evans and Cameron 1986). A second mechanism involves a paracellular transfer of strong ions, mainly Na^+ and Cl^-, with acidic equivalents of NH_4^+, H+, HCO_3^- or OH^-. This mechanism requires carbonic-anhydrase (CAH) that drives the acidification process of the gill water boundary layer and facilitates the diffusion of un-ionized ammonia (Wilson *et al.* 1994). However, the evidence is much weaker for this mechanism as an important role in the excretion of un-ionized ammonia in fish (McDonald *et al.* 1989, Wright *et al.* 1993).

The mechanisms facilitating ammonium ion exchange and the passive diffusion of un-ionized ammonia are obviously relevant to toxicity tests that evaluate the effects of hardness on ammonia toxicity. However, the majority of the mechanistic studies focusing on ammonia excretion and osmoregulation have been conducted at ambient ammonia concentrations much lower than those typically used in toxicology studies (Ankley *et al.* 1995). Thus, uncertainty resides in the efficiency of these mechanisms when ambient ammonia levels are much greater, or when the hardness/ionic strength of the water is much greater.

There are few studies that have specifically examined the role of hardness on ammonia toxicity to aquatic organisms (Tomasso et al. 1980, Yesaki and Iwama 1992, Borgmann 1994, Sarda 1994, Ankley et al. 1995, Borgmann and Borgmann 1997) and even fewer that have met USEPA requirements for appropriate calculation of LC50s or EC50s in acute and chronic toxicity tests used in derivation of AWQC (see review of studies below). The majority of the studies have used two species of crustacea – *Hyalella azteca* and *Ceriodaphnia dubia* – though one study used the channel catfish (*Ictalurus punctatus*). The amphipod – *Hyalella azteca* – has often been selected for ammonia toxicity tests because of its selected habitat, the benthos, and its sensitivity to ammonia. There is a greater potential for ammonia exposure at the sediment–water interface due to the accumulation of decomposing organic matter. However, when *H. azteca* has been subjected to water-column toxicity tests, it has exhibited poor control survival in both acute and chronic toxicity experiments (Borgmann 1994, Ankley et al. 1995, Borgmann and Borgmann 1997), which raises concern when developing water quality criteria (Chadwick Ecological Consultants Inc. 2004).

There has been special interest in the anadromous salmonids and their adaptive ability to process/excrete nitrogenous wastes in both saline and freshwater environments (Soderberg and Meade 1991, Wilson and Taylor 1992). However, most are physiological studies that do not examine the toxic response to ammonia. Nonetheless, these experiments provide insight into the role of Na^+, K^+, and Ca^{+2} in the fish's ability to process ammonia (Soderberg and Meade 1991, Paley *et al.* 1993). The Lahontan cutthroat trout has also received special attention regarding its specialized ability to excrete nitrogenous wastes in the extremely alkaline environment (pH 9.4) of Pyramid Lake, Nevada (Yesaki and Iwama 1992). However, the range of this species is quite limited, and the site-specific studies are of limited use in the development of regional or national criteria.

With regard to ammonia toxicity and hardness relationships for *H. azteca*, Ankley *et al.* (1995) reported a general increase in the organisms' tolerance to TA-N at high hardness levels (240 mg $CaCO_3$/L) at pH levels of 6.5 and 7.5, though this trend is not apparent at pH 8.5. Ankley *et al.* (1995) evaluated the effect of hardness on the toxicity of ammonia to *H. azteca* using three treatments: Soft Water (Lake Superior) = 42 mg $CaCO_3$/L, Moderately Hard Water (amended Lake Superior water with $CaSO_4$, $CaCl_2$, $MgSO_4$, $NaHCO_3$, and KCl) = 100 mg $CaCO_3$/L, and Hard Water (Millipore Deionized Water amended with the above constituents) = 240 mg $CaCO_3$/L. The authors noted that the three waters differed not only in hardness but in concentrations of specific anions. Thus, the authors could not conclusively state that the effects of water type on ammonia toxicity were caused solely by hardness (Ankley *et al.* 1995).

Ammonia toxicity in the soft water was independent of pH, because TA-N LC50s were essentially the same across all pH levels (Figure 2-1 A). However, when based on NH_3-N LC50s, ammonia toxicity decreased as hardness increased. Total ammonia toxicity (TA-N LC50s) in the MHW was more variable across pH and increased (i.e., LC50s decreased) approximately 2.5-fold at the higher pH level (Figure 2-1 A), although when expressed as un-ionized ammonia, toxicity decreased approximately 30-fold. Similar patterns of ammonia toxicity were observed for the hard water treatment, though the differences were not as great. Based on experimental conditions, the majority of ammonia was ammonium ion; thus, given the relative constancy of TA-N LC50s for moderately hard water across the range of pH, these data indicate that *H. azteca* may be responding more to NH_4^+ rather than NH_3.

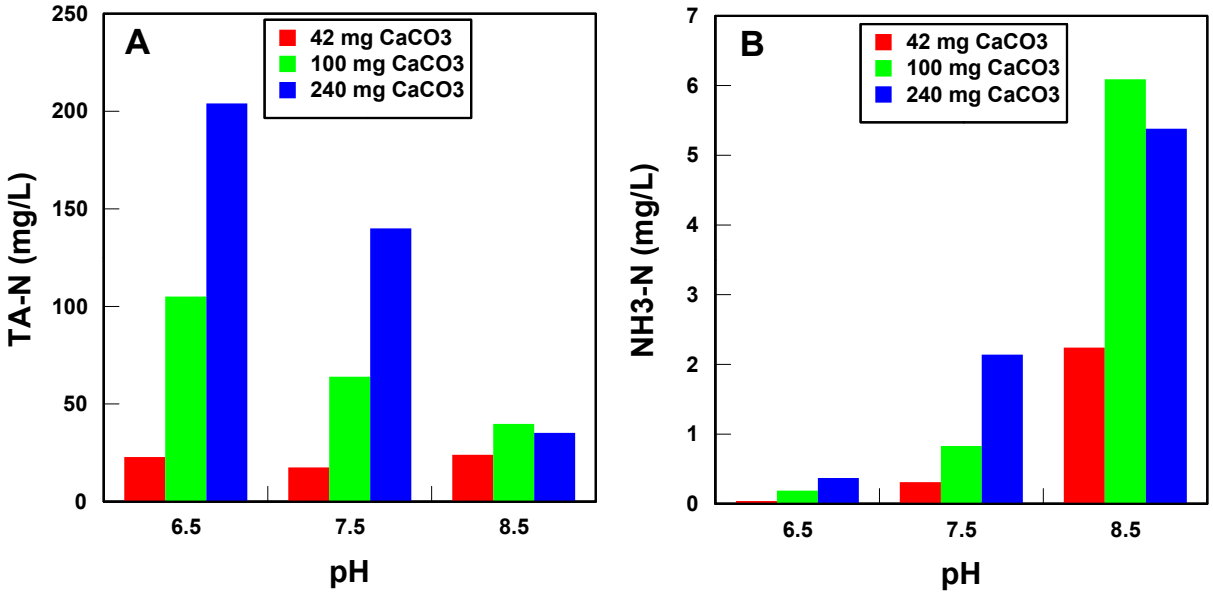

Figure 2-1. A) Total ammonia (TA-N), and B) un-ionized ammonia (NH3-N) LC50s for *Hyalella azteca* at three hardness treatments and three pH factors (Ankley *et al.* 1995).

At pH levels of 6.5 and 7.5, total ammonia toxicity (TA-N LC50s) to *H. azteca* decreased as the hardness of the test waters increased. At pH 8.5, total ammonia toxicity to *H. azteca* was constant across the range of hardness. *H. azteca* exhibited a decreased sensitivity to un-ionized ammonia toxicity (NH_3-N LC50s) when hardness increased at each nominal pH. These trends suggest that the low sodium concentrations in soft waters may affect the organism's ability to excrete ammonium ion via the Na^+ - NH_4^+ exchange mechanism.

Borgmann (1994) observed a similar increased sensitivity of *H. azteca* to total ammonia toxicity in soft water treatments at pH 7.4, and also concluded that ammonium ion appeared to be the more toxic form for the amphipod. Borgmann's (1994) primary objective was to evaluate the effects of chronic ammonia toxicity to *H. azteca* in dechlorinated tap water originating from Lake Ontario (pH = 8.0 – 8.4, hardness = 130 mg $CaCO_3$/L) for future comparison to sediment bioassays. Secondary objectives evaluated chronic ammonia toxicity in Lake Ontario water with reduced pH (either by acid addition or dilution with distilled water) to determine the relative importance of ionized/un-ionized ammonia toxicity. There were no specific objectives to evaluate the effects of hardness (i.e., experimental design with varying hardness treatments) on ammonia toxicity to *H. azteca*. The reduced pH

experiments, however, provided anecdotal information into potential hardness (ionic strength) and ammonia toxicity relationships.

Mortality was relatively constant on a TA-N basis for all tests performed at the same hardness (i.e., acidified treatment and dechlorinated tap water experiment [primary objective]). Amphipod survival was lower in the control acidified treatment and for the low ammonia solutions (acidified treatment), though TA-N LC50s were indistinguishable between treatments. Total ammonia was more toxic in the 90% DIW treatment, with TA-N LC50s being significantly lower than the dechlorinated tap water experiment. A reduction in hardness and other ions was associated with an increased sensitivity of *H. azteca* to ammonia toxicity. Notably, when TA-N LC50s for both the acidified and 90% DIW treatments were converted to NH_3 LC50s, both values were significantly lower than the dechlorinated tap water experiment. Ammonia toxicity as a function of un-ionized ammonia was not constant among the experiments, indicating that NH_3 is not a good predictor of chronic ammonia toxicity to *H. azteca*, even in waters with the same hardness.

Borgmann concluded that chronic toxicity was more a function of ionized ammonia rather than un-ionized ammonia in waters with the same hardness (i.e., 130 mg $CaCO_3$/L), and that a 90% reduction in hardness resulted in the increased sensitivity of *H. azteca* to ammonia. Given *H. azteca*'s apparent sensitivity to ionized ammonia, the Na^+ – NH_4^+ exchange mechanism described for fish may in part account for the "hardness" effect.

These observations that ammonia toxicity can decrease as a function of increasing hardness (Borgmann 1994, Ankley *et al.* 1995) may be explained by the enhanced ability of *H. azteca* to actively exchange NH_4^+ in the presence of elevated external Na^+ concentrations. However, the increased cationic strength of the hard water is mirrored by an increase in anionic strength (alkalinity – OH^- and HCO_3^-), which may also facilitate the exchange of H^+ and NH_3.

For *C. dubia*, the relationship between ammonia toxicity and hardness is reversed (Sarda 1994). Sarda (1994) evaluated the effects of hardness, alkalinity and pH on the toxicity of ammonia to *C. dubia* by using waters of three different hardness: Hard Water (Massie's Creek) = 364 mg $CaCO_3$/L, Reconstituted Hard Water (RHW) = 180 mg $CaCO_3$/L, and Moderately Hard Water = 100 mg $CaCO_3$/L. The high hardness treatment (360 mg $CaCO_3$) decreased *C. dubia* survival at all ammonia concentrations in comparison to the reconstituted and moderately hard water treatments. Calculated LC50s were similar for the MHW and RHW treatments. However, the LC50 for the HW treatment was approximate 30% lower than either the MHW or RHW treatment.

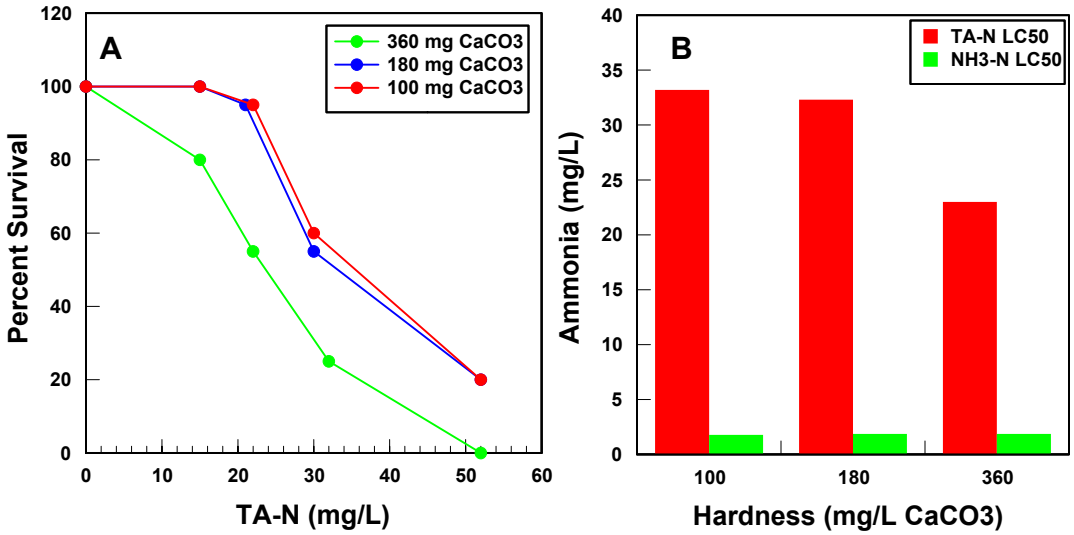

Figure 2-2. A) Effects of hardness on *Ceriodaphnia dubia* survival, and B) total ammonia and un-ionized ammonia LC50s (Sarda 1994).

When the total ammonia LC50 concentrations were fractioned into un-ionized ammonia concentrations using an experimental pH and temperature relationship, the un-ionized ammonia LC50 remained constant among all treatments. At pH levels between 7.8 and 8.3, *C. dubia* showed an increased sensitivity to TA-N toxicity at higher hardness treatments (360 mg $CaCO_3$/L). However, when TA-N LC50s were converted to NH_3 LC50s, the organism responded similarly to all hardness treatments. Un-ionized ammonia was, therefore, the more toxic form of ammonia for this organism.

