UNIVERSITY OF CALIFORNIA, DAVIS

BERKELEY • DAVIS • IRVINE • LOS ANGELES • MERCED • RIVERSIDE • SAN DIEGO • SAN FRANCISCO SANTA BARBARA • SANTA CRUZ

SCHOOL OF VETERINARY MEDICINE
ANATOMY, PHYSIOLOGY AND CELL BIOLOGY
UNIVERSITY OF CALIFORNIA
(530) 752-1174
FAX (530) 752-7690

ONE SHIELDS AVENUE
DAVIS, CALIFORNIA 95616-8734

FINAL REPORT

Full Life-Cycle Bioassay Approach to Assess Chronic Exposure of *Pseudodiaptomus forbesi* to Ammonia/Ammonium

Submitted to:

Chris Foe and Mark Gowdy
State Water Board / UC Davis Agreement No. 06-447-300
SUBTASK No. 14

By

Swee Teh, Ida Flores, Michelle Kawaguchi, Sarah Lesmeister, and Ching Teh
Aquatic Toxicology Program
Department of Anatomy, Physiology, and Cell Biology
School of Veterinary Medicine
University of California-Davis, Davis CA 95616

Date: August 31, 2011

Executive Summary

This study investigates the effects of ammonia to the calanoid copepod *Pseudodiaptomus forbesi* using a full-life cycle bioassay approach. The overall objectives of this study are to 1) investigate the acute and chronic toxic effects of ammonia on the estuarine copepod *P. forbesi*, and 2) determine whether environmentally relevant concentrations of ammonia in the SFE potentially contribute to the decline in the abundance of *P. forbesi*. This study was initiated based on available information on *P. forbesi* abundance and average pH and temperature levels in the Cache Slough regions. Two studies were conducted on acute ammonia toxicity using pH 7.4 and 7.8 at 20^{o}C. Because pH and temperature can modulate the toxicity of the ionized (IA) and unionized (UIA) forms of ammonia, the toxicity of both fractions was evaluated as a function of pH. Importantly, a full life-cycle static-renewal bioassay was conducted to estimate the effects of total ammonia concentrations on the growth and reproduction of *P. forbesi*. This assay assessed the chronic effects of total ammonia by using environmentally relevant concentrations measured from several locations in the Sacramento Rivers and Cache Slough region. The sensitivity of 3 day-old nauplii stages of *P. forbesi* to ammonia was also tested following hatching from gravid females previously exposed to ammonia. This study demonstrates the adverse effects of ammonia, as exacerbated by pH levels, on the growth, reproduction, and survival of parents and progenies of *P. forbesi*.

Results:

1. In Task 3-1A and Task 3-1B, time to 50% mortality of *P forbesi* was approximately 1.75 times faster at pH 7.4 and 20^{o}C (76 hr) at 5.47 mg/L total ammonia nitrogen (TAN) when compared to pH 7.8 (135 hr) at 5.25 mg TAN /L. The 4d-LC50 was 2.96 mg TAN /L and 0.029 mg UIA/L at pH 7.4 and the 6d-LC50 was 6.014 mg TAN /L and 0.150 mg UIA /L at pH 7.8. These results suggest that *P. forbesi* is more sensitive to TAN at low pH**.**

2. In Task 3-2, time to 50% mortality of *P forbesi* was faster at pH 8.6 (66 hr) than pH 8.2 (87 hr) at 3.71 mg TAN /L. The 4d-LC50 was 0.303 mg UIA/L at 20 ^{o}C. These results suggest that *P. forbesi* sensitivity to a constant TAN concentration increases with increasing pH once the toxic threshold level of UIA is reached.

3. In Task 3-3, there is a dose-response relationship of ammonia exposure and the number of nauplii, juvenile, and adult *P. forbesi* production in the chronic 31-d study at pH 7.8 and 20^{o}C. A 31-d life-cycle study indicated that gravid females either produced significantly lower numbers of nauplii and juveniles or survival of nauplii and juveniles were significantly lower when exposed to 0.79, 1.62, and 3.36 mg TAN/L compared to both the control and the 0.36 mg TAN/L treatment. In addition, significant differences in mean total number of adult *P. forbesi* production were observed between the treatment groups (0.36, 0.79, 1.62, and 3.36 mg TAN/L) and control ($P<0.05$). These results demonstrate that ammonia significantly impacts populations of *P. forbesi* as analyzed by one-way ANOVA and Kruskal-Wallis (p-values 0.0004, 0.0075 respectively). The ANOVA analysis of differences in population decline between the control and 0.36 mg TAN /L treatment group shows significant difference at p-value = 0.0256 while the Kruskal-Wallis test yields marginal significance (p-value = 0.059). Therefore, we estimated the Lowest Observed Effect Level (LOEL) to be 0.36 mg TAN /L.

4. In Task 3-4-1, there is significantly lower number of newborn nauplii surviving to 3-day old when exposed to 0.38 mg TAN /L as compared to the control group. Using Dunnett's Multiple Comparison Test, the LOEL was 0.38±0.011 mg TAN/L for *P. forbesi* reproduction. These independent results support the conclusion of the earlier 31-d life cycle study and confirm that ammonia concentration of ≥ 0.36 mg TAN/L affect the survival and reproduction of *P. forbesi*.

5. In Task 3-4-2, the lethal concentrations (LC) of TAN for *P. forbesi* nauplii at day 4 pH 7.8 and 20°C were: LC5 = 0.591 mg TAN/L; LC10 = 0.731 mg TAN/L; LC50 = 1.547 mg TAN/L. Using Fisher Exact/Bonferroni-Holm Test, the No Observed Effect Level (NOEL) and LOEL were 0.62 and 0.95 mg TAN/L respectively for *P. forbesi* nauplii. These results indicated that 3-day-old nauplii (4d-LC50 = 1.547 mg/L) are more sensitive than juveniles (6d-LC50 = 6.014 mg/L at pH 7.8 and 4d-LC50 = 2.96 mg/L at pH 7.4) but less sensitive than newly hatched nauplii.

In summary, our first objective indicated *P. forbesi* is more sensitive to TAN at lower pH and the nauplii stage is more sensitive to TAN than juveniles. Results of the acute toxicity testing suggested that the environmentally relevant concentrations of TAN measured in water samples collected from Sacramento River at Hood and Cache Slough region between April and July 2009 do not affect the survival of nauplii and juvenile *P. forbesi*. However, results of our second objective indicated that the chronic 31-d life-cycle study and subsequent reproductive performance by acute toxicity testing of newborn nauplii confirmed lower recruitment rate of *P. forbesi* exposed to 0.36 and 0.79 mg TAN/L and lower survival of newborn nauplii acutely exposed to 0.38 mg TAN/L when compared to the control. In the 31-d chronic toxicity study, the survivals of nauplii to juvenile stage were lower in the control (63.2%) than 0.36 mg TAN/L (74.6%) while the survival of juvenile to adult stage were higher in the control (22.6%) than in 0.36 mg TAN/L (13.6%). These results suggested that chronically exposure to TAN can affects adult recruitment despite the fact that females exposed to 0.36 mg TAN/L had initial high number of nauplii production. Our research demonstrates that concentrations of TAN in the Sacramento River at and downstream of Hood are at potentially toxic levels to *P. forbesi*. The LOEL for *P. forbesi* at environmentally realistic River and Slough pH and temperatures values is between 0.36 and 0.38 mg TAN/L. Average TAN concentrations in summer at Hood were 0.46 with one value as high at 0.65 mg/L. Concentrations in the Sacramento River as far downstream as Isleton periodically reached 0.40 mg TAN/L during the summer of 2009. Isleton is 30 river miles below Hood. In contrast, average TAN concentrations during the same time period in the Cache Slough complex were between 0.01 and 0.1 mg/L. The highest TAN value measured in the Slough was 0.23 mg TAN/L on April 27, 2009 in the lower Ship Channel at the entrance to Cache Slough. This value is about 60 percent of the acute LOEL value for 3-day old nauplii. The present study did not determine a NOEL for this life stage. So, it is impossible to ascertain whether toxic episodes of TAN extend to the Cache Slough complex or not.