Similar to the amphipod, channel catfish appears to become more tolerant to ammonia at elevated hardness and neutral pH (Tomasso *et al.* 1980). Tomasso *et al.* (1980) evaluated 24-hr LC50s of total ammonia toxicity to channel catfish (*Ictalurus punctatus*) at three pH levels (7, 8, and 9) in soft water (40 mg/L $CaCO_3$) and at one pH level (7) for hard water (440 mg/L $CaCO_3$). In the soft water treatment, the channel catfish became more acutely sensitive to total ammonia toxicity as pH increased. The 24-hr TA-N LC50s at pH 7, 8, and 9 were 263, 38.3, and 4.5 mg/L, respectively. However, when the TA-N LC50s were converted to un-ionized ammonia fractions using experimental pH and temperature, the LC50 at pH 8 (1.82 mg NH_3/L) was significantly higher than the LC50 of either the pH 7 or 9 treatment (1.39 and 1.49 mg NH_3/L).

The increased hardness treatment at pH 7 significantly reduced the TA-N toxicity to channel catfish by approximately 26% when compared to the soft water treatment (Tomasso *et al.* 1980). There was similar reduction (22%) in sensitivity when toxicity was expressed as a function of un-ionized ammonia. Elevated calcium appears to increase the tolerance of fish to ammonia toxicity at neutral pH (Tomasso *et al.* 1980). However, calcium stimulates adenosine triphosphatase activity at the gill membrane which is responsible for Na^+ - K^+ exchange, thereby increasing the influx of Na^+ at the gill membrane (Fleming 1974). Thus, the decreased sensitivity to ammonia, at elevated hardness and neutral pH, may be explained by Na^+ - NH_4^+ exchange mechanism that facilitates the exchange of ammonium ion or un-ionized ammonia at the gill membrane.

The studies reviewed above suggest changes in the ion composition of freshwaters can indeed decrease ammonia toxicity for some (but not all) species, but this is not likely to be a consistent function of hardness *per se*. Varying responses to elevated hardness may instead be more of a function of changes in sodium ion concentrations rather than calcium or magnesium ions. Similarly, acute ammonia toxicity tests of Atlantic salmon (*Salmo salar*) and lake trout (*Salvelinus namaycush*) demonstrated that elevated calcium levels did not decrease the sensitivity of Atlantic salmon fry or smolt to un-ionized ammonia, though it increased the tolerance of lake trout fingerlings (Soderberg and Meade 1991). Elevated sodium levels were also more protective of older age class fish for both species, but did not increase the tolerance of fry (both species) to un-ionized ammonia.

3. MATERIALS AND METHODS

3.1 HARDNESS/pH ACUTE TOXICITY TESTS

3.1.1 General Test Conditions

Acute toxicity tests were conducted at the Parametrix Environmental Research Laboratory (PERL, Corvallis, OR, USA) with two freshwater fish species: *Pimephales promelas* (fathead minnow) and *Oncorhynchus mykiss* (rainbow trout), and two freshwater invertebrate species: *Hyalella azteca* and *Ceriodaphnia dubia*. All toxicity tests followed appropriate USEPA and American Society for Testing and Materials guidance (ASTM 2000, USEPA 2002). *P. promelas*, *O. mykiss*, and *H. azteca* were tested under 96-h static-renewal conditions (80% volume replacement at 48 h), while *C. dubia* were tested under 48-h static conditions. The biological endpoint used for all toxicity tests was immobilization following gentle flushing via transfer pipette. Organism mortality was determined at 24 h intervals and dead organisms were immediately removed. Due to the potential of testing fathead minnow that were only one to two days old, all fathead minnow tests were fed 0.2 ml *Artemia* nauplii 2 hours prior to the 48 h renewal. To prevent cannibalism, *H. azteca* were fed 400 µl of a combination of yeast, trout chow, and cereal leaves (YTC) at the 48 h renewal. *C. dubia* and rainbow trout were not fed during testing. A 16:8 h light:dark photoperiod was maintained in an environmental chamber using cool white fluorescent tubes that provided 50 – 100 foot-candles at the test chamber surface. Temperature was maintained at 20 ± 1°C for fathead minnow and *C. dubia*, 25 ± 1°C for *H. azteca*, and 12 ± 1°C for rainbow trout. Dissolved oxygen concentration was > 80% of saturation.

For each species, six toxicity tests were conducted at three nominal hardness levels (100, 300, and 600 mg/L as $CaCO_3$) and two nominal pH levels (7, 8). Different hardness levels were achieved by preparing reconstituted laboratory waters using reagent grade salts ($CaSO_4 \cdot 2H_2O$, $MgSO_4$, KCl, and $NaHCO_3$; Fisher Scientific, Pittsburg, PA, USA). Reconstituted water recipes were determined using a Ca:Mg molar ratio of 1.82 to better approximate natural water composition (Welsh *et al.* 2000). Alkalinity was not allowed to fluctuate with hardness and was held constant (although pH adjustment did result in a decrease in alkalinity in the pH 7 treatments). Reconstituted waters used in fathead minnow, *H. azteca*, and *C. dubia* tests were adjusted to pH 7 using hydrochloric acid (HCl) and maintained using a CO_2 atmosphere (Mount and Mount 1992). Due to the large volume of water required for rainbow trout tests, a CO_2 atmosphere could not be used to control pH. Instead, a pH of 7 was achieved by adjusting reconstituted waters using 750 mg/L 3-N-morpholino propansulfonic acid (MOPS) and 10.0 N sodium hydroxide (NaOH). A similar approach was taken to adjust and maintain reconstituted waters at pH 8. That is, fathead minnow, *H. azteca*, and *C. dubia* tests were conducted in a sealed box (without CO_2), and MOPS and NaOH were used to control pH in rainbow trout tests.

Exposure treatments were prepared by adding appropriate volumes of ammonia stock (NH_4Cl; Fisher Scientific, Pittsburg, PA, USA) to dilution chambers and were distributed to exposure chambers wherein organisms were added no later than 30 minutes after spiking. Tests were conducted in 250 ml glass beakers containing 200 ml of test solution (fathead minnows), 5-gallon aquaria containing 10 L of test solution (rainbow trout), 100 ml glass beakers containing 50 ml of test solution (*H. azteca*), or 30-ml polypropylene cups containing 25 ml of test solution (*C. dubia*). For fathead minnow, *H. azteca*, and *C. dubia* tests, four replicate exposure chambers were prepared for each of five or six toxicant concentrations (50% dilution series) and a negative control (unspiked dilution water), while only two

replicate exposure chambers were prepared for rainbow trout tests. Exposures were initiated by randomly assigning organisms (ten fathead minnow/rainbow trout or five *H. azteca*/*C. dubia*) directly into test solutions.

3.1.2 Chemical Analyses

Water quality parameters measured in the toxicity tests included dissolved oxygen (DO; mg/L), temperature (°C), pH (SU), hardness (mg/L as $CaCO_3$), alkalinity (mg/L as $CaCO_3$), conductivity (µS/cm), total residual chlorine (TRC; mg/L), and total ammonia (mg/L as N). All parameters were measured at test initiation. Measurements of DO, pH and temperature were made daily. Samples for total ammonia were also taken from renewal water and at test termination or complete mortality, whichever occurred first. Un-ionized ammonia (mg/L as NH_3-N) was calculated based on equations from Emerson (1975) using treatment-specific average pH and temperature data.

DO was measured using a dissolved oxygen probe (Yellow Springs Instruments, Yellow Springs, OH, USA); pH was measured with an Orion-Ross (Orion Research, Beverly, MA, USA) combination pH electrode, connected to a multi-channel pH/mV meter (Orion 720A); hardness and alkalinity were determined by colorimetric titration; conductivity was measured using a Hach sensION 5 conductivity meter (Hach Company, Loveland, CO, USA); TRC was measured using a Hach Pocket Colorimeter™ II (Hach Company, Loveland, CO, USA); and total ammonia was measured using an Orion ammonia electrode (Thermo Electron Corporation, Beverly, MA, USA) connected to a multi-channel pH/mV meter (Orion 520A) calibrated using a NIST Standard Reference Material ammonia standard.

3.1.3 Culture Methods

Larval fathead minnows and *C. dubia* neonates used for toxicity tests were offspring from in-house PERL cultures maintained using standard methods (USEPA 2002). Fathead minnow brood stock were reared in a flow-through system using moderately-hard well water, saturated in dissolved oxygen, pH 7.8, 25 ± 2 °C, hardness and alkalinity 100 and 100 mg/L as $CaCO_3$, respectively. Poly-vinyl chloride tiles within culture tanks were checked daily for the presence of eggs; tiles containing eyed eggs were removed, cleaned of debris using deionized water and placed in polypropylene pans containing moderately-hard reconstituted laboratory water that was continuously aerated and renewed every over day. Larval fish were fed *Artemia* nauplii three times daily until use in toxicity tests. Cultures were maintained in an environmental chamber having a 16:8 h light:dark photoperiod at 25 ± 1°C. All fathead minnows used for testing were between one and seven days old.

C. dubia mass cultures were grown in moderately-hard water. Mass cultures consisted of approximately 100 individual organisms in 2.0 L of moderately-hard water that were fed 10 ml of a YTC/algae (*Raphidocelis subcapitata*) suspension (USEPA 2002) daily and transferred to new solution every 2 – 3 days. Cultures were maintained in a water bath having a 16:8 h light:dark photoperiod at 23 ± 2°C. *C. dubia* less than 24 h old were used in all studies.

Juvenile *H. azteca* were obtained from a commercial supplier (Chesapeake Cultures, Hayes, VA, USA or Aquatic Biosystems, Ft. Collins, CO, USA) and rainbow trout were obtained from the Oregon State University Sinnhuber Aquatic Research Laboratory (SARL; Corvallis, OR, USA). Invertebrates were held for at least 24 hours and fish were held for at least two weeks at PERL prior to testing. *H. azteca* were held in 2.0 L of moderately-hard water and were fed 10 ml of a YTC/algae (*Raphidocelis subcapitata*) suspension (USEPA 2002) daily

and transferred to new solution every 2 – 3 days. Cultures were maintained in a water bath having a 16:8 h light:dark photoperiod at 23 ± 2°C.

Rainbow trout were held in 500 gallon circular water baths with continuously flowing moderately-hard water maintained at 13 ± 1°C. Fish were fed trout chow two times daily and were subject to a 16:8 h light:dark photoperiod. All rainbow trout used for testing were less than 30 days post swim-up.

3.1.4 Data Analysis

Median-lethal concentrations (LC50) and 95% confidence limits were calculated from observed mortalities and measured ammonia concentrations using the trimmed Spearman-Karber method (Hamilton *et al.* 1977). The toxicant concentrations used in these calculations were averages of two or three measurements for each treatment within each toxicity test.

Relationships were examined for their statistical significance by conducting hypothesis tests wherein the null hypothesis was that the slope of the regression was equal to zero. An analysis of variance (ANOVA) was also conducted to determine whether the mean LC50s (expressed as un-ionized or total ammonia) were significantly different among the three hardness/two pH levels tested for each species. The significance level for both analyses was a P-value ≤ 0.05.

3.2 WER STUDIES

Chadwick Ecological Consultants Inc., (CEC), in conjunction with the aquatic biological laboratory, Chadwick & Associates, Inc. (C&A) conducted a series of acute ammonia toxicity tests using paired site-water and reconstituted laboratory water as dilution water. Four effluent-dependent waters including Las Vegas Wash (LVW), Salt River (SR), Santa Ana River (SAR), and the South Platte River (SPR), were chosen for this study due to the wide range of water hardness present at these sites. These waters are also currently being used for copper WER tests in the BLM validation study (Parametrix 2005), and were previously studied in the Habitat Characterization Study (HCS; URS 2002) and Extant Criteria Evaluation (ECE) projects (Parametrix 2003).

Differences in ammonia toxicity between sites and laboratory water were evaluated by calculating water-effect ratios (WERs) for each of the acute tests. WERs were calculated by dividing the measured ammonia LC50 in site water by the measured ammonia LC50 in hardaness and pH matched laboratory water (Equation 1; USEPA 1994).

$$\mathrm{WER} = \frac{\mathrm{LC50}_{\text{Site Water}}}{\mathrm{LC50}_{\text{Lab Water}}} \tag{1}$$

General guidance for test methods and calculating the WER was obtained from the USEPA documents, *Interim Guidance on Determination and Use of Water-Effect Ratios for Metals* (USEPA 1994) and *Streamlined Water-Effect Ratio Procedure for Discharges of Copper* (USEPA 2001).

3.2.1 General Test Conditions

WER testing involves side-by-side laboratory-water and site-water acute toxicity tests. Forty eight-hour static renewal acute toxicity tests were conducted with *C. dubia* and 96-hour static renewal acute toxicity tests were conducted with *P. promelas*. Acute toxicity test procedures followed methods described in USEPA documentation (USEPA 2002). The toxicity testing

method was modified in order to adjust pH for the ammonia-spiked treatments to that of the original site water. The pH of the moderately hard reconstituted laboratory water used in toxicity tests was also adjusted, after spiking, to the original pH of the water. Adding potassium hydroxide (KOH) and air sparging the headspace with 1% CO_2 was the method that resulted in the least pH drift without causing toxicity interference. All toxicity tests were conducted at 20°C with lab water pH being stabilized at 7.5 – 8.0. A light intensity of 50 – 100 foot candles was delivered on a 16:8 hr light:dark photoperiod for the duration of the tests. DO remained above concentrations greater than 4 mg/L.