Background and Introduction

The San Francisco Estuary (SFE) ecosystem and its aquatic organisms are currently facing emerging challenges due to the potential impacts of ammonia from point and non-point sources (http://www.science.calwater.ca.gov/events/workshops/workshop_ammonia.html http://science.calwater.ca.gov/pdf/workshops/workshop_ammonia_bckgrnd_paper_nh4-nh3_030209.pdf). However, little information is available to evaluate the impact of ammonia on copepods. Viewed as one of the primary food sources to higher trophic level organisms (Kimmerer 2004), calanoid copepods comprise 60-80% of all metazoan zooplankton hence the main zooplankton component in the SFE (Lopez et al., 2006). Gut contents of larval fish further reveal the calanoid copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* to be the dominant prey organisms highlighting their importance in the pelagic foodweb of the SFE (Steve Slater, California Department of Fish and Game (CDFG), unpublished data). Unfortunately, little is known on the effects of ammonia on early life stages of copepods particularly *P. forbesi* and *E. affinis* that many larval fish species rely on for food. Both of these zooplankton species are experiencing population declines that further threaten the availability of food sources to native or endangered species in the SFE such as the Delta smelt and longfin smelt (Sommer et al., 2007). Preliminary studies by CDFG indicated *P. forbesi* as the major food item in all four POD (Age-0) fish species (delta smelt, longfin smelt, striped bass, and threadfin shad) between April and September. Since Age-0 delta smelt and longfin smelt switch their prey preference from *E. affinis* to *P. forbesi* after April and May, we selected *P. forbesi* as the target organism in the current study. Additional investigations with *E. affinis* will be proposed when more funding becomes available.

The overall objectives of this study are to 1) investigate the acute and chronic toxic effects of ammonia on the estuarine copepod *P. forbesi,* and 2) determine whether environmentally relevant concentrations of ammonia in the SFE potentially contribute to the decline in the abundance of *P. forbesi.* Study results are first discussed in terms of the tasks in the contract and then integrated in the discussion and conclusions section to address both objectives.

Task 1: Integrating ammonia/pH data with abundance of *P. forbesi* in the SFE

Task 1 is focused on acquiring recent *P. forbesi* abundance and distribution data collected by the California Department of Fish and Game (CDFG)'s 20mm survey and integrating the locations of *P. forbesi* abundance with pH/ammonia data provided by the program manager (Dr. Chris Foe) of the State Water Resources Control Board (SWRCB).

Task 1A pH, temperature, and ammonia values at Hood and at the North Delta

There is no SWRCB water quality data available for Sacramento River at Hood in 2008. In addition, except for temperature there is no pH data prior to 7 November 2008 from the California Data Exchange Center for Sacramento River at Hood http://cdec.water.ca.gov/cgi-progs/staMeta?station_id=SRH. **Table 1A** shows the pH, temperature and ammonia data for the Sacramento River at Hood collected by the SWRCB between April 13 and July 14 2009 (Foe et al., 2010). The mean ± standard deviations of pH, temperature, total ammonia nitrogen (TAN), and unionized ammonia (UIA) are 7.39±0.12, 18.34±2.44^{o}C, 0.46±0.16 mg/L, and 0.004±0.0006 mg/L respectively. The 2009 California Data Exchange Center temperature and pH values for the Sacramento River at Hood are summarized in **Table 1B**. The average temperature and pH between April and August 2009 are 19.71±4.64^{o}C and 7.33±0.12. In addition, the 2009 pH, temperature, TAN monitoring data by the SWRCB (Dr. Chris Foe) in several sites (Sacramento River Deepwater Ship Channel @ Cache Slough, lower flooded Liberty Island, Lindsey Slough, and Toe Drain @ Dredger Cut) within the Cache Slough region are shown in **Table 2.** The average pH, temperature, and TAN between March and July of 2009 are 8.00±0.26, 18.91±4.21, and 0.079±0.066 mg/L respectively.

Table 1A 2009 pH, temperature, and ammonia for Sacramento River at Hood collected by the State Water Resources Control Board

Date	pH	Temperature (^{0}C)	$NH4^{+}$-N(TAN) mg/L	NH3 (UIA) mg/L
4/13/2009	7.6	14.80	0.53	0.006
4/27/2009	7.4	15.80	0.54	0.004
5/11/2009	7.3	17.20	0.28	0.002
5/26/2009	7.3	19.10	0.65	0.005
6/8/2009	7.5	19.80	0.4	0.005
6/22/2009	7.3	21.00	0.59	0.005
7/14/2009	7.3	20.70	0.24	0.002
Mean±Stdev	**7.39±0.12**	**18.34±2.44**	**0.46±0.16**	**0.004±0.002**

Table 1B 2008 and 2009 temperature and pH values for Sacramento River at Hood from the California Data Exchange Center

Date	pH	Temperature (^{o}C)
2009		
04/01-04/30/2009	7.35±0.13	15.87 ± 2.47
05/01-05/31/2009	7.38±0.12	18.35 ± 3.75
06/01-06/30/2009	7.34±0.09	21.20 ± 1.79
07/01-07/31/2009	7.22±0.08	21.41 ± 1.22
08/0108/31/2009	7.37±0.10	21.65 ± 1.01
04/01-08/31/2009	Mean=7.33±0.12	Mean=19.71 ± 4.64
2008		
04/01-04/30/2008	N/A	16.46 ± 2.04
05/01-05/31/2008	N/A	20.14 ± 3.31
06/01-06/30/2008	N/A	21.44 ± 1.54
07/01-07/31/2008	N/A	22.86 ± 1.27
08/01-08/31/2008	N/A	23.46 ± 1.10
0401-08/31/2008	N/A	Mean= 20.90 ± 4.89

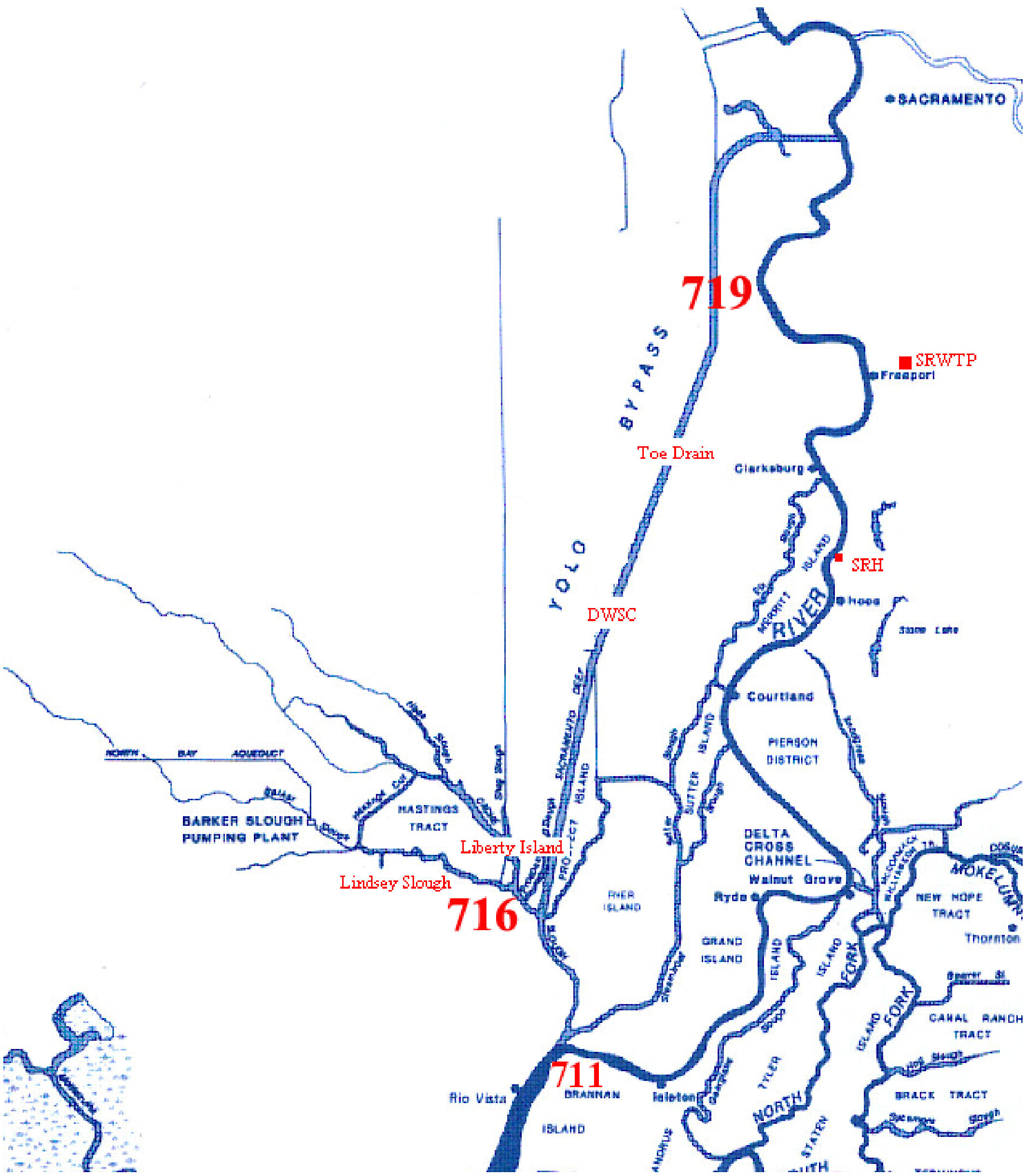
Table 2 2009 pH, temperature, and TAN within Cache Slough complex

Sacramento Deep Water Ship Channel				Liberty Island			Lindsey Slough			Toe Drain @Dredger Cut		
Date	**pH**	**Temperature**	**TAN**	**pH**	**Temperature**	**TAN**	**pH**	**Temperature**	**TAN**	**pH**	**Temperature**	**TAN**
3/16/2009	7.93	12.88	0.012	8.14	13.23	0.007	7.74	13.07	0.005	8.12	14.3	0.004
3/30/2009	8.07	14.28	0.170	8.27	13.67	0.139	8.12	14.45	0.059	8.42	15.81	0.027
4/13/2009	7.84	15.12	0.224	8.09	14.41	0.194	8.38	15.48	0.018	8.18	17.11	0.032
4/27/2009	7.86	16.94	0.225	8.06	16	0.188	8.63	17.07	0.011	8.35	18.61	0.161
5/11/2009	7.58	18.86	0.121	7.91	18.91	0.083	8.6	20.36	0.014	8.02	22.08	0.040
5/26/2009	7.75	20.83	0.121	7.84	18.87	0.113	7.73	21.07	0.087	7.87	21.92	0.056
6/8/2009	7.8	19.71	0.124	8	18.99	0.123	7.99	19.51	0.078	7.94	21.33	0.034
6/22/2009	7.86	21.34	0.059	8.04	21.14	0.051	8.01	21.67	0.016	7.8	22.83	0.040
7/14/2009	7.39	23.45	0.113	7.89	26.34	0.034	7.76	23.87	0.048	7.89	25.4	0.022
Mean ± Stdev	7.87±0.20	18.16±3.57	0.013±0.07	8.03±0.14	17.95±4.19	0.10±0.07	8.11±0.36	18.51±3.66	0.04±0.03	8.07±0.22	19.93+3.67	0.05+0.05

Task 1B *P. forbesi* Abundance in North Delta

The 2007-2009 CDFG 20 mm survey for *P. forbesi* and *E. affinis* in the North Delta are summarized in **Tables 3A-3C** (see **Figure 1** for location and description of stations 711, 716 and 719). (http://www.dfg.ca.gov/delta/projects.asp?ProjectID=20mm). The surveys indicated that for all 3 years, *P. forbesi* increased in abundance at stations 711, 716 and 719 beginning in April and peaked in June while *E. affinis* abundance decreased to zero on or after April and May.

Figure 1 Map of North Delta of CDFG 20 mm survey for *P. forbesi* and *E. affinis*



Site 716 is in Cache Slough North of Cable Ferry 1 and 51 near Boat Sheds (Latitude 38-14'-28.8"N and Longitude 121-41'-8.4"W) and Site 719 is in Sacramento Deep water shipping channel between lights 59 and 60 (Latitude 38-19'-98.5"N and Longitude 121-38'-84.7"W). (Sites information was obtained from Kelly Souza, DFG).

Table 3A 2007 Zooplankton Counts at Station 711, 716, and 719*

Site 716	*Pseudodiaptomus forbesi*	*Eurytemora affinis*	Temperature (°C)
3/16/2007	20	9	16.6
3/27/2007	40	22	15.5
4/10/2007	28	2	17.1
4/24/2007	36	7	16.4
5/10/2007	257	4	20
5/23/2007	354	0	18.7
6/5/2007	2467	0	18.9
6/19/2007	2877	0	22.9
7/3/2007	2020	0	21.8
			Mean 18.7±2.5
Site 711			
3/16/2007	1	11	16.4
3/27/2007	1	12	15.2
4/10/2007	2	0	17.3
4/24/2007	1	18	16.7
5/10/2007	ND	ND	19.7
5/23/2007	73	4	19.3
6/5/2007	279	1	21
6/19/2007	78	1	23.7
7/3/2007	ND	ND	22
			Mean 19.0±2.9
Sampling at **site 719** did not start until 2008			

ND indicates no data

Table 3B 2008 Zooplankton Counts at Station 711, 716, and 719*

Site 716	*Pseudodiaptomus forbesi*	*Eurytemora affinis*	Temperature (^{O}C)
4/1/2008	147	26	15
4/15/2008	200	7	16
4/29/2008	157	25	17.5
5/28/2008	717	0	17.7
6/9/2008	1193	0	ND
6/23/2008	1282	0	22
7/7/2008	468	0	24.5
			Mean 18.8±3.7
Site 719			
4/1/2008	109	6	14.5
4/14/2008	66	31	16.3
4/29/2008	117	9	17.7
5/12/2008	127	3	18.3
6/9/2008	636	1	ND
6/23/2008	2561	0	22.1
7/7/2008	787	0	23.9
			Mean 18.8±3.5
Site 711			
4/1/2008	3	10	14.4
4/14/2008	1	18	16.2
4/29/2008	81	59	16.9
5/28/2008	368	5	18.3
6/9/2008	15	0	ND
6/23/2008	1635	0	21.7
7/7/2008	13	0	24.8
			Mean 18.7±3.9

Table 3C 2009 Zooplankton Counts at Station 711, 716, and 719*

Site 716	*Pseudodiaptomus forbesi*	*Eurytemora affinis*	Temperature (^{o}C)
4/6/2009	13	1	16.2
4/22/2009	36	4	19.3
5/6/2009	119	0	17.1
5/20/2009	520	0	20.3
6/1/2009	602	0	18.9
6/15/2009	555	0	19.6
6/29/2009	1250	0	23.6
			Mean 19.3±2.4
Site 711			
4/6/2009	2	0	14.9
4/22/2009	0	7	18.5
5/20/2009	2	0	19.6
6/2/2009	196	8	20.4
6/15/2009	515	0	20.8
6/30/2009	815	0	23.1
			Mean 19.6±2.7
Site 719			
4/6/2009	20	1	15.5
4/22/2009	13	1	19.6
5/6/2009	28	1	18.2
5/20/2009	322	1	21
6/1/2009	324	0	20.4
6/15/2009	ND	ND	20.9
6/29/2009	1240	0	22.2
			Mean 19.7±2.2

*We acknowledge Erin Gleason, Julio Adib-Samii, and Bob Fujimura of California DFG for providing the zooplankton data.