C. dubia toxicity tests were conducted using neonates less than 24-hours old which were fed YTC/algae (*R. Subcapitata*). at least two hours prior to the tests. Five neonates were placed in each 30 ml polypropylene test cup containing 25 ml of test water. For each test concentration, including a control, there were 4 replicate cups. The test cups were placed in an empty aquarium, which was sealed with vacuum grease and a glass cover. Headspace was purged with 1% CO_2 (Mount and Mount 1992, Elphick *et al.* 2005) from a pressurized gas cylinder for 10 seconds at the initiation of the tests and upon treatment renewals.

Chemical analyses were performed on the initial, renewal, and final test solutions to determine ammonia concentration, DO, conductivity, and pH. Upon renewal and termination of the toxicity tests, mortality and survival of test organisms was recorded and dead organisms were removed. DO was measured using a dissolved oxygen probe (YSI Model 50B); pH was measured with an Orion Ross 8165 probe (Orion Research, Beverly, MA, USA) connected to a Model 225 pH-ISE meter (Denver Instruments Company); hardness and alkalinity were determined by colorimetric titration; conductivity was measured using an Orion 125A Plus conductivity meter (Orion Research, Beverly, MA, USA); TRC was measured using a Hach DR 100 Colorimeter (Hach Company, Loveland, CO, USA); and total ammonia was measured using a Denver Instruments ammonia probe 300740.1 connected to a Model 225 pH-ISE meter and is calibrated using 2 standard solutions (0.5mg/L and 5.0 mg/L) made from the 100 ppm Orion stock solution.

P. promelas toxicity tests were conducted using larvae less than 14-days old, which were fed *Artemia* nauplii at least two hours prior to conducting toxicity tests. Ten fish were placed in each 250 ml test cup containing 200 ml of test water. For each test concentration, including a control, there were 4 replicate cups. Test cups were sealed with Parafilm® to allow aeration of the headspace with 1% CO_2 (Mount and Mount 1992, Elphick *et al.* 2005). Tests were checked for mortality every 24 hours and renewed at 48 hours.

Chemical analyses were performed on the initial, renewal, and final test solutions for all tests to determine ammonia concentration, DO, conductivity, and pH.

Using the methods above, initial range-finding toxicity tests were conducted with both *C. dubia* and *P. promelas* to determine the test concentration ranges that would provide adequate data required for determination of toxicity endpoints (i.e. LC50). These tests were also used to determine the best method of statistical determination of acute endpoints.

3.2.2 Aquatic Insect Toxicity Testing

3.2.2.1 Field-collected Mayflies

The original goal of the study was to conduct ammonia WER testing with a field-collected aquatic insect. The mayfly species, *Tricorythodes minutus,* was selected as the native species to be tested in the laboratory, based on results of collections from streams near the laboratory (See Appendix A) and for toxicity comparisons to resident species of arid West streams (URS 2002).

Specimens of *T. minutus* were collected on July 15, July 22, and July 29, 2005, for tests to begin on July 18, July 25, and August 1, respectively. Samples were collected using a rectangular kick net. The contents of the kick net were emptied into a plastic tray, and the *T. minutus* were sorted from the debris using wide-mouth disposable plastic pipettes to minimize handling stress. *T. minutus* specimens were placed into a collection container and aerated for transport to Chadwick & Associates, Inc. Transport generally required <1 hour.

Upon arrival at the laboratory, the organisms were transferred to an incubator at 25°C, with a light:dark photoperiod of 16:8 hrs. The organisms were acclimated to laboratory water by replacing 50% of the site water with reconstituted water over a three day period.

Acclimation of *T. minutes* to laboratory conditions was generally not successful. A majority of the specimens died within 72 hours of being placed in the incubator. Organisms that did survive the three-day acclimation period were used to initiate 96-hour acute toxicity tests, using 10 replicates containing 1 organism per replicate. However, those toxicity tests had to be terminated due to high mortality of the mayflies in all treatments, including controls, within 48 hours of test initiation.

3.2.2.2 Laboratory-cultured Midges

Based on the inability to acclimate this native species to laboratory conditions, aquatic insect ammonia toxicity testing was conducted with a more conventional test organism, the midge *Chironomus tentans*. Using ASTM (2000) method E729-96, 48-hour acute toxicity tests were conducted with chironomids cultured by Aquatic Biosystems (ABS), Fort Collins, CO.

Chironomid tests were treated in the same manner as fathead minnow tests. To prevent pH drift, test cups were covered with Parafilm© and the headspace was sparged with 1% CO_2. Tests differed from fathead minnow tests in that 175 ml of water was used and approximately 10 ml of washed sand was added in each treatment to minimize test stress for the midges (USEPA 2000).

3.2.3 Data Analysis

All acute toxicity data were analysed using the Comprehensive Environmental Toxicity Information System, CETIS (Tidepool Scientific Software 2000-2004). Un-ionized ammonia concentrations were calculated using total ammonia and treatment-specific mean pH and temperature (Emerson 1975). Both total ammonia and un-ionized ammonia concentrations were entered into the CETIS program to obtain LC50 values for each constituent. The total and un-ionized ammonia LC50s were used to calculate WERs of site water LC50s to reconstituted water LC50s.

The CETIS program determines if the data meets the requirements of the different toxicity data evaluation programs. The Probit test method was generally used to evaluate toxicity tests unless the data did not support the method. For many of the tests, the Trimmed-Spearman Karber (TSK) program was used, providing similar LC50 calculations, but with "tighter" confidence limits.

4. RESULTS

4.1 HARDNESS/PH ACUTE TOXICITY TESTS

Toxicity data are presented below both as a function of pH and of hardness. Data are expressed as a function of pH in order to evaluate whether toxicity was most likely due to un-ionized vs. ionized ammonia. This is important for comparison to the ammonia toxicity literature for the species tested, and to help evaluate potential mechanisms explaining the influence of other cations (e.g., Na^+ or Ca^{2+}) on ammonia toxicity. Data are expressed as a function of hardness to evaluate the strength and consistency of possible hardness-toxicity relationships. Data are presented and regression analyses were conducted with both PERL and CEC data where possible (i.e. for species tested by both laboratories). ANOVA were conducted with PERL data only (i.e. where groups required for ANOVA were defined by the study design).

4.1.1 Fathead Minnow

Although the mean unionized ammonia LC50 of the pH 7 treatment was significantly lower than that of the pH 8 treatment (P = 0.05), the toxicity of ammonia to fathead minnows expressed on the basis of un-ionized ammonia was relatively constant (i.e. the slope of the regression was not significantly different than zero, P = 0.06) over the range of pH values tested (Table 4-1, Figure 4-1 A). However, a significant, negative slope was observed between pH and fathead minnow ammonia toxicity when LC50 values were expressed on a total ammonia basis (P = 0.0002; Figure 4-1 B). Correspondingly, the mean total ammonia LC50 was significantly higher at pH 7 than at pH 8 ($P < 0.0001$).

While fathead minnow un-ionized ammonia LC50 values increased slightly with increasing hardness at pH 7 and 8, these relationships were not significant (P = 0.09, 0.19 respectively; Figure 4-2 A, B). There were no other significant regressions observed between ammonia toxicity (expressed as un-ionized or total) and hardness, at any pH level for fathead minnow. Furthermore, there were no significant differences detected among the mean LC50s (expressed as un-ionized or total ammonia) observed at the three hardness levels.

Table 4-1. Ammonia toxicity test results for fathead minnow

		Measured		Total Ammonia		Un-ionized Ammonia	
Testing Laboratory	**Test Conditions**	**pH**	**Hardness**	**LC50 (mg TA-N/L with 95% CI)**	**WER**	**LC50 (mg NH_3-N/L with 95% CI)**	**WER**
PERL	pH=7 H=600	7.16	680	113.90 (102.88 – 126.11)	-	0.73 (0.66 – 0.81)	-
	pH=7 H=300	6.94	324	135.31 (122.44 – 149.54)	-	0.57 (0.52 – 0.63)	-
	pH=7 H=100	6.95	112	124.31 (109.16 – 141.55)	-	0.52 (0.46 – 0.59)	-
	pH=8 H=600	7.87	670	26.96 (24.11 – 30.15)	-	0.88 (0.81 – 0.95)	-
	pH=8 H=300	7.86	316	26.14 (23.49 – 29.10)	-	0.83 (0.77 – 0.89)	-
	pH=8 H=100	7.90	108	21.66 (19.83 – 23.65)	-	0.75 (0.70 – 0.79)	-
CEC	SPR - lab	7.56	94	24.2 (22.7 – 25.7)	-	0.40 (0.38 – 0.42)	-
	SR - lab	7.37	102	37.0 (33.9 – 40.4)	-	0.62 (0.58 – 0.67)	-
	LVW - lab	7.57	100	36.1 (33.1 – 39.3)	-	0.38 (0.36 – 0.40)	-
	SAR - lab	7.46	100	37.1 (33.7 – 40.8)	-	0.41 (0.37 – 0.45)	-
	SPR - site	7.52	198	53.5 (45.4 – 67.2)	2.2	0.71 (0.61 – 0.87)	1.8
	SR - site	7.65	374	41.2 (37.1 – 45.7)	1.1	0.74 (0.67 – 0.82)	1.2
	LVW - site	7.34	480	64.1 (55.9 – 73.6)	1.8	0.72 (0.64 – 0.80)	1.9
	SAR - site	8.06	258	20.2 (18.3 – 22.2)	0.5	0.92 (0.85 – 1.01)	2.2

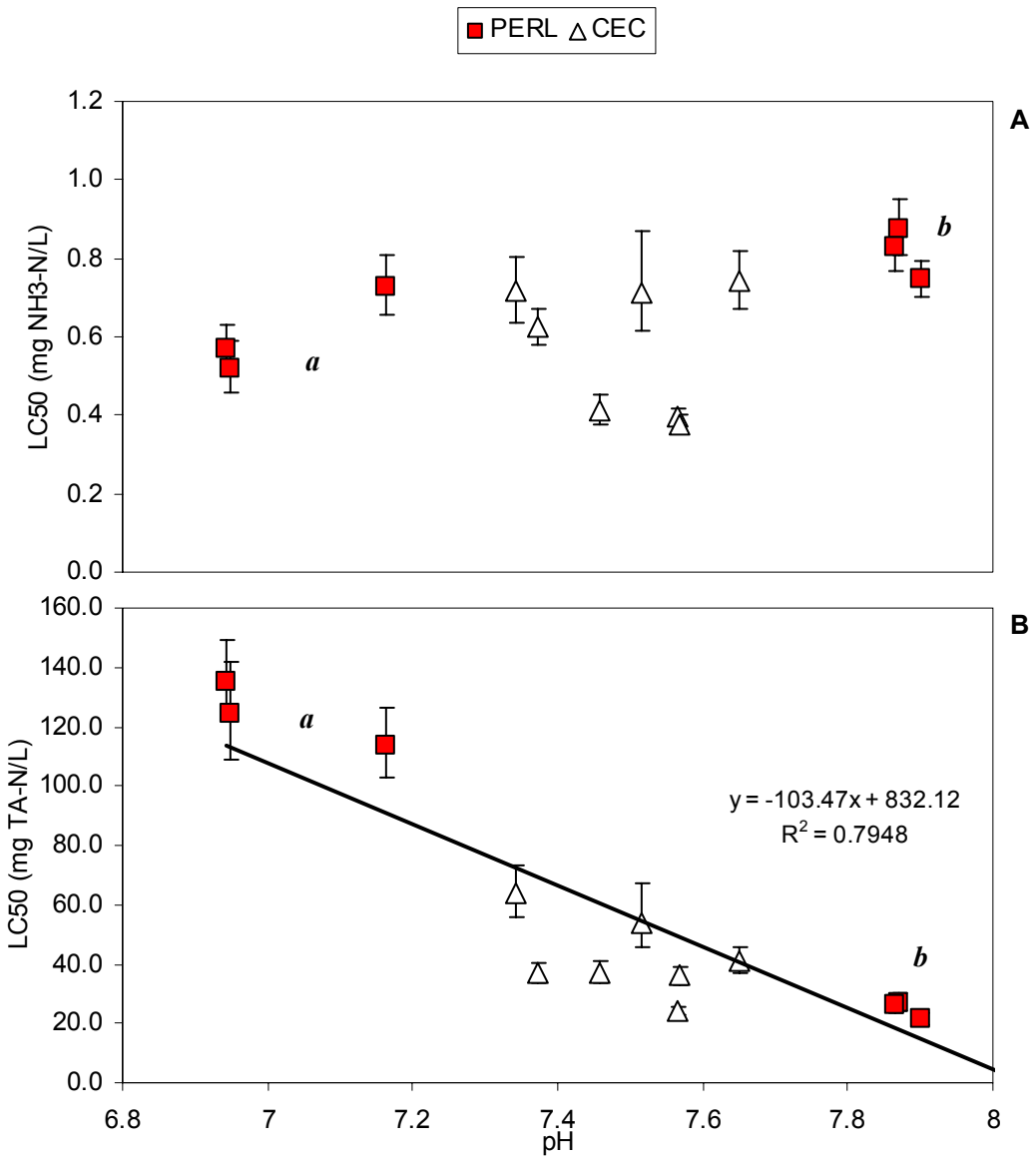

Figure 4-1. Relationship between fathead minnow toxicity expressed as un-ionized ammonia (A) and total ammonia (B) and pH. Significant regressions are identified by trendline, significant differences between means are identified by lower case letter.