Task 2A: Establish laboratory culture of *P. forbesi* for the bioassays

Because *P. forbesi* is the preferred test species and is not commercially available, maintaining the integrity, health, and adequate population of the copepod cultures under selected pH and temperature conditions is extremely important. Task 2 is focused on establishing optimum culture conditions to ensure that high quality and quantity of all life stages of the copepods will be available for the acute and chronic bioassays described in Task 3. Copepods were collected from Rio Vista and Suisun Marsh in the San Francisco Estuary using a 174µm zooplankton tow net in June 2007. Cultures of *P. forbesi* were acclimated and maintained in aerated 120L tanks with standard moderately hard fresh water (pH 7.8 at 20 ± 1ºC). Water quality were monitored weekly and maintained as follows: alkalinity (80 mg/L), dissolved oxygen (>8 mg/L), salinity (0.5 ppt or 2.0 ppt), and ammonia (<1 µg/L) (Hach, USA). An equal biovolume of the instant algae (IA) mix were given as food at 500 µg $C.L^{-1}day^{-1}$. The IA diet comprised of highly nutritious and pure concentrated forms of the phytoplankton *Nannochloropsis* and *Pavlova* (Instant Algae, Reed Mariculture, USA). Approximately 50% of the total culture medium was replaced weekly with aerated and pH/temperature acclimated medium. The culture system was maintained under a natural photoperiod (16L:8D) and covered with a semi-transparent black tarp.

The life cycle of *P. forbesi* is similar to other calanoid copepods. The adults copulate, followed by deposition of the eggs into two egg sacs carried by the female. The eggs hatch to release the typical crustacean larva called a nauplius (plural nauplii). The nauplius undergoes six naupliar stages. After the last naupliar stage, the subsequent molt remodels the animal into a juvenile copepod, called a copedodite. Copepodites have distinct segmentation, which is lacking in the naupliar stages, and the body regions become apparent. Species of *Pseudodiaptomus* copepods have five copepodite (juvenile) stages, after which the animal molt into an adults, and becomes reproductive. There are no further molts after the adult stage (Johnson 1948). Depending on environmental conditions, the nauplius and copepodite stages can last up to two weeks each, until the copepods turn into adults. The life span of *P. forbesi* under laboratory conditions is approximately two months, females can be reproductive active at approximately 30 days old, and new egg sacs production is variable ranging from one to seven days depending on environmental conditions. Based on our observations in the laboratory (unpublished data), females can produce new egg sacs in the absence of males suggesting the possibility of females storing male sperm. Staging of live copepods is difficult because of their size and speed in swimming. In this study, four main factors were used to assess mortality: lack of movement of any limbs, antennae fold down into their appendages, bodies are found on the bottom of the beakers, and turned a darker shade of gray. To verify mortality, copepods were observed under a dissection microscope for at least one minute to look for any movement. Any slightest movement by a copepod will be scored as a live copepod. Mortality data developed in this study is based on the initial number (n=20) minus the number of live copepod counted at the end of the experiment.

2A-1 Methods for acute and chronic toxicity testing

As *P. forbesi* is a non-standard resident species, test methods are considered developmental. Toxicity testing followed our published laboratory culture techniques and conditions (Ger et al, 2009a, 2009b, and 2010) as well as US EPA methods. Protocols were based on standard acute toxicity testing procedures, as outlined in US EPA (EPA/821/R-02/012) while chronic test protocols were based on standard chronic toxicity testing procedures as outlined in US EPA (EPA-821/R-02/013). Both protocols were modeled after these test conditions, in terms of the number of replicates, number of organisms per replicate, frequency of feeding and water renewals, water quality measurements, temperature and photoperiod used. Moderately hard synthetic freshwater was prepared according to methods published in EPA-821/R-02/013 and was used as the culture and testing medium for all tests. Ammonium chloride (NH_4Cl, 99.5% purity) was purchased from EMD Chemicals, Gibbstown, NJ USA.

Acute test methods were comprised of four replicate 600 ml test chambers, each containing 500 ml of moderately hard synthetic control water (0.5 ppt) and 20 organisms per replicate. Tests were initiated with juvenile-stage *P. forbesi*. Eighty percent of the test solution was renewed daily, at which time debris and dead organisms were removed. pH were measured daily before and after water renewals. Ammonia measurements were taken on test solutions daily prior to feeding and water renewal. Organisms were fed 500 µg $C.L^{-1}day^{-1}$ of IA diet daily before water renewal. Test chambers were incubated in a temperature controlled environmental chamber/water bath maintained at 20 ± 0.1°C with a 16h L: 8h D photoperiod under natural and fluorescent light. Mortality was measured daily.

Chronic full life-cycle test methods consisted of four replicate 1L test chambers, each containing 900 ml of moderately hard synthetic control water (2.0 ppt) and three organisms per replicate. Three gravid-stage females were employed per replicate chamber at test initiation and allowed to reproduce over the 31-day testing period, during which time the life stages of nauplii, juvenile, and adult were monitored and recorded. Organisms were fed 500 µg $C.L^{-1}day^{-1}$ of IA diet daily. Eighty percent of the test solution was renewed and organisms were identified and enumerated every 2-3 days for each life stages. Water quality such as pH, hardness, salinity and ammonia was measured daily prior to feeding. At test termination organisms were preserved for identification and enumeration of life stages.

2A-2Test Acceptability and Ammonia analysis

The referenced EPA manual was used to determine the appropriate test protocols, which were optimized for use with *P. forbesi*. As *P. forbesi* is a sensitive species, we would expect that overall survival would be lower than what is listed in the acute manual for *C. dubia*, *D. pulex* and *D. magna* species. There is no standard ammonia toxicity test method that has been developed for *P. forbesi* in the revised U.S. EPA water quality criteria document for ammonia (USEPA 1999; 2009). Augspurger and co-authors (2003) evaluated data from all sources for acceptability using modified USEPA methods (Stephan et al 1985) for freshwater mussel ammonia toxicity. In their studies, survival in control treatments (≥80%) is acceptable as long as measured ammonia test concentrations, pH, temperature were documented. Since there is no acceptability data available for was for *P. forbesi*, we set the acceptance criteria at ≥80% control *P. forbesi* survival for the acute 96-hour test.

In the ammonia tests, TAN of each treatment concentration was measured every 1 to 3 days using an Orion Ammonia ion selective electrode (ISE) and Orion 4STAR meter following the U.S. EPA method 350.3 (Thermo Scientific, Beverly, MA USA). The equipment was calibrated each time before measuring samples with purchased stock solutions prepared and certified by Thermo Fisher Scientific. For TAN in water samples, a minimum reporting limit of <0.1 mg N/L was selected based on the method detection limit of 0.02 mg N/L.

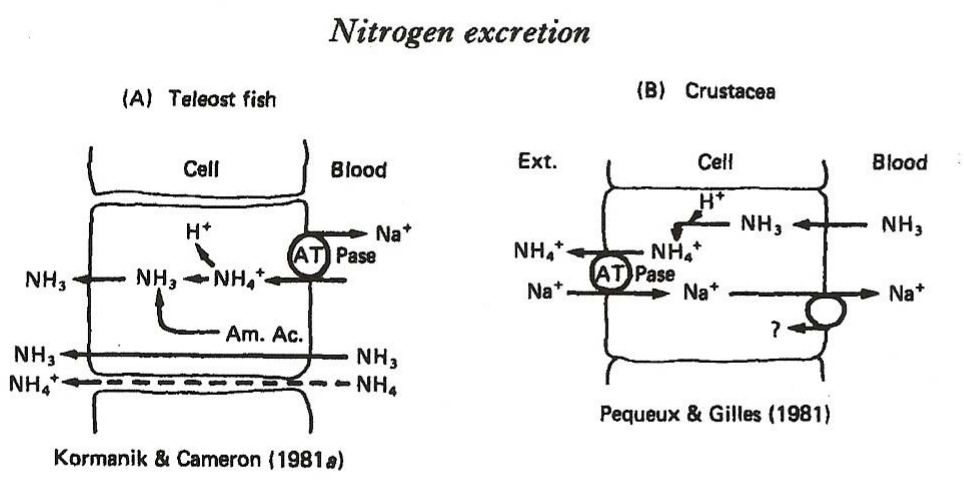
Unionized ammonia concentrations (UIA) were calculated based on the following equations:

$IA = TAN/(1+ 10^{(pH-pK)})$ (Wood 1993)
Where
UIA = Total ammonia – IA
$pK = 0.09018 + (2729.92/(273.2 + T))$ (Emerson et al. 1975)

Task 3: Acute and chronic effects of ammonia on *P. forbesi*

For many decades, unionized ammonia (UIA) was known to be the most toxic form to fish. This led to several studies reporting UIA as the major cause of toxicity to other types of aquatic organisms including invertebrates. The recently revised USEPA water quality criteria document for ammonia – freshwater (USEPA 2009) indicated that freshwater mussels are more sensitive to ammonia than other freshwater aquatic organisms. The new recommended criteria to prevent acute toxicity are 2.9 or 5.0 mg TAN/L (at pH 8 and 25°C) depending on the presence or absence of freshwater mussels. Chronic toxicity is prevented at either 0.26 or 1.8 mg TAN/L (at pH 8 and 25°C) again depending on the presence or absence of freshwater mussels (U.S. EPA 2009). The primary toxic agent for mussels was still assumed to be UIA at pH 8.

Most of the studies conducted to date have focused on determining lethal concentration of UIA to invertebrates. Only a few studies have evaluated the toxicity of both UIA and IA despite the fact that it is well known that pH and temperature modulate both the relative fraction of the two ammonia forms in water and the toxicological response of invertebrates. There are differences between fish and zooplankton in the excretion of nitrogen waste. Briefly, fish excrete UIA through their gills while copepods have no gills and excrete IA through the maxillary glands. The following schematic illustrates the physiological processes involved in ammonia excretion through epithelial cell of fish gill and copepod maxillary glands as modified from Regnault 1987. The different excretory mechanisms and products may influence the relative sensitivity of UIA and IA to the different types of aquatic organisms. Armstrong et al (1978), Borgmann (1994), and Ankley et al (1995) have all demonstrated that the toxicity of TAN increase with decreasing pH. This is the reverse of what has been observed with fish (U.S. EPA 1999) and is consistent with the difference in excretory mechanism.



The equilibrium equation of total ammonia (TAN) based on the presence of ionized and unionized forms are outlined below:

$$NH_4^+ \text{ (Ionized Ammonia)} + H_20 \leftrightarrow NH_3 \text{ (Unionized Ammonia)} + H_30^+$$

TAN (Total ammonia) = Ionized ammonia (IA) + Unionized ammonia (UIA). The dissociation of ammonia in aqueous solutions is dependent on water quality such as pH, salinity and temperature.

Controversial arguments are being debated regarding the effects of pH on ammonia toxicity in aquatic organisms (Armstrong et al 1978; Szumski et al 1982; Borgmann, 1994; Ankley et al 1995, U.S. EPA 1999). To quote a statement from page 284 of Szumski et al (1982) "*This result is not consistent with classical bioassay theory. The scientific basis for the tests leads to the conclusion that if 0.18 mg/1 of un-ionized ammonia was determined to be the LC50 for Daphnia at pH 6, then Daphnia exposed to 0.18 mg/1 of un-ionized ammonia at any other pH will exhibit about 50% mortality. Such is not the case, however. Instead, at pH 7.0, 50% mortality occurs with 1.4 mg UIA/1; and at pH 8.0, 50% mortality occurs with 4.9 mg UIA/1***".** These results concur with our previous findings in *E. affinis* where 4 day LC50 values of UIA were: 0.068 mg/l at pH 7.2, 0.12 mg/l at pH 7.6, and 0.78 mg/l at 8.1 (Teh et al 2009). As is shown in the above schematic diagram, high concentrations of IA in the culture medium may compete with sodium ions influx thereby diminishing body concentrations of this important sodium salt. In addition, disruption of the Na^+/NH_4^+ transport system also cause body levels of ammonia body levels to rise in copepods, riding the transport mechanism in or preventing metabolic NH_4^+ from riding it out that may result in autointoxication. Because of this mechanism and results of Armstrong (1978), Borgmann (1994) and Ankley (1995) on prawn larvae and *Hyallella azteca*, we postulated that copepods are more sensitive to IA than to UIA.

To the best of our knowledge, the acute and chronic effects of contaminants on *P. forbesi* have not been examine except for three recent studies (Ger et al. 2009a, 2009b and 2010). Therefore, toxicity testing conditions in this study will closely follow the US-EPA standard toxicity testing procedures (EPA-821-R-02-012; EPA-821-R-02-013) including culture techniques and conditions of copepod cultures that we developed in our laboratory.

Because pH can modulate the toxicity of IA and UIA forms of ammonia, the toxicity of both ammonia fractions to *P. forbesi* was evaluated as a function of pH. Furthermore, the SWRCB field monitoring data revealed ambient TAN in the Delta ranging from undetectable (<0.005) to 0.65 mg/L (Foe et al., 2010). It is unknown however, whether these ambient ammonia concentrations have any adverse long-term effects on survival and reproduction of *P. forbesi*. The objectives of Task 3 are to:

1. Determine the lethal concentration (LC) of ammonia on juvenile *P. forbesi* at 20ºC and pH 7.4 and 7.8
2. Evaluate the lethal concentration of UIA as a function of pH on juvenile *P. forbesi* at 20ºC
3. Assess the chronic effect of ammonia on growth and survival of *P. forbesi*
4. Investigate the reproductive fitness of adult female *P. forbesi*

Task 3 is separated into 4 subtasks (3-1 to 3-4) based on the following hypotheses:

$\mathbf{H_1}$: *P. forbesi* is sensitive to ionized ammonia therefore is more sensitive to total ammonia nitrogen at lower pH

$\mathbf{H_2}$: *P. forbesi* is sensitive to unionized ammonia at high pH when the threshold toxic concentration of UIA is reached

$\mathbf{H_3}$: The fecundity and fertility of adult copepods is likely affected by exposure to TAN at environmentally relevant concentration when compared to controls

$\mathbf{H_4}$: The survival of nauplii to juvenile or adult is likely affected by exposure to TAN at environmentally relevant concentration when compared to controls.