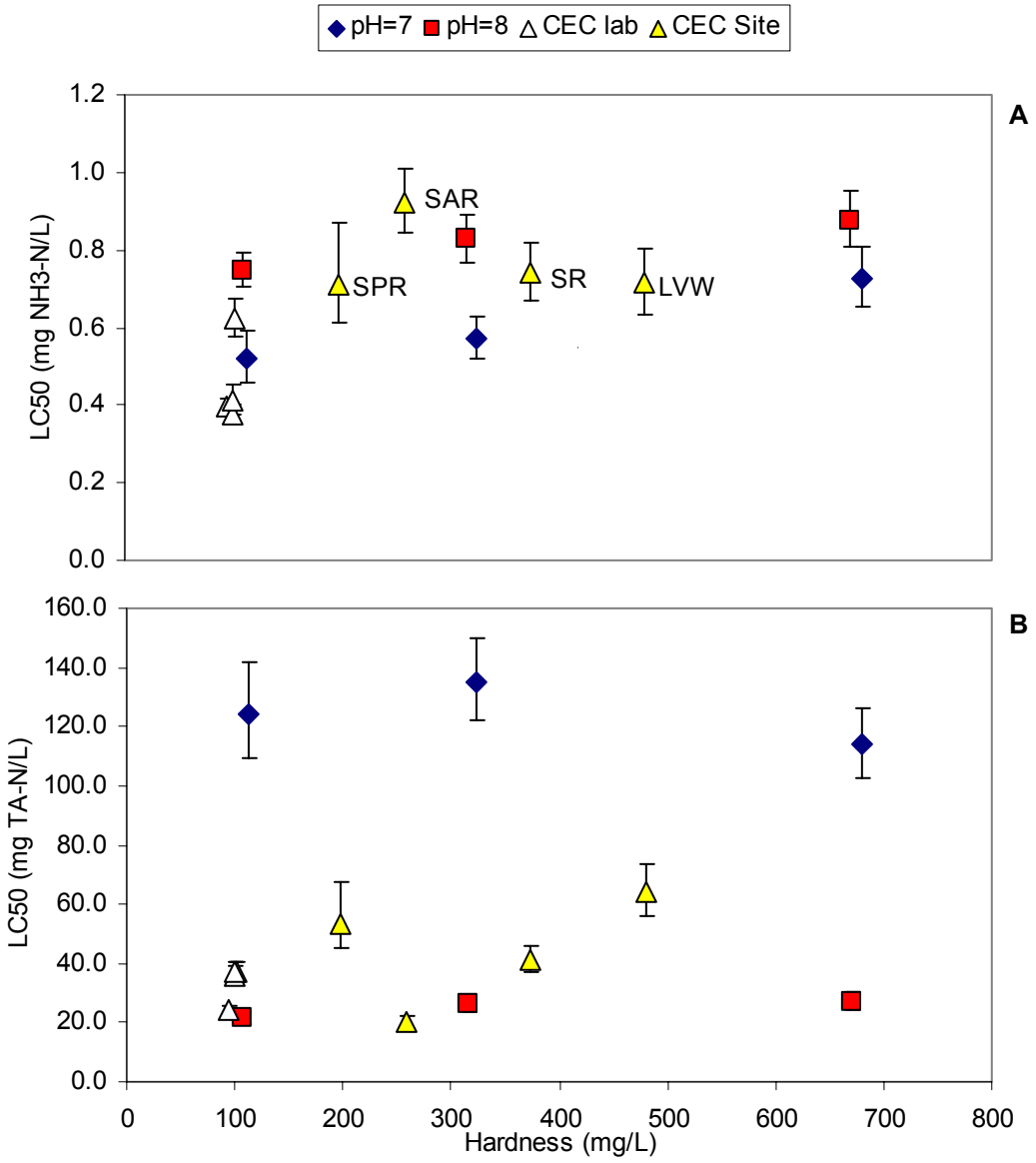

Figure 4-2. Relationship between fathead minnow toxicity expressed as un-ionized ammonia (A) and total ammonia (B) and hardness at various pH levels for lab and site waters. Significant regressions are identified by trendline, significant differences between means are identified by lower case letter.

4.1.2 Rainbow Trout

Rainbow trout LC50 values, expressed on an unionized basis, only varied by a factor of 1 across the range of pH levels tested (Table 4-2). Correspondingly, there was no significant regression observed between un-ionized ammonia toxicity and pH ($P = 0.96$, Figure 4-3 A), and the mean un-ionized ammonia LC50s were not significantly different between the pH 7 and 8 treatments ($P = 0.78$). However, a significant, negative relationship was observed between pH and LC50 values, expressed as total ammonia ($P < 0.0001$, Figure 4-3 B) and, likewise, the mean total ammonia LC50 measured at pH 7 was significantly greater than the mean total ammonia LC50 measured at pH 8 ($P < 0.0001$).

There were no significant regressions observed between hardness and the toxicity of ammonia (expressed as un-ionized or total) to rainbow trout at any pH level (Figure 4-4 A, B). However, the mean un-ionized LC50 observed at H=300 was significantly lower than the mean un-ionized LC50 observed at H=100 ($P = 0.02$) and H=600 ($P = 0.04$).

Table 4-2. Ammonia toxicity test results for rainbow trout

	Measured		LC50 (with 95% CI)	
Testing Laboratory	**pH**	**Hardness**	**Total NH_3 (mg N/L)**	**Un-ionized NH_3 (mg NH_3-N/L)**
PERL	7.09	625	121.77 (109.17 – 135.82)	0.41 (0.37 – 0.45)
	6.97	312	124.29 (108.80 – 141.98)	0.30 (0.26 – 0.34)
	7.10	104	119.82 (103.44 – 138.80)	0.37 (0.32 – 0.42)
	7.89	620	17.16 (14.18 – 20.77)	0.37 (0.30 – 0.45)
	7.79	304	17.51 (15.57 – 19.69)	0.28 (0.25 – 0.31)
	7.89	104	19.70 (18.70 – 20.75)	0.38 (0.36 – 0.41)

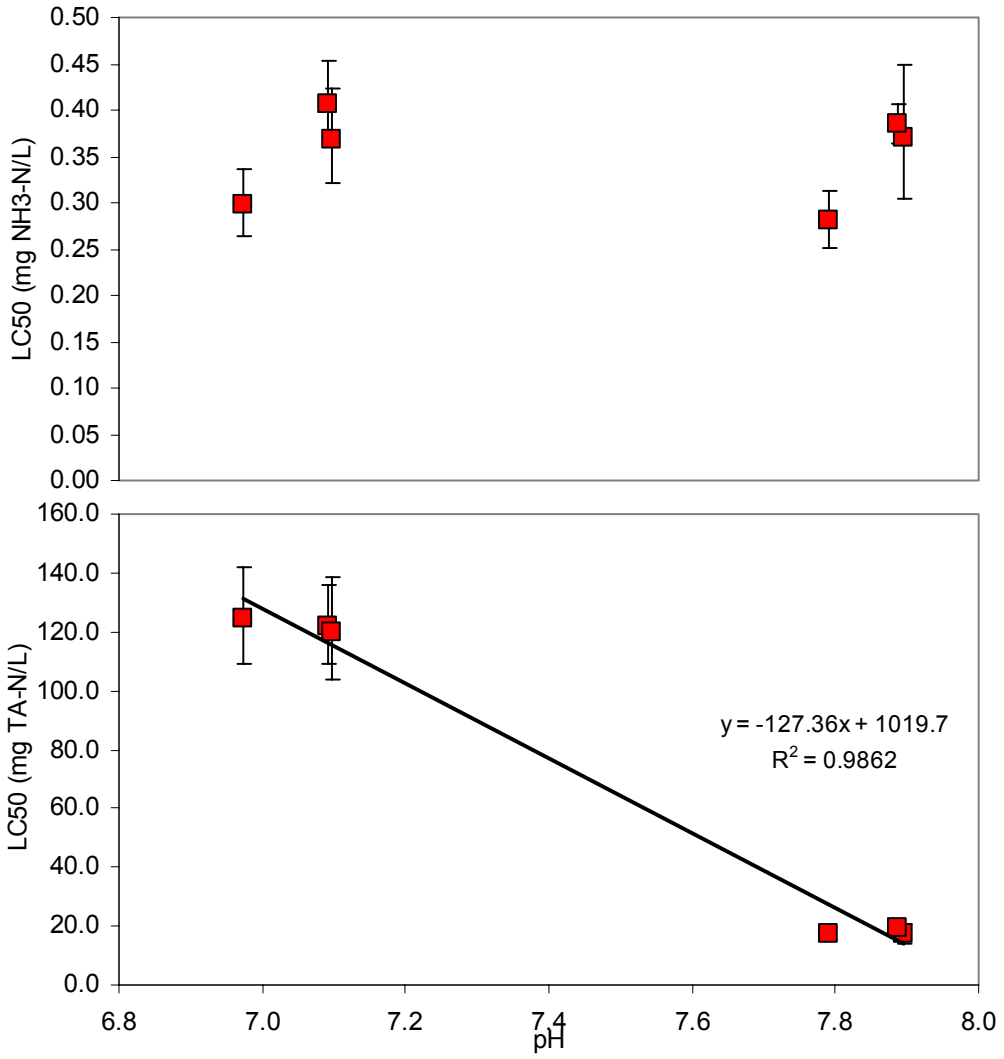

Figure 4-3. Relationship between rainbow trout toxicity expressed as un-ionized ammonia (A) and total ammonia (B) and pH. Significant regressions are identified by trendline, significant differences between means are identified by lower case letter.

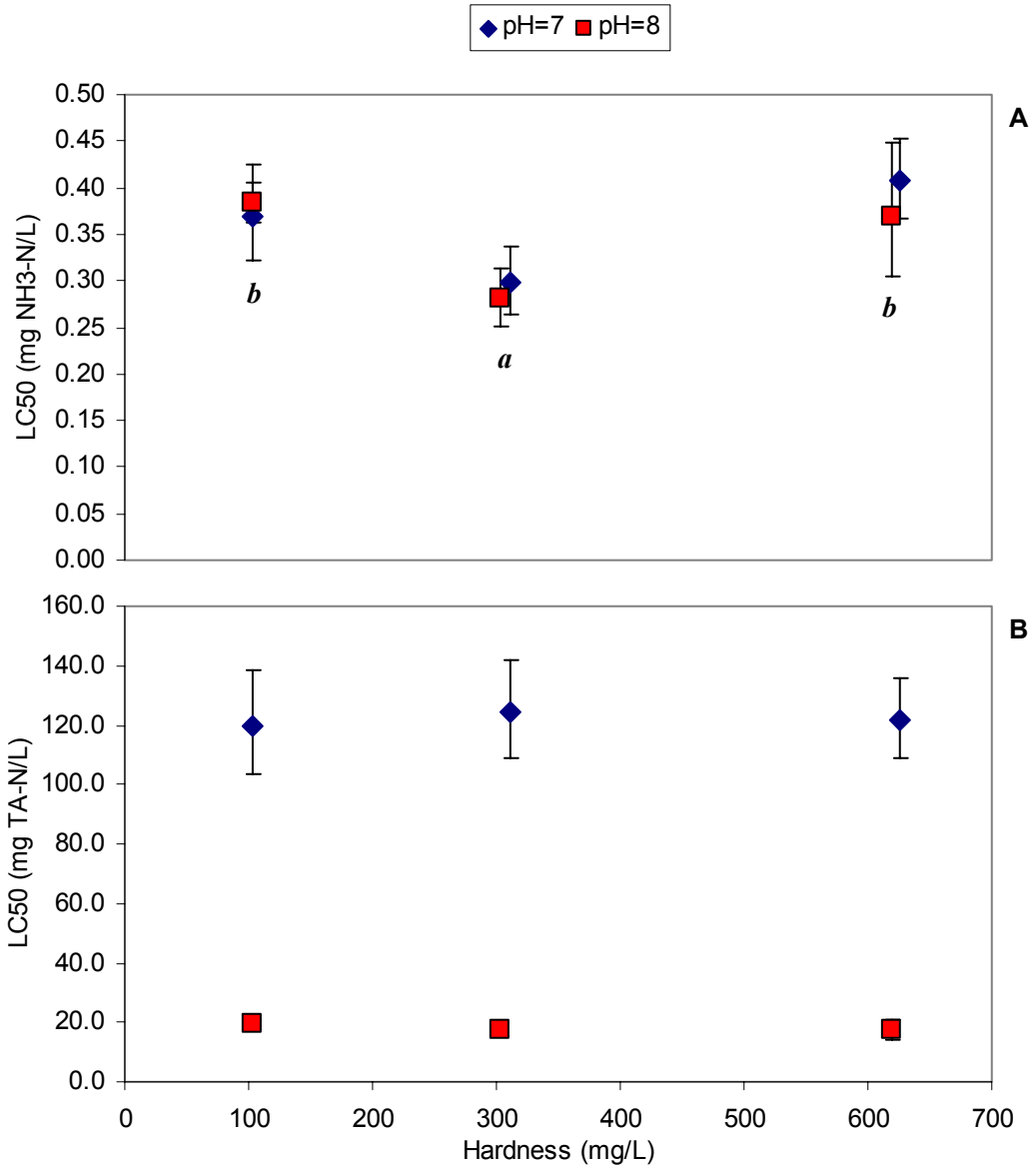

Figure 4-4. Relationship between rainbow trout toxicity expressed as un-ionized ammonia (A) and total ammonia (B) and hardness at various pH levels. Significant regressions are identified by trendline, significant differences between means are identified by lower case letter.

4.1.3 *C. dubia*

For *C.* dubia, un-ionized ammonia LC50 values varied 3-fold across the range of pH levels tested, and total ammonia LC50 values varied 12-fold (Table 4-3) As such, a significant, positive relationship was observed between pH and ammonia toxicity, expressed on an un-ionized ammonia basis ($P = 0.02$, Figure 4-5 A), and a significant, negative relationship was observed between pH and ammonia toxicity, expressed on a total ammonia basis ($P = 0.0001$, Figure 4-5 B). Similarly, the mean un-ionized ammonia LC50 was significantly lower at pH 7 than at pH 8 ($P = 0.02$) and the total ammonia LC50 was significantly higher at pH 7 than at pH 8 ($P = 0.01$).

For both un-ionized and total ammonia, *C. dubia* toxicity slightly increased with increasing hardness at pH 7 and slightly decreased with increasing hardness at pH 8 (Figure 4-6 A, B). However, the only statistically significant regression was that total ammonia LC50 values increased with hardness at pH 8 ($P = 0.02$). Furthermore, there were no significant differences detected among the mean LC50s (expressed as un-ionized or total ammonia) observed at the three hardness levels.

Table 4-3. Ammonia toxicity test results for *C. dubia*

		Measured		Total Ammonia		Un-ionized Ammonia	
Testing Laboratory	**Test Conditions**	**pH**	**Hardness**	**LC50 (mg TA-N/L with 95% CI)**	**WER**	**LC50 (mg NH_3-N/L with 95% CI)**	**WER**
PERL	pH=7 H=600	7.09	680	98.38 (85.94 – 112.62)	-	0.61 (0.53 – 0.69)	-
	pH=7 H=300	6.86	320	175.72 (160.91 – 191.89)	-	0.61 (0.56 – 0.67)	-
	pH=7 H=100	6.95	112	161.23 (141.04 – 184.32)	-	0.71 (0.63 – 0.79)	-
	pH=8 H=600	7.81	670	26.70 (23.05 – 30.92)	-	1.13 (1.04 – 1.23)	-
	pH=8 H=300	7.83	316	21.50 (17.22 – 26.84)	-	0.97 (0.86 – 1.10)	-
	pH=8 H=100	7.85	108	18.82 (15.32 – 23.11)	-	0.84 (0.75 – 0.94)	-
CEC	SPR, SR - lab	7.72	102	80.9 (69.6 – 94.0)	-	1.41 (1.23 – 1.62)	-
	LVW - lab[‡]	7.85	100	80.0 (65.9 – 97.1)	-	1.80 (1.55 – 2.09)	-
	SAR - lab	7.44	100	65.0 (55.8 – 75.7)	-	0.66 (0.57 – 0.76)	-
	SPR - site	7.67	198	86.2 (78.7 – 94.4)	1.1	1.39 (1.28 – 1.50)	1.0
	SR - site	7.79	374	49.0 (43.2 – 55.6)	0.6	1.18 (1.09 – 1.29)	0.8
	LVW - site	7.60	480	87.3 (79.4 – 96.0)	1.1	1.49 (1.41 – 1.59)	0.8
	SAR - site	8.31	258	14.9 (12.6 – 17.5)	0.2	1.25 (1.08 – 1.44)	1.9

[‡]In the 15 mg TAN/L nominal concentration, there was only 85% survival, while both of the next lowest and highest concentrations had 100% survival. The concurrent site water toxicity test exhibited 100% survival in the control and first three concentrations as well. The 15 mg TAN/L value was removed before data analysis.

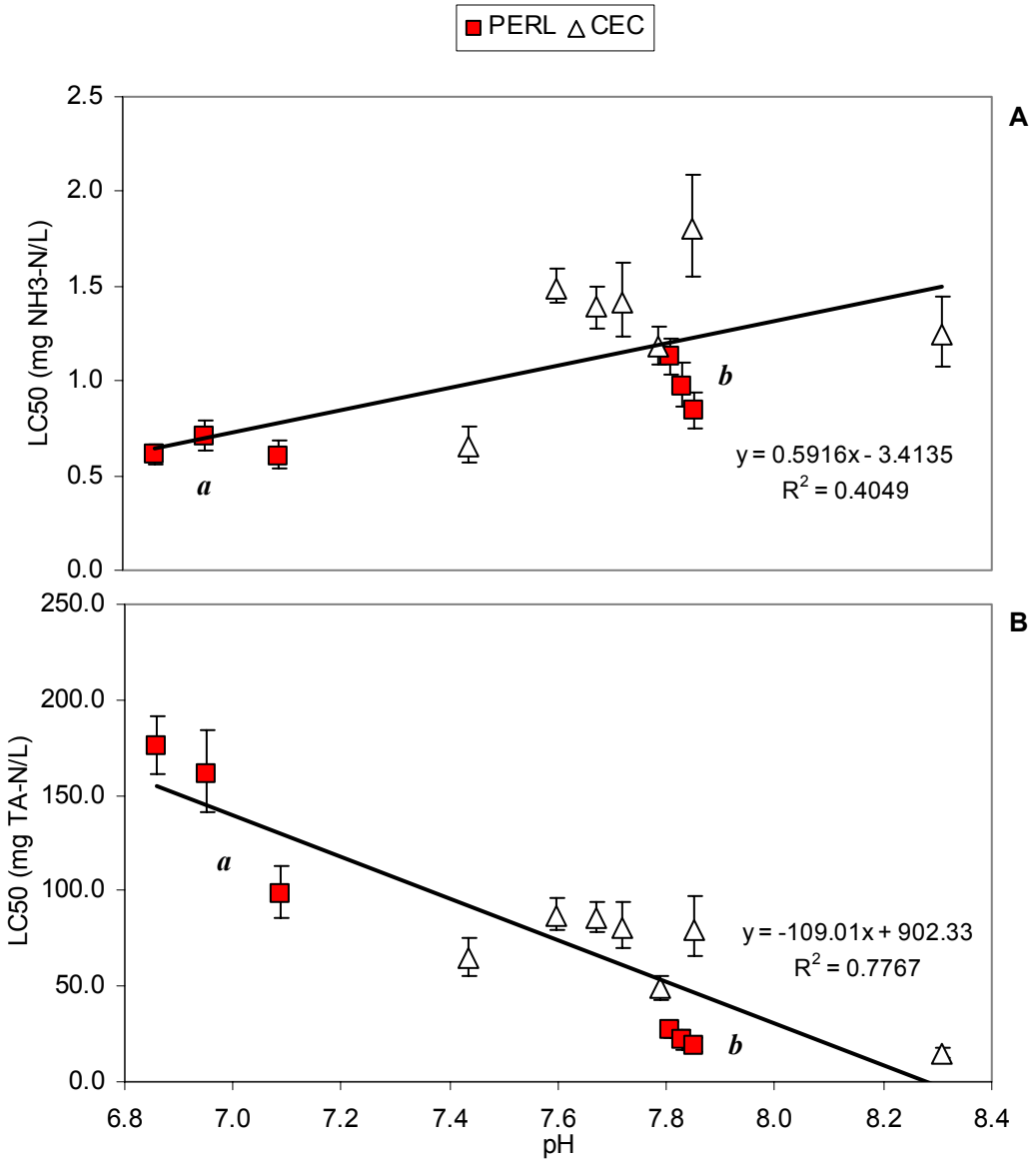

Figure 4-5. Relationship between *C. dubia* toxicity expressed as un-ionized ammonia (A) and total ammonia B) and pH. Significant regressions are identified by trendline, significant differences between means are identified by lower case letter.

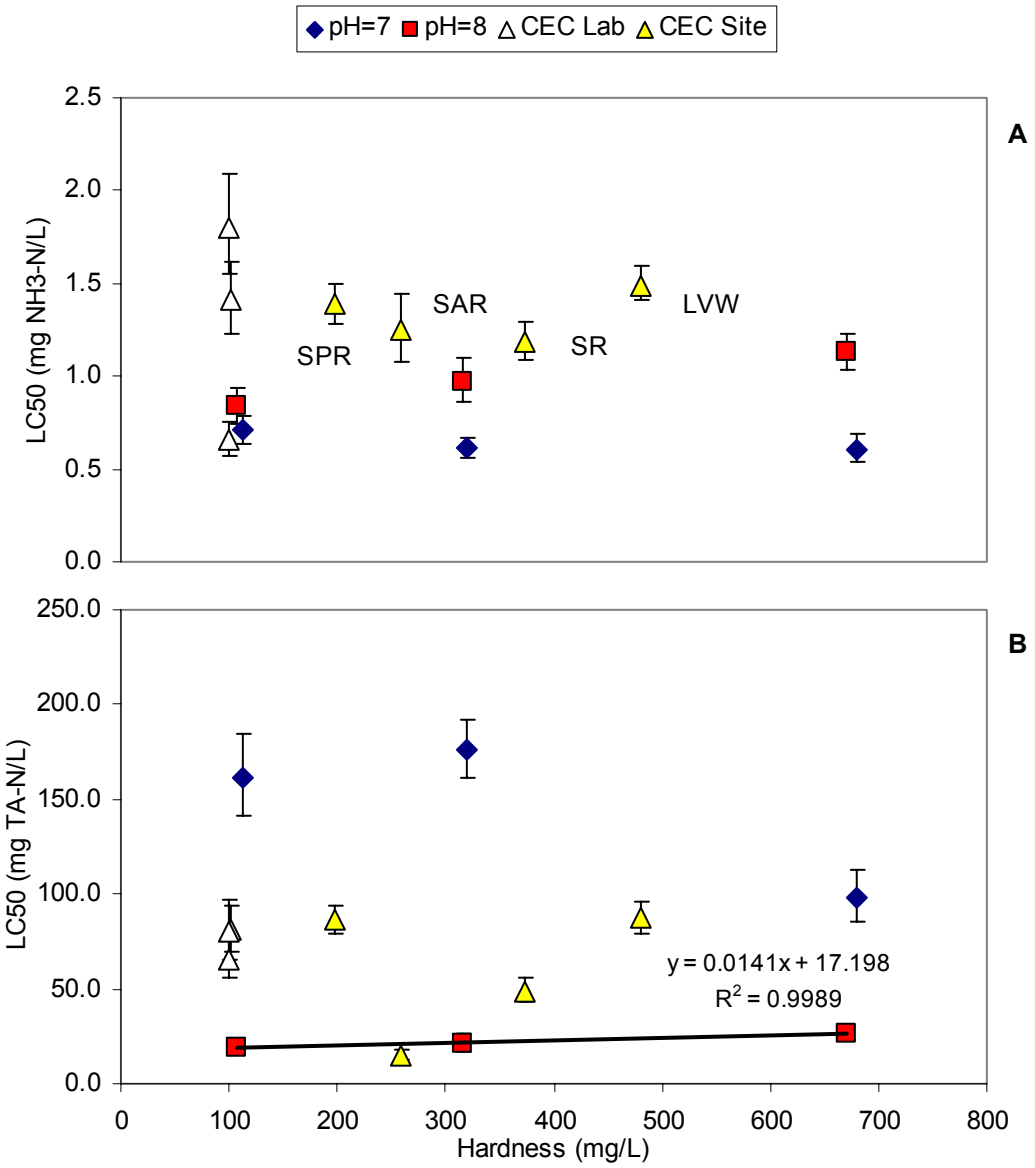

Figure 4-6. Relationship between *C. dubia* toxicity expressed as un-ionized ammonia (A) and total ammonia (B) and hardness at various pH levels for lab and site waters. Significant regressions are identified by trendline, significant differences between means are identified by lower case letter.

4.1.4 *H. azteca*

Unlike fish, *H. azteca* ammonia toxicity decreased significantly with increasing pH when expressed on an un-ionized basis (P = 0.001, Table 4-4, Figure 4-7 A). As such, the mean un-ionized ammonia LC50 observed at pH 7 was significantly lower than that observed at pH 8 (P = 0.002). There was no significant trend observed between pH and *H. azteca* total ammonia LC50 values either as a regression relationship (P = 0.10, Figure 4-7 B), or as a difference among means (P = 0.10).

In contrast to *C. dubia*, the only significant regression observed was that *H. azteca* total ammonia LC50 values decreased slightly with increasing hardness at pH 8 (P = 0.04, Figure 4-8 A, B). Additionally, there were no significant differences detected among the mean LC50s values (expressed as un-ionized or total ammonia) measured at the three hardness levels (Figure 4-8 A, B).