Subtask 3-1A: Estimating 4 and 6-d lethal concentration (LC) of ammonia on juvenile P. forbesi at 20ºC and pH 7.8

Groups of juvenile *P. forbesi* (N = 20 per replicate; 4 replicates per concentration) were exposed separately to ammonia for 6 days at 20ºC and pH 7.8 on December 4, 2009. The nominal and measured concentrations of TAN for 6-d acute toxicity testing at pH 7.8 and water chemistry measurements are shown in **Table 4**. Water samples were collected at 0 and 24 hr for 6 days from each concentration and analyzed with an ion selective electrode (ISE) and meter. The ISE meter was calibrated using the EPA method 350.3 with stock solutions prepared and certified by Fisher Scientific. Three replicates per water sample per treatment were prepared as per the EPA method and analyzed after the calibration. Daily ammonia variation in control treatment at 0 and 24 hr is <0.1 mg/L (i.e., between 0.04-0.06 mg TAN /L).

The mean survival (%) of *P. forbesi* at the end of day 4 and 6 is shown in **Table 5**. The estimated lethal concentrations (LC) causing 5%, 10% and 50% mortality of the *P. forbesi* were calculated using the U.S. Environmental Protection Agency Probit Analysis Program v1.5 (http://www.epa.gov/nerleerd/stat2.htm).

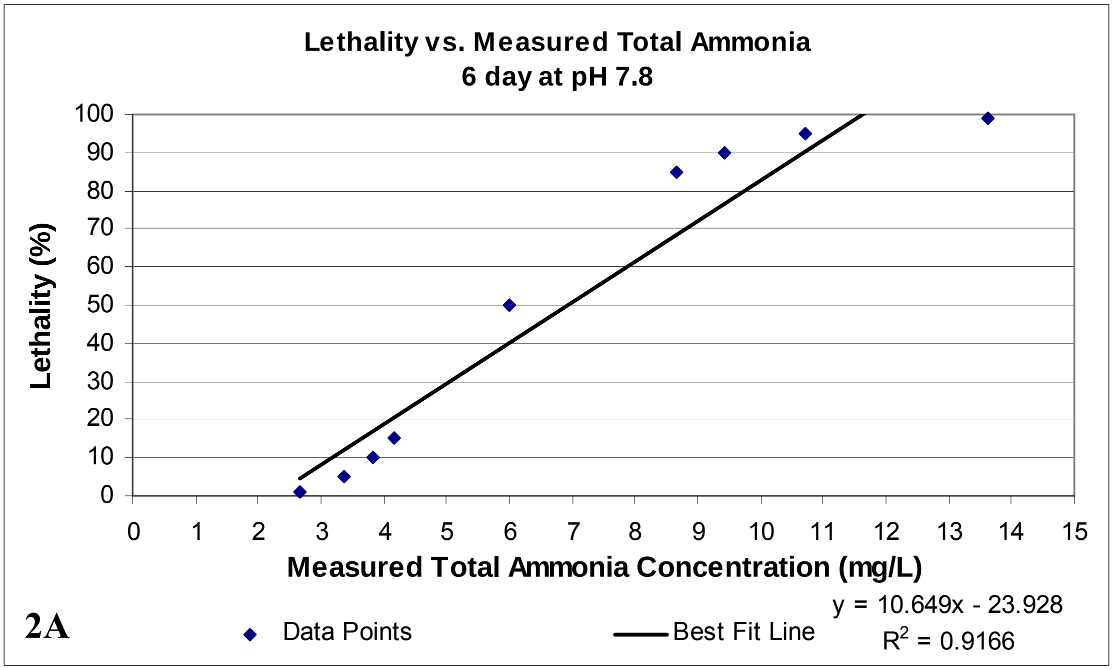
Results indicated no significant mortality in *P. forbesi* at day 4 of exposure to ammonia at pH 7.8. However at day 6, significant mortalities were observed in *P. forbesi* exposed to measured ammonia concentrations of 4.75 and 6.25 mg TAN/L. The measured LC of TAN and UIA concentrations with 0.95 confidence intervals for *P. forbesi* at day 6 were: LC5 = 3.374 (1.774, 4.110) mg TAN/L and 0.085 (0.044, 0.103) mg UIA/L; LC10 = 3.834 (2.345, 4.484) mg TAN/L and 0.096 (0.059, 0.113) mg UIA/L (UIA); LC50 = 6.014 mg (5.468, 6.998) TAN /L and 0.150 (0.137, 0.175) mg UIA /L. The LC5, 10, and 50 of measured TAN concentrations are shown in **Figures 2A and 2B**.

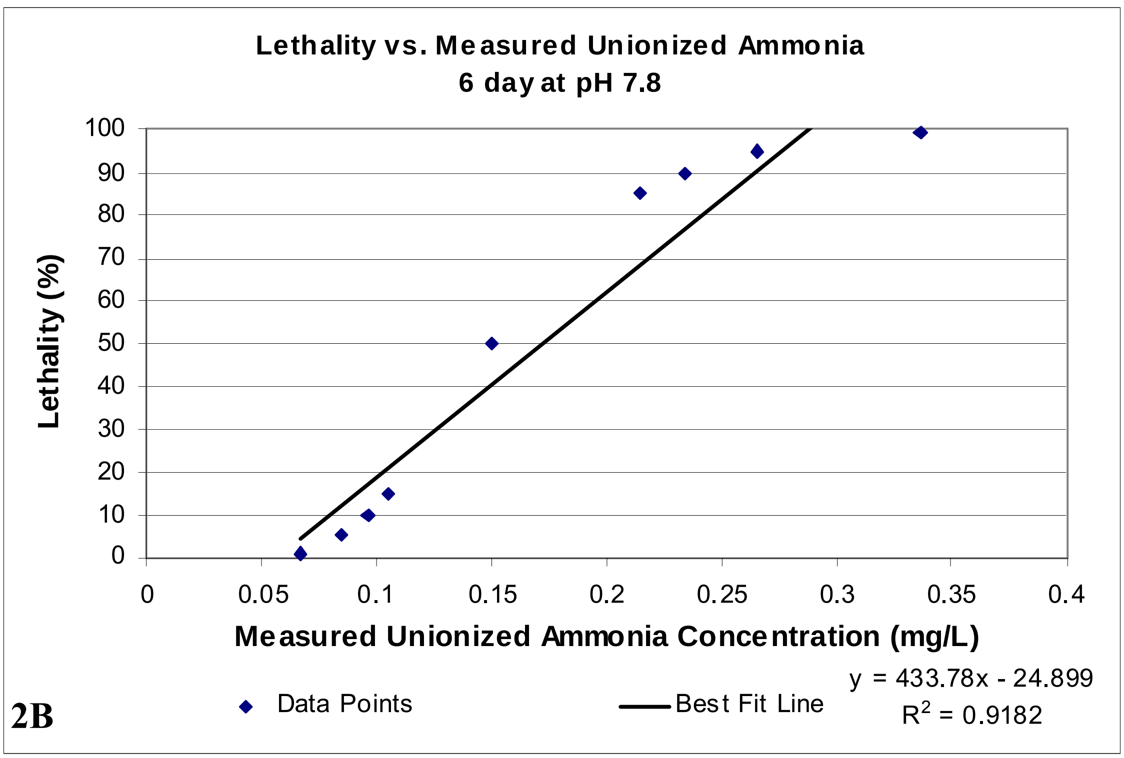
Table 4. Summary of water chemistry and ammonia concentrations during acute toxicity testing