Table 4-4. Ammonia toxicity test results for *H. azteca*

	Measured		LC50 (with 95% CI)	
Testing Laboratory	**pH**	**Hardness**	**Total NH_3 (mg N/L)**	**Un-ionized NH_3 (mg NH_3-N/L)**
PERL	6.95	660	92.50 (69.83 - 122.52)	0.50 (0.39 - 0.65)
	7.01	324	125.03 (110.35 - 141.67)	0.75 (0.66 - 0.84)
	7.00	112	90.21 (75.19 - 108.23)	0.55 (0.46 - 0.65)
	7.62	672	57.15 (44.57 – 73.27)	1.21 (1.10 - 1.33)
	7.62	324	75.71 (59.78 – 95.88)	1.37 (1.21 - 1.56)
	7.54	104	84.91 (72.26 – 99.78)	1.29 (1.23 - 1.34)

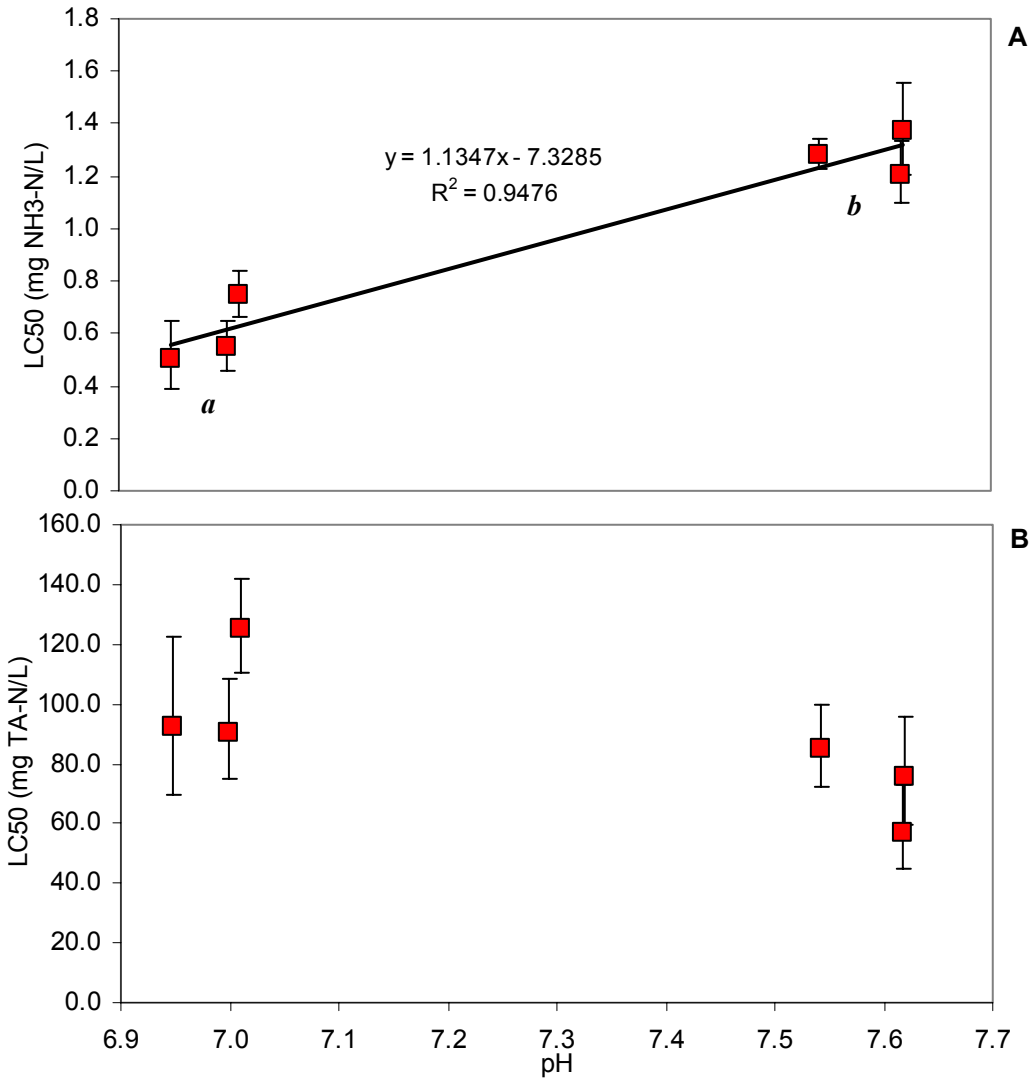

Figure 4-7. Relationship between *H. azteca* toxicity expressed as un-ionized ammonia (A) and total ammonia (B) and pH. Significant regressions are identified by trendline, significant differences between means are identified by lower case letter.

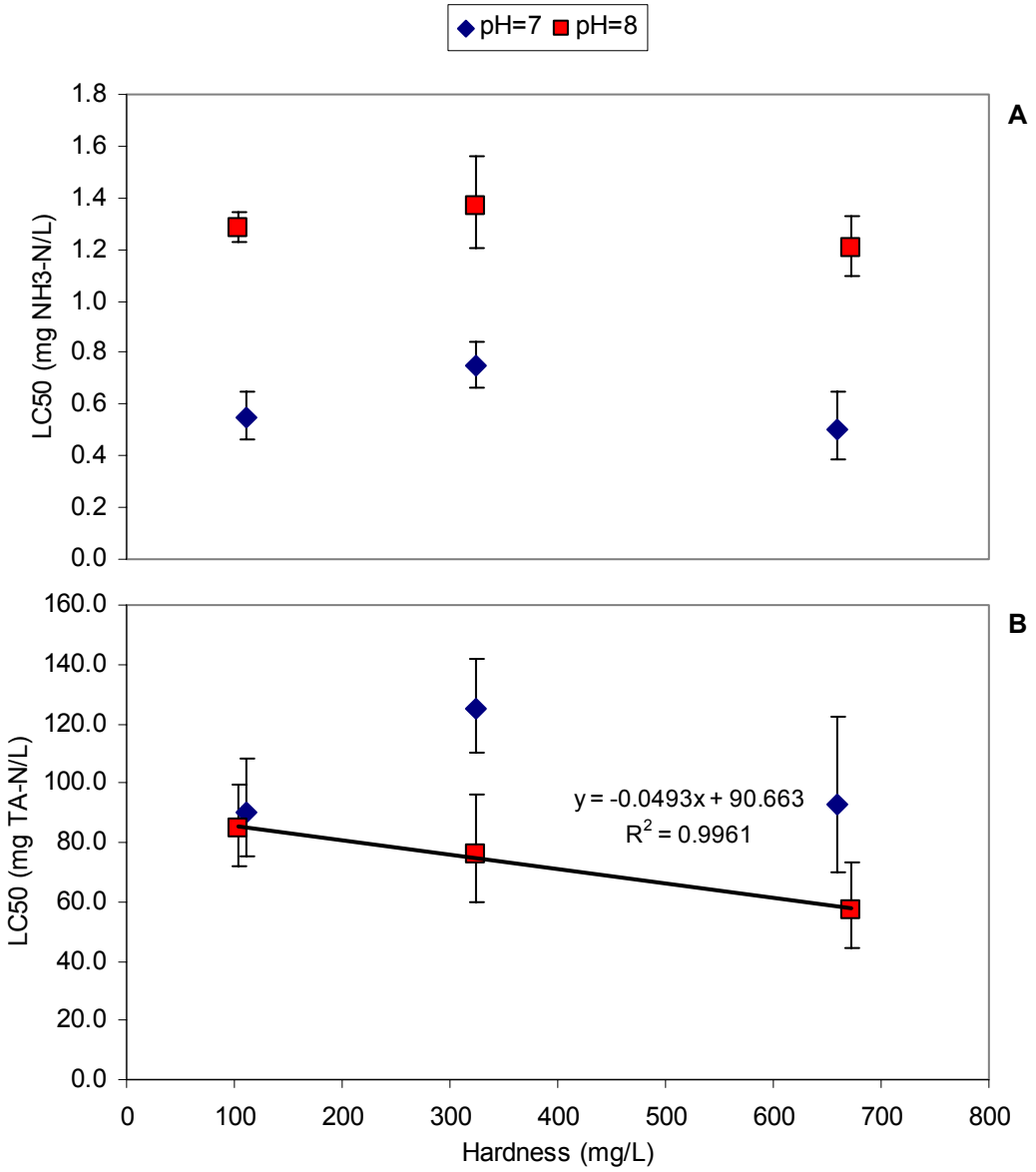

Figure 4-8. Relationship between *H. azteca* toxicity expressed as un-ionized ammonia (A) and total ammonia (B) and hardness at various pH levels. Significant regressions are identified by trendline, significant differences between means are identified by lower case letter.

4.1.5 *C. tentans*

There were no significant correlations between *C. tentans* ammonia toxicity, expressed as un-ionized or total) and pH (Figure 4-9 A, B) or hardness (Figure 4-10 A, B).

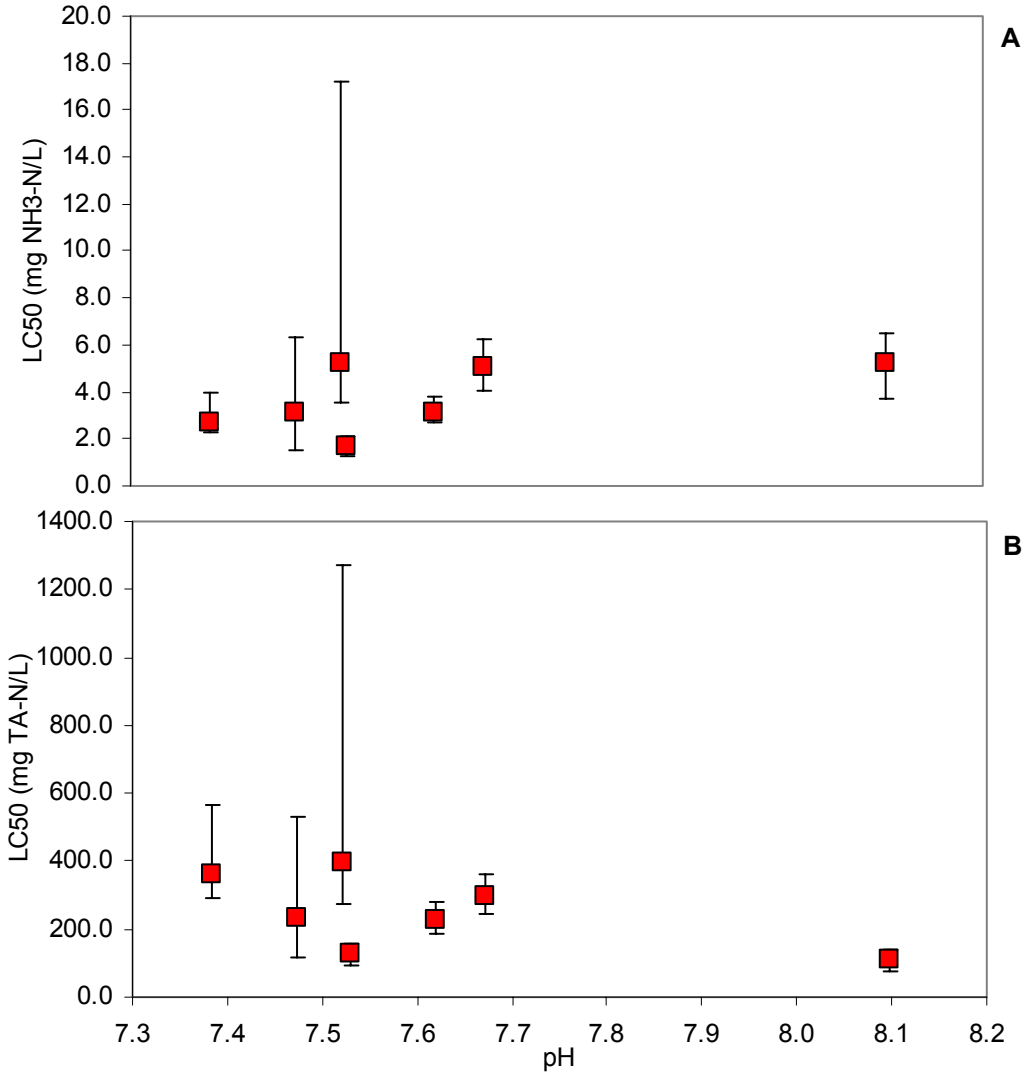

Figure 4-9. Relationship between *C. tentans* toxicity expressed as un-ionized ammonia (A) and total ammonia (B) and pH. Significant regressions are identified by trendline.

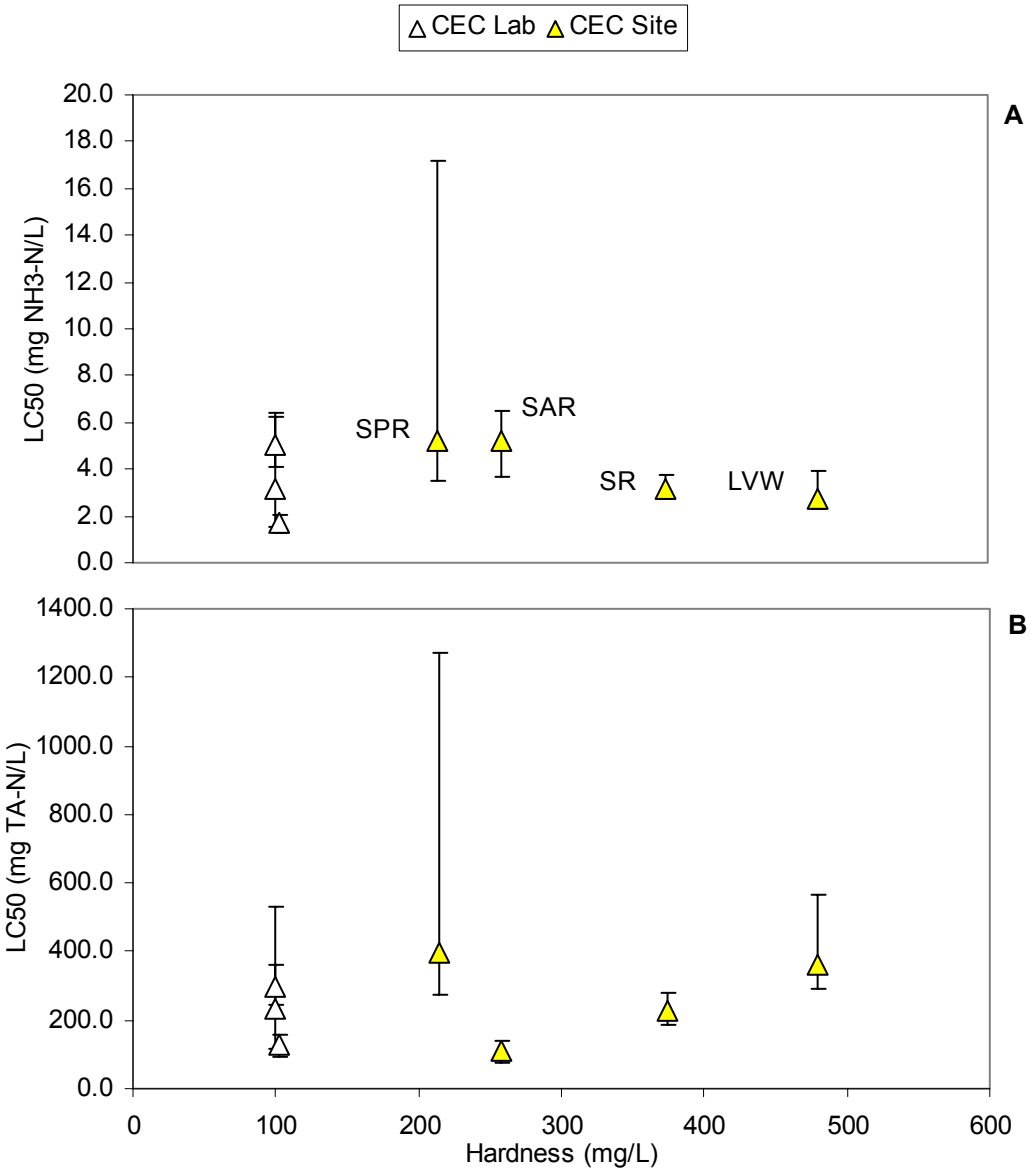

Figure 4-10. Relationship between *C. tentans* toxicity expressed as un-ionized ammonia (A) and total ammonia (B) and hardness for lab and site waters. Significant regressions are identified by trendline.