Nominal mgTAN/L	Temp (°C)	Alkalinity (mg/L)	conductivity (μHMOS)	DO (mg/L)	Hardness mg/L	pH	Salinity ppt	Measured mg TAN/L	Measured mg UIA/L
0	20	50	975 ± 17	>8	206.7	7.80±0.09	0.5	<0.1	0.000
1	20	50	975 ± 17	>8	206.7	7.80±0.04	0.5	0.87±0.01	0.022±0.000
2	20	50	975 ± 17	>8	206.7	7.79±0.03	0.5	1.60±0.00	0.039±0.000
4	20	50	975 ± 17	>8	206.7	7.79±0.02	0.5	3.20±0.00	0.078±0.000
6	20	50	975 ± 17	>8	206.7	7.79±0.02	0.5	4.75±0.05	0.116±0.001
8	20	50	975 ± 17	>8	206.7	7.79±0.01	0.5	6.25±0.05	0.156± 0.001

Table 5 *P. forbesi* survival at day 4 and 6 of exposure to total ammonia at pH7.8
(N=20 per replicate beaker and a total of 80 juveniles per treatment concentration)

Treatments	Number survivors 4-day	% survival at 4-day	Number survivors 6-day	% survival at 6-day
control – A	20	**98.75**	20	**91.25**
control – B	19		15	
control – C	20		19	
control – D	20		19	
1ppm – A	18	**91.25**	16	**82.50**
1ppm – B	19		18	
1ppm – C	18		16	
1ppm – D	18		16	
2ppm – A	19	**90**	18	**78.75**
2ppm – B	14		11	
2ppm – C	20		17	
2ppm – D	19		17	
4ppm – A	19	**91.25**	17	**76.25**
4ppm – B	17		14	
4ppm – C	18		15	
4ppm – D	19		15	
6ppm – A	19	**87.50**	14	**66.25**
6ppm – B	18		14	
6ppm – C	15		11	
6ppm – D	18		14	
8ppm – A	19	**75.00**	6	**36.25**
8ppm – B	12		6	
8ppm – C	16		8	
8ppm – D	13		9	





Figures 2A and 2B Six days lethal measured TAN and UIA concentrations (LC) of *P. forbesi* at pH 7.8. The TAN (2A) and UIA (2B) concentration for LC5 = 3.374 (1.774, 4.110) mg TAN/L and 0.085 (0.044, 0.103) mg UIA/L; LC10 = 3.834 (2.345, 4.484) mg TAN/L and 0.096 (0.059, 0.113) mg UIA/L; LC50 = 6.014 (5.468, 6.998) mg TAN/L and 0.150 (0.137, 0.175) mgUIA/L.

Subtask 3-1B: Estimating 4-d lethal concentration (LC) of ammonia on juvenile P. forbesi at 20ºC and pH 7.4

Groups of juvenile *P. forbesi* (N = 20 per replicate; 4 replicates per concentration) were exposed separately to ammonia for 4 days at 20ºC and pH 7.4 on February 1, 2010. The nominal and measured concentrations of TAN and UIA for 4-d acute toxicity testing at pH 7.4 and water chemistry measurements are shown in **Table 6**. The mean survival (%) of *P. forbesi* at the end of 4-d are shown in **Table 7**. The 4 days LC5, 10, and 50 of measured TAN and UIA values as calculated using the USEPA Probit Analysis program are shown in **Figures 3A** and **3B**.

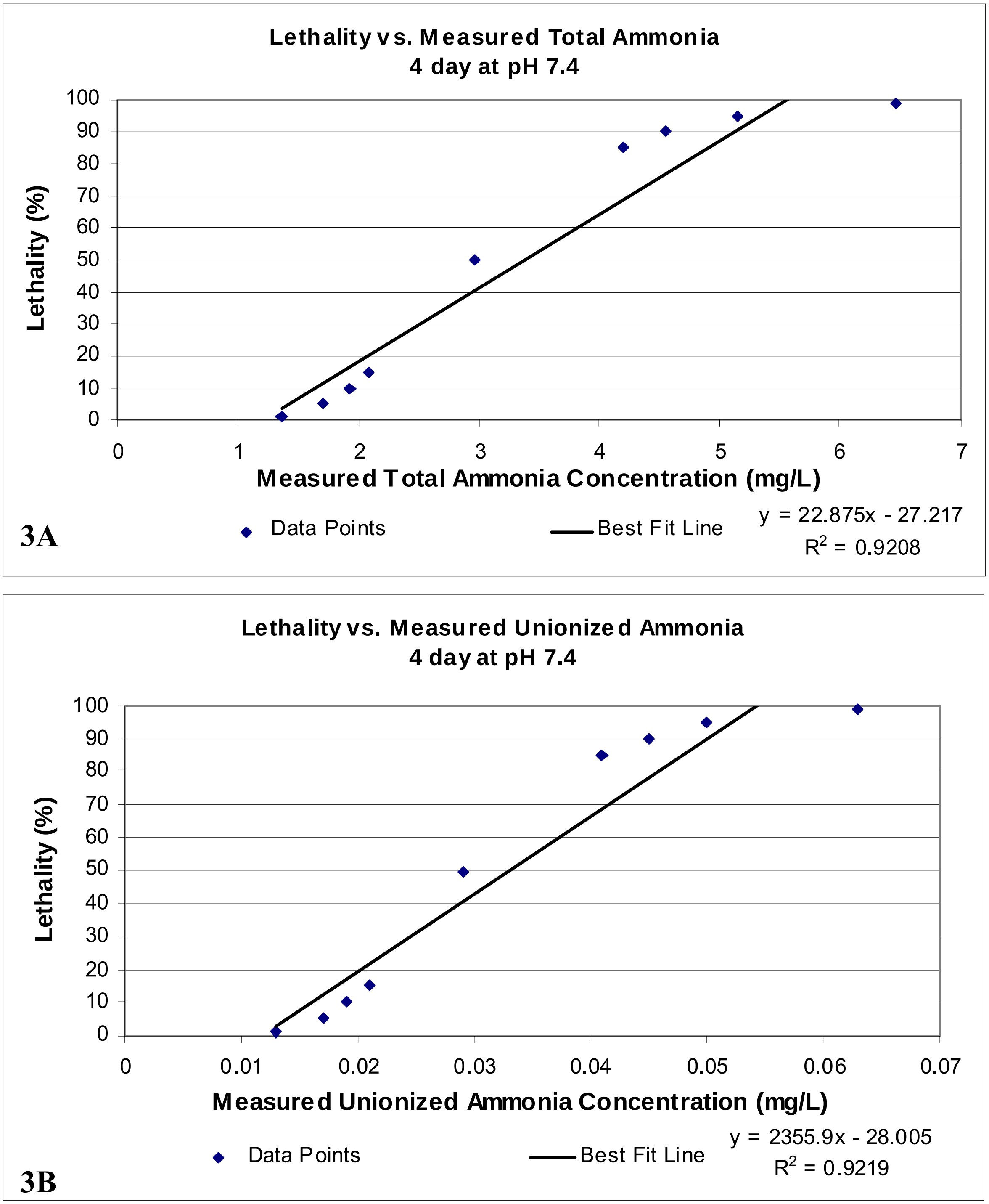
Results indicated significant mortality in *P. forbesi* at 4-d of exposure to ammonia at pH 7.4. The measured LC of TAN and UIA concentrations with 0.95 confidence intervals for *P. forbesi* at day 4 were: LC5 = 1.703 (1.191, 2.064) mg TAN/L and 0.017 (0.012, 0.020) mg UIA /L; LC10 = 1.924 (1.420, 2.270) mg TAN/L and 0.019 (0.014, 0.022) mg UIA/L; LC50 = 2.960 (2.601, 3.228) mg TAN/L and 0.029 (0.026, 0.032) mg UIA/L. Comparison of acute toxicity results for pH 7.4 and 7.8 indicate the *P. forbesi* are more sensitive to increasing ammonia concentrations at a lower pH. No measureable 96hr-LC50 toxicity was observed at pH 7.8 while the 96hr-LC50 at 7.4 was 2.960 mg/L. Result of this study supports our hypothesis that copepods are more sensitive to ammonia at low pH.