4.2 WER STUDIES

The WERs derived and measured hardness for each of the four sites are given below. No correlation was found between site water hardness and WERs.

4.2.1 South Platte River

The South Platte River had the lowest hardness of the site waters being tested, ranging from 198-214 mg/L $CaCO_3$. The WERs for this river were 1.1 for total ammonia and 1.0 for un-ionized ammonia, in the *C. dubia* tests (Table 4-3, Figure 4-11). The WERs in the fathead minnow tests were 2.2 for total and 1.8 for un-ionized ammonia (Table 4-1), and the *C. tentans* WERs were 3.1 and 3.0 for total and un-ionized ammonia, respectively (Table 4-5).

4.2.2 Santa Ana River

The Santa Ana River had a hardness of 258 mg/L $CaCO_3$. The WERs for this river were 0.2 for total ammonia and 1.9 for un-ionized ammonia, in the *C. dubia* tests (Table 4-3, Figure 4-11). The WERs in the fathead minnow tests were 0.5 for total and 2.2 for un-ionized ammonia (Table 4-1), and the *C. tentans* WERs were 0.5 and 1.7 for total and un-ionized ammonia, respectively (Table 4-5).

4.2.3 Salt River

The Salt River had a hardness of 374 mg/L $CaCO_3$. The WERs for this river were below one at 0.6 for total ammonia and 0.8 for un-ionized ammonia, in the *C. dubia* tests (Table 4-3, Figure 4-11). The WERs in the fathead minnow tests were very close to one at 1.1 for total and 1.2 for un-ionized ammonia (Table 4-1), and the *C. tentans* WERs were 1.7 and 1.8 for total and un-ionized ammonia, respectively (Table 4-5).

4.2.4 Las Vegas Wash

The Las Vegas Wash had a hardness of 480 mg/L $CaCO_3$. The WERs for this river were very close to one at 1.1 for total ammonia and 0.8 for un-ionized ammonia, in the *C. dubia* tests (Table 4-3, Figure 4-11). The WERs in the fathead minnow tests were 1.8 for total and 1.9 for un-ionized ammonia (Table 4-1), and the *C. tentans* WERs were 1.2 and 0.5 for total and un-ionized ammonia, respectively (Table 4-5).

Table 4-5. Ammonia toxicity test results for *C. tentans*

		Measured		Total Ammonia		Un-ionized Ammonia	
Testing Laboratory	**Test Conditions**	**pH**	**Hardness**	**LC50 (mg TA-N/L with 95% CI)**	**WER**	**LC50 (mg NH_3-N/L with 95%CI)**	**WER**
CEC	SPR, SR – lab*	7.53	102	129.3 (92.5 – 159.1)	-	1.72 (1.24 – 2.09)	-
	LVW – lab† ‡	7.67	100	296.2 (242.9 – 361.3)	-	5.04 (4.08 – 6.22)	-
	SAR - lab†	7.47	100	231.9 (116.2 – 533.4)	-	3.14 (1.55 – 6.37)	-
	SPR - site	7.52	214	397.6 (273.8 – 1269.9)	3.1	5.20 (3.52 – 17.20)	3.0
	SR - site	7.62	374	225.0 (188.0 – 281.0)	1.7	3.15 (2.71 – 3.78)	1.8
	LVW - site	7.38	480	359.6 (289.1 – 565.4)	1.2	2.72 (2.30 – 3.94)	0.5
	SAR – site*	8.10	258	109.8 (78.1 – 138.0)	0.5	5.19 (3.68 – 6.50)	1.7

*Tests possibly invalid due to poor control performance (tests did not contain any concentrations in which 90% survival occurred). LC_{50} calculations were determined with concurrently run controls from other tests.

‡In the 120 mg TAN/L nominal concentration, there was only 65% survival, while the next highest concentration contained 67.5% survival. The lower concentration was removed because there was greater survival shown at the next greatest concentration.

†Controls had <90% survival – yet, the next highest concentration had >90% survival. Word *et al.* (2002) reported ammonia hormesis may be the cause of poor survival in toxicity testing controls that are not spiked with ammonia for *Hyalella* (i.e., controls may perform poorly compared to low-level ammonia treatments due to the lack of ammonia). Since this may also be the case in this study, we believe it would be reasonable to accept a toxicity test in which survival of the lowest test concentration, spiked with low levels of ammonia, exhibited at least 90% survival.

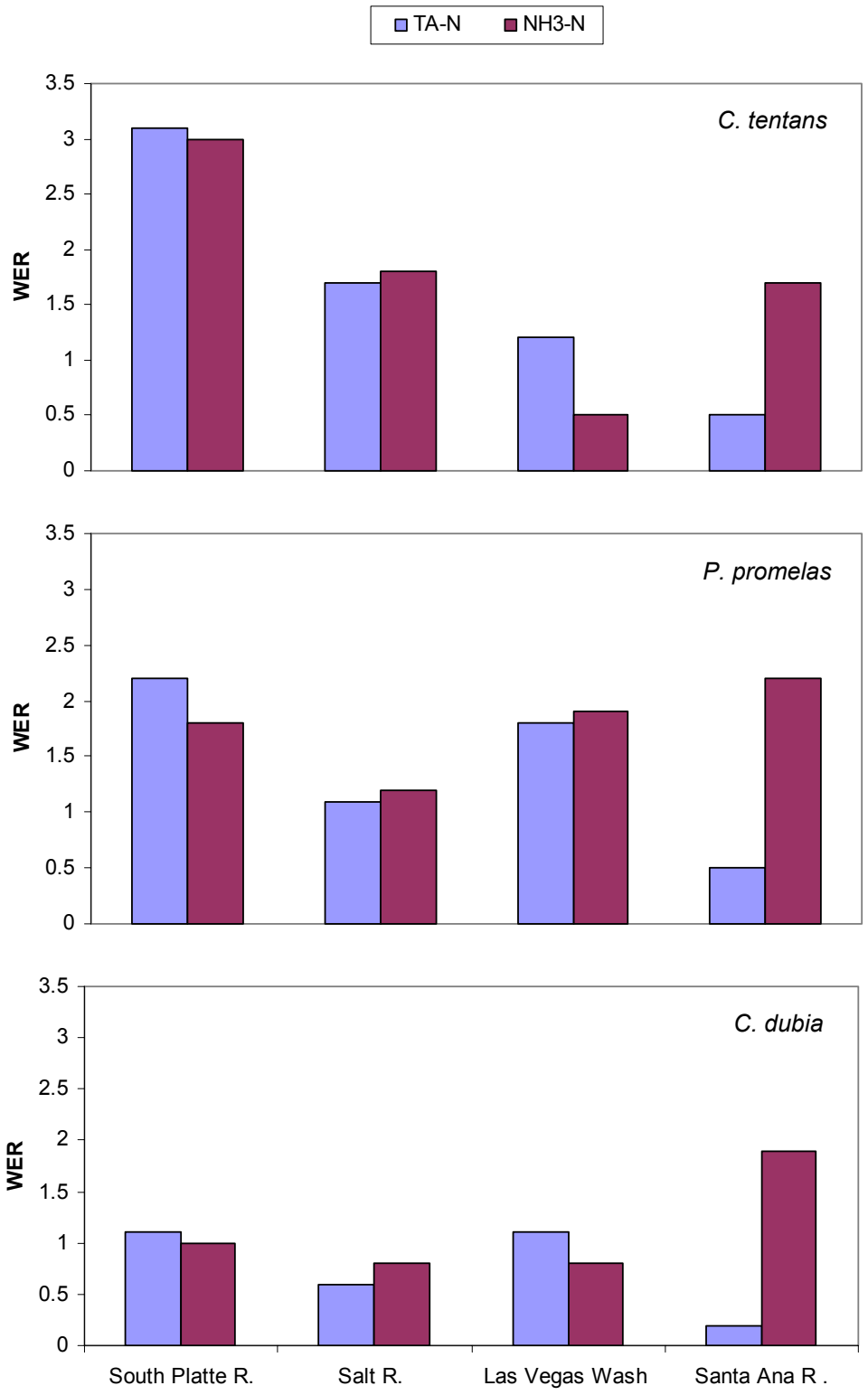

Figure 4-11. Total ammonia and un-ionized ammonia water effect ratios (WERs) for *C. tentans*, *P. promelas*, and *C. dubia*

5. DISCUSSION

5.1 pH-AMMONIA RELATIONSHIP

For both fish species examined, ammonia toxicity was relatively constant with increasing pH when expressed on an un-ionized basis. In contrast, ammonia toxicity significantly increased with pH when expressed on the basis of total ammonia-N. These results are consistent with those from previous studies with channel catfish (Tomasso *et al.* 1980), fathead minnow and rainbow trout (USEPA 1999), that the effect of pH on ammonia toxicity in fish is best explained by the pH-dependent speciation of un-ionized ammonia (i.e., these taxa respond to the more toxic, un-ionized form of ammonia). This relationship is likely a function of the mechanism of ammonia transfer across epithelial membranes in fish. Unlike the charged ammonium ion, which requires an active transport mechanism, the neutral un-ionized ammonia molecule can readily diffuse across epithelial cells causing ammonia concentrations to increase in gill tissue and in the blood (Evans and Cameron 1986).

In contrast, for both invertebrate species tested, ammonia toxicity (expressed on an un-ionized basis) decreased with increasing pH. These results suggest that the toxicity of ammonia to invertebrates may, in fact, be best explained by a joint model wherein both the ionized and un-ionized fractions play an important role in ammonia toxicity. This conclusion agrees with those made by Ankley (1995) and Borgman (1994) who suggested that, because both acute and chronic toxicity was more constant when expressed as total rather than un-ionized ammonia, *H. azteca* were responding predominantly to the ionized form of ammonia.

5.2 HARDNESS-AMMONIA RELATIONSHIP

No significant relationships were observed between hardness and the toxicity of ammonia to either of the fish species examined. These findings contradict the conclusions of several physiological studies, that an ammonia/hardness relationship does exist as evidenced by an increase in ammonia excretion with increasing hardness (Yesaki and Iwama 1992, Iwama *et al.* 1997).

Several factors distinguish these physiological studies from the acute toxicity tests conducted for this project, however. First, the physiological experiments were conducted at ambient ammonia concentrations much lower than those tested in the acute toxicity tests. Second, the physiological studies were conducted in natural waters where the ionic composition was likely very different from that of the acute toxicity test waters used in the present study in which calcium and magnesium where the only ionic constituents manipulated. This is particularly important given the strong evidence of a direct link between various ions (Na^+, H^+ and Cl^-) and ammonia excretion (Evans and Cameron 1986, Wilson *et al.* 1994). Finally, even though a relationship between hardness and ammonia excretion may exist under ambient conditions in natural waters, this condition may not necessarily elicit a toxicological response.

For the invertebrate species tested, the only significant hardness/ammonia toxicity relationships observed were that at pH 8, ammonia toxicity increased with increasing hardness for *H. azteca* and decreased with increasing hardness for *C. dubia* when expressed on the basis of total ammonia-N. These results were not in agreement with Ankley *et al.* (1995), who concluded that total ammonia toxicity decreased with increasing hardness across a pH range of 6.5 – 8.5. Several differences in experimental design may help explain why we were unable to repeat the hardness trends observed by Ankley *et al.* (1995). First, whereas hardness in the acute toxicity tests conducted for this project ranged from 104 – 680 mg/L as

$CaCO_3$, Ankley *et al.* only tested hardness levels up to 240 mg/L as $CaCO_3$. Despite the fact that two of the hardness levels tested in our studies (100 and 300 mg/L $CaCO_3$) were close to or overlapped with those tested by Ankley *et al.* (100 and 240 mg/L as $CaCO_3$), we were still unable to detect significant regressions or differences between the mean LC50s of these hardness levels that paralleled their findings.

The Ankley *et al.* (1995) studies were also confounded by the fact that alkalinity (and, likely, sodium) co-varied with hardness, while alkalinity was held constant in the acute toxicity tests conducted for this project. Given the suggestion that elevated external Na^+ concentrations may enhance the ability of *H. azteca* to actively exchange NH_4^+, and an increase in anionic strength (alkalinity – OH^- and HCO_3^-) may also facilitate the exchange of H^+ and NH_3, the differences in alkalinity adjustment between this and Ankley *et al.*'s study may explain the corresponding disparity in the hardness/ammonia toxicity relationships observed. This is because in our studies, hardness was independently manipulated and alkalinity and sodium were held constant, and in Ankley *et al.*'s studies, alkalinity and sodium concentrations increased with hardness. Therefore, our inability to identify a hardness/ammonia toxicity relationship for *H. azteca* suggests that alkalinity and/or sodium, rather than hardness cations, could have been responsible for the trends Ankley *et al.* observed.

To further investigate this hypothesis, a series of acute *H. azteca* studies were conducted wherein sodium was independently manipulated in conjunction with hardness and alkalinity (Parametrix 2006). Four reconstituted waters were made in which sodium concentrations were determined either by direct addition of sodium chloride (NaCl) or as a result of increasing alkalinity (sodium addition as sodium bicarbonate – $NaHCO_3$). In the first study, hardness and alkalinity were maintained at 100 and 70 mg/L as $CaCO_3$, respectively. The natural amount of sodium associated with this alkalinity (32.2 mg/L) was not altered in this first experiment. Hardness and alkalinity were held at the same levels in the second study as the first; however, 190 mg/L sodium (as NaCl) was added to mimic the natural amount of sodium associated with the alkalinity of the third study (420 mg/L), in which hardness was maintained at 100 mg/L as $CaCO_3$. The fourth study was identical to the third, except that hardness was increased to 600 mg/L as $CaCO_3$. The target pH of all four studies was 8.

Table 5-1. Results of acute *H. azteca* sodium studies (Parametrix 2006)

	Nominal Na (mg/L)	Hardness (mg/L as $CaCO_3$)	Alkalinity (mg/L as $CaCO_3$)	TA-N LC50 (mg N/L with 95% CI)	UA-N LC50 (mg NH_3-N/L with 95% CI)
1.	32.2	104	72	58.4 (48 – 71.1)	1.9 (1.6 – 2.1)
2.	193.1*	108	78	102.1 (83.8 – 124.5)	2.2 (1.9 – 2.4)
3.	193.1	112	376	65.8 (57 – 75.9)	2.1 (1.9 – 2.4)
4.	193.1	570	360	146.2 (126.1 – 169.5)	3.4 (3.0 – 3.8)

* Sodium concentration due to NaCl addition. All other sodium concentrations due to alkalinity (as $NaHCO_3$).

Comparing the results of the first and fourth studies, total ammonia toxicity decreased significantly with increasing hardness. This was the same trend observed by Ankley *et al.* and, thus, we can support the hypothesis that increasing hardness will only decrease ammonia toxicity to the amphipod when accompanied by a concurrent increase in sodium along with alkalinity. Furthermore, these studies also suggest that increasing sodium as NaCl alone (i.e. without also increasing hardness) also decreases ammonia toxicity, although increasing

sodium as alkalinity (i.e. without also increasing hardness) does not affect ammonia toxicity. It is clear from these studies that elevated sodium levels offer considerable protection to *H. azteca* against ammonia toxicity, especially when coupled with elevated hardness. However, it is not yet apparent how sodium may influence the ammonia/hardness relationship at other pH levels, intermediate hardness concentrations, or for other species. Therefore, additional research is needed to confirm the role of sodium in controlling acute ammonia toxicity in very hard or ion rich waters.

The negative relationship observed between hardness and total ammonia toxicity for *C. dubia* at pH 8 was also not in agreement with Sarda (1994) who found the toxicity of total ammonia to *C. dubia* was higher in a natural hard water (364 mg/L as $CaCO_3$) when compared to a reconstituted hard water (180 mg/L as $CaCO_3$), and a moderately hard water (100 mg/L as $CaCO_3$). However, while the observed hardness/toxicity regression was significantly greater than zero, there were no significant differences detected among the mean LC50s calculated at the three hardness levels, and so the effect we observed was relatively minor. Furthermore, the ionic composition of the waters used in Sarda (1994) is unknown, and so it is also as yet not possible to determine whether other ions such as sodium may influence relationships between hardness and acute toxicity in *C. dubia*.

5.3 WER STUDIES

WERs, expressed as total ammonia, were fairly consistent among species. In particular, fathead minnow WERs generally ranged from 0.5 – 2 among all sites, WERs were consistently highest for *C. tentans* among all sites (0.5 – 3), and WERs for *C. dubia* were ≤ 1 for all sites. It is noteworthy, however, that the LC50s of the lab water *C. dubia* tests were unusually high (65 – 81 mg TA-N/L), when compared to the updated database Species Mean Acute Value (SMAV; 22.2 mg TA-N/L @ pH 8). Thus, the elevated lab water LC50s may have artificially lowered the WERs below what would be expected for *C. dubia*. Given the fact that the LC50s of the PERL *C. dubia* tests conducted at pH 8 were very similar to the SMAV (19 – 27 mg TA-N/L), and the only difference between these tests and the acute toxicity tests conducted by CEC was the use of sodium, rather than potassium, hydroxide for pH adjustment, this discrepancy in LC50s may have been due to the presence of elevated potassium levels in the WER studies. This finding further supports the hypothesis that cations other than Ca^{2+} and Mg^{2+} may influence the acute toxicity of ammonia to invertebrates.

WERs, expressed as total ammonia, were also fairly consistent among sites. The highest WERs were generally found in the South Platte River, the lowest WERs were generally found in the Santa Ana River. The Salt River and Las Vegas Wash WERs were intermediate. WERs at these sites were not a function of hardness given that the South Platte River had the lowest hardness (198-214 mg/L $CaCO_3$), the Santa Ana River had the second lowest hardness (258 mg/L $CaCO_3$), and the Salt River and Las Vegas Wash had the two highest hardness values measured at any of the sites (374 and 480 mg/L $CaCO_3$, respectively).

However, as previously discussed, other water quality parameters (i.e., alkalinity and sodium) may affect the toxicity of ammonia in natural waters; thus, the lack of a clear relationship between hardness and the WERs measured at these sites may be due to the fact that some other factor(s) was contributing more heavily to the toxicity of ammonia to the species tested. Alkalinity trends in the site waters did not explain the WER patterns observed, however, as the Santa Ana River (lowest WER) had the highest measured alkalinity (204 mg/L) and the South Platte River (highest WER) had the lowest measured alkalinity (126 mg/L). This is the exact opposite of what would be expected if, as hypothesized, an increase in anionic strength facilitates the exchange of H^+ and NH_3, thereby decreasing toxicity. In addition pH

differences between lab and site waters may also have been large enough to significantly affect the WERs, especially at the Santa Ana River.

To evaluate the potential role of sodium in helping explain WER results, we examined sodium concentrations measured for same site waters for the copper BLM validation study (Parametrix 2005) because analytical data were unavailable for waters used in the present study. Similar to alkalinity, sodium concentrations measured in these site waters several months prior also did not adequately explain the WER trends. The Salt River and Las Vegas Wash (intermediate WERs) had the highest measured sodium concentrations, while the Santa Ana and South Platte Rivers (lowest and highest WERs, respectively) had the lowest measured sodium concentrations. These trends are also counterintuitive given the hypothesis that elevated external sodium concentrations enhance the active transport of the ammonium ion, thereby decreasing toxicity. Under this assumption, the Salt River and Las Vegas Wash WERs should have been the highest, and the South Platte River WER should have been the lowest. However, these Copper BLM study sodium measurements are likely very different than what would have been measured at the time the ammonia WER studies were conducted, and so these conclusions should be viewed with caution, and should be validated by further study.

5.4 CONCLUSIONS & FUTURE RESEARCH

This study has supported the limited toxicity literature available which suggests that hardness (and/or related cations) may influence acute ammonia toxicity. However, these effects are species-specific, (i.e., no one ion composition will exert the same influence) and at this time appear to only be valid for invertebrates, not fish. Although we were able to identify hardness-toxicity relationships for *C. dubia* and *H. azteca*, major ion composition other than hardness needs independent experimental manipulation to further elucidate the mechanisms governing these effects. The influences of sodium and potassium on acute ammonia toxicity are of particular interest given the results of the invertebrate studies from both the present study and from Parametrix (2006).

The current AWQC for ammonia (USEPA 1999) suggests that ammonia WERs >1 are unlikely unless there is an interaction with other pollutants, a difference in ionic composition in conjunction with pH or hardness, or if WERs are derived at pH <6.5 or pH >9.0. While it is impossible to rule out an interaction with other pollutants, this study has shown that WERs >1 are possible in effluent-dependent waters of pH >6.5 and pH <9.0 for both fish and invertebrates. The WERs found to be >1 may have been the result of a difference in ionic composition between the site and laboratory waters, but it is clear that the protective effect associated with these significant WERs was not due to hardness cations alone. Therefore, until ion effects and/or mechanisms are better understood, empirical tests would be recommended for a particular site prior to undertaking a full WER study.

Based on the results of these studies and their relation to those found in the toxicity literature, there are a number of issues that would benefit from future research. First, in order to more fully understand the effects of ionic composition on acute ammonia toxicity, a series of additional experiments are needed. In these tests, major ions, such as sodium and potassium, would be independently manipulated and the effects of these ions on ammonia toxicity compared with the effects of hardness on ammonia toxicity. The results of these additional studies may help us better understand the trends observed in this project, as well as any differences or similarities between those reported by other researchers. The acute *H. azteca* sodium studies conducted by Parametrix (2006) represent a start to this research, but to more fully understand the role of sodium on the hardness/ammonia toxicity relationship, more testing at additional pHs and with additional species is needed.

Second, evaluating the major ion composition of the lab and site waters tested, especially sodium and potassium versus calcium and magnesium, may help us to better interpret WER results. In future WER studies, detailed water quality data are needed to help determine what ionic constituents were most likely responsible for the species and site-specific WER trends that might be observed. Additional studies with both fish and invertebrates would be useful, and these same four waters would benefit from repeat study owing to the variety of hardness levels present at these sites, and because a range of WERs for both fish and invertebrates were observed in the present study.

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APPENDIX A

Native Species Selection Efforts

Based on review of resident species in arid West streams (PCWWM 2005), common taxa also potentially present in Colorado were chosen - specifically, the mayflies *Tricorythodes minutus* and *Callibaetis* sp., the black fly *Simulium* sp., the caddisfly *Hydropsyche* sp., and damselflies Coenagrionidae. Sixteen sites in the Cherry Creek, Plum Creek, and Monument Creek drainages (Table A-1) were surveyed for possible source populations of aquatic insects for use in ammonia bioassays.

At each site, habitat conditions were assessed (e.g., flow, submerged and emergent aquatic vegetation) to determine if the site might be suitable for test organism collection. A water sample was also collected from acceptable sites in a cubitainer® for analysis of total ammonia to determine the potential of pre-exposure.

At each potentially acceptable site, except on West Plum Creek at Dakan Road because of current road improvement activities, a subsample of the sweep sample was placed in a container and preserved in ethanol to verify field identifications (Table A-2). An additional community sample was collected at the Site West Plum Creek at Red Rock Road, although this site was later rejected due to the lack of *T. minutus* at this site (Table A-2).

Based on these initial collections, four sites were chosen as possible locations for collection of organisms for future ammonia bioassays, including West Plum Creek at Jackson Creek Road, West Plum Creek at Dakan Road, East Plum Creek at Plum Creek Drive in Larkspur, and Cherry Creek at State Highway 86 in Franktown (Table A-2). Water samples collect from these sites had negligible ammonia concentrations. Sites that were not deemed acceptable were rejected due to poor habitat and lack of appropriate selected aquatic insects.

Table A-1. Potential native species sampling site screening results.

Site	Decision	Bug Sample to be taken?
Upper Lake Gulch at Garton Road	Rejected	N
West Cherry Creek at Greenland Road	Rejected	N
East Cherry Creek at Russelville Road	Rejected	N
West Cherry Creek at SH83 near Russelville Road	Rejected	N
Cherry Creek at SH86 in Franktown	Acceptable (#4)	Y
* N39°23.539' W104°45.680'		
Cherry Creek at Scott Road	Rejected	N
West Plum Creek at Pine Cliff Road	Rejected	N
West Plum Creek at Jackson Creek Road	Acceptable (#1)	Y
* N39°20.845' W104°58.257'		
West Plum Creek at Dakan Road	Acceptable (#2)	N
* GPS coordinates not taken		
West Plum Creek at Red Rock Road	Rejected	Y
East Plum Creek at SH105 (=Perry Park Road)	Rejected	N
Cook Creek at SH105 (=Perry Park Road)	Rejected	N
North Monument Creek at Palmer Lake	Rejected	N
Monument Creek at FR320 in Monument	Rejected	N
Carpenter Creek at Spruce	Rejected	N
East Plum Creek at Plum Creek Drive in Larkspur	Acceptable (#3)	Y
* N39°13.699' W104°53.010'		

*GPS coordinates are listed for “acceptable sites”, below sites to which they pertain

Table A-2. Organisms collected in qualitative sweep samples or observed at sites in Plum Creek and Cherry Creek drainages, 12 July 200

Species	W. Plum Cr. @ Jackson Cr. Rd.	E. Plum Cr. @ Larkspur	Cherry Cr. @ Franktown	W. Plum Cr. @ Red Rock Rd.
Ephemeroptera				
Ephemerella dorothea	X			
Baetis tricaudatus	X		X	
Fallceon quilleri				X
Tricorythodes minutus	X	X	X	
Caenis sp.		X		
Plecoptera				
Isoperla sp.			X	
Sweltsa sp.			X	
Odonata				
Ophiogomphus severus	X		X	
Aeschna sp.		X		
Argia sp.	X			
Hemiptera				
Aquarius remigis		X		
Ambryssus mormon				X
Trichoptera				
Leptoceridae	X			
Cheumatopsyche sp.	X		X	X
Hydropsyche sp.			X	
Coleoptera				
Peltodytes sp.		X		
Dytiscidae	X			
Diptera				
Chironomidae	X	X	X	X
Tipula sp.	X	X	X	
Simulium sp.			X	X
Ceratopogininae	X			
Crustacea				
Gammarus sp.		X		
Caecidotea sp.		X		
Orconectes immunis				X
Annelida				
Erpobdella punctata		X		X
Eiseniella tetraedra			X	
Mollusca				
Physa sp.		X		
Sphaerium sp.		X		
Anura				
Rana sp.	X			X
Pisces				
Rhinichthys sp.				X
Pimephales promelas				X
Samotilus atromaculatus	X			X