Table 6 Summary of water chemistry and ammonia concentrations during acute toxicity testing

Nominal mgTAN/L	Temp (°C)	Alkalinity mg/L	conductivity µHMOS	DO mg/L	Hardness mg/L	pH	Salinity ppt	Measured mgTAN/L	Measured mgUIA/L
0	20	20	580 ± 24	>8	140	7.40±0.000	0.5	<0.1	0.000
1	20	20	580 ± 24	>8	140	7.40±0.004	0.5	0.60±0.008	0.007±0.000
2	20	20	580 ± 24	>8	140	7.42±0.003	0.5	1.33±0.021	0.015±0.000
4	20	20	580 ± 24	>8	140	7.41±0.000	0.5	2.75±0.022	0.031±0.000
6	20	20	580 ± 24	>8	140	7.40±0.000	0.5	3.88±0.070	0.043±0.000
8	20	20	580 ± 24	>8	140	7.40±0.003	0.5	5.47±0.029	0.060±0.000

Table 7 *P. forbesi* survival at the end of 4-d exposure to total ammonia at pH7.4 (N=20 per replicate beaker and a total of 80 juveniles per treatment concentration)

Treatment	Number survivors	Total Survivors	% survival
control - A	20	**72**	**90.00**
control - B	19		
control - C	16		
control - D	17		
1ppm - A	14	**61**	**75.00**
1ppm - B	16		
1ppm - C	14		
1ppm - D	17		
2ppm - A	16	**59**	**73.75**
2ppm - B	14		
2ppm - C	16		
2ppm - D	13		
4ppm - A	8	**37**	**46.25**
4ppm - B	9		
4ppm - C	10		
4ppm - D	10		
6ppm - A	5	**16**	**20.00**
6ppm - B	2		
6ppm - C	4		
6ppm - D	5		
8ppm - A	0	**1**	**1.25**
8ppm - B	0		
8ppm - C	1		
8ppm - D	0		



Figures 3A and 3B 4-d lethal measured TAN and UIA concentrations (LC) of *P forbesi* at pH 7.4. The TAN (3A) and UIA (3B) concentration for LC5 = 1.703 (1.191, 2.064) mg TAN/L and 0.017 (0.012, 0.020) mg UIA/L; LC10 = 1.924 (1.420, 2.270) mg TAN/L and 0.019 (0.014, 0.022) mg UIA/L; LC50 = 2.960 (2.601, 3.228) mg TAN/L and 0.029 (0.026, 0.032) mg UIA/L.

Subtask 3-2: Effects of pH on ammonia toxicity in juvenile P. forbesi at 20ºC

Based on the results of Subtask 3.1A and 3.1B, groups of juvenile *P. forbesi* (N = 20 per replicate; 4 replicates per concentration) were exposed separately to 0 mg/L at pH 7.8 (control) and 5 mg/L nominal TAN concentration at pH 7.0, 7.4, 7.8, 8.2, and 8.6 for 4 days on April 1, 2010. The objective of this study is to determine the toxic threshold concentration of UIA to *P. forbesi*. Except for pH, water chemistry for all treatments were maintained at 0.5 ppt (salinity), 75.6 ± 0.3 mg/L (alkalinity), 983.3 ± 2.7 µHMOS (conductivity), 163 ± 1.8 mg/L (hardness), and >8 mg/L dissolved oxygen. The measured concentrations of TAN and UIA corresponding to each pH levels are shown in **Table 8 and Figure 4**. The average measured TAN among the pH treatments varied a little over a 24h (Table 8). Despite minor fluctuation of TAN and pH over the 24 hr period, concentration gradients of UIA existed and were maintained by 80% water changes every 24 hours during the 96-hour exposure. The mean survival (%) of *P. forbesi* at the end of 4-d are shown in **Table 9**. The 4 days LC5, 10, and 50 of measured UIA values as calculated using the USEPA Probit Analysis program is shown in **Figure 5**.

Results indicated lower copepod survival at higher pH and UIA concentration (**Table 9**; **Figure 5**). The 4-d measured LC of UIA concentrations with 0.95 confidence intervals for *P. forbesi* were: LC5 = 0.021 (0.002, 0.052) mg/L; LC10 = 0.038 (0.006, 0.079) mg/L and LC50 = 0.303 (0.192, 0.472) mg/L. This is consistent with our second hypothesis that *P. forbesi* is sensitive to UIA at high pH when the toxic threshold concentration of UIA is reached and the IA fraction is relatively constant. The result also demonstrates that both UIA and IA contribute to the overall toxicity of TAN. The relative toxicity of the two fractions varies as a function of pH. IA is more toxic at low pH while UIA is more toxic at high pH.

Table 8 Measured TAN and UIA at each pH level

pH	0 hour		pH	24 hour	
	TAN	UIA		TAN	UIA
7.8 ± 0.00	<0.1	<0.1	7.85 ± 0.02	<0.1	<0.1
7.0 ± 0.00	3.41 ± 0.06	0.014 ± 0.000	7.29 ± 0.10	3.58 ± 0.07	0.029 ± 0.001
7.4 ± 0.00	3.61 ± 0.06	0.037 ± 0.001	7.59 ± 0.07	3.66 ± 0.07	0.058 ± 0.001
7.8 ± 0.00	3.71 ± 0.06	0.094 ± 0.002	7.89 ± 0.03	3.66 ± 0.10	0.114 ± 0.003
8.2 ± 0.00	3.69 ± 0.07	0.227 ± 0.004	8.15 ± 0.02	3.68 ± 0.10	0.204 ± 0.005
8.6 ± 0.00	3.63 ± 0.04	0.514 ± 0.005	8.43 ± 0.06	3.58 ± 0.02	0.359 ± 0.002

Table 9 Four days survival of *P. forbesi* exposed to 5 mg/L nominal TAN at various pH levels (N=20 per replicate beaker and a total of 80 juveniles per treatment concentration)

Treatments	Number survivor	Total survivors	% Survival
control pH7.8- A	**15**	**66**	**82.5**
control pH7.8- B	**17**		
control pH7.8- C	**16**		
control pH7.8- D	**18**		
5ppm pH 7.0 - A	**18**	**61**	**76.25**
5ppm pH 7.0 - B	**15**		
5ppm pH 7.0 - C	**12**		
5ppm pH 7.0 - D	**16**		
5ppm pH 7.4 - A	**10**	**45**	**56.25**
5ppm pH 7.4 - B	**10**		
5ppm pH 7.4 - C	**14**		
5ppm pH 7.4 - D	**11**		
5ppm pH 7.8 - A	**10**	**45**	**56.25**
5ppm pH 7.8 - B	**10**		
5ppm pH 7.8 - C	**17**		
5ppm pH 7.8 - D	**8**		
5ppm pH 8.2 - A	**7**	**34**	**42.5**
5ppm pH 8.2 - B	**11**		
5ppm pH 8.2 - C	**10**		
5ppm pH 8.2 - D	**6**		
5ppm pH 8.6 - A	**7**	**24**	**30**
5ppm pH 8.6 - B	**5**		
5ppm pH 8.6 - C	**8**		
5ppm pH 8.6 - D	**4**		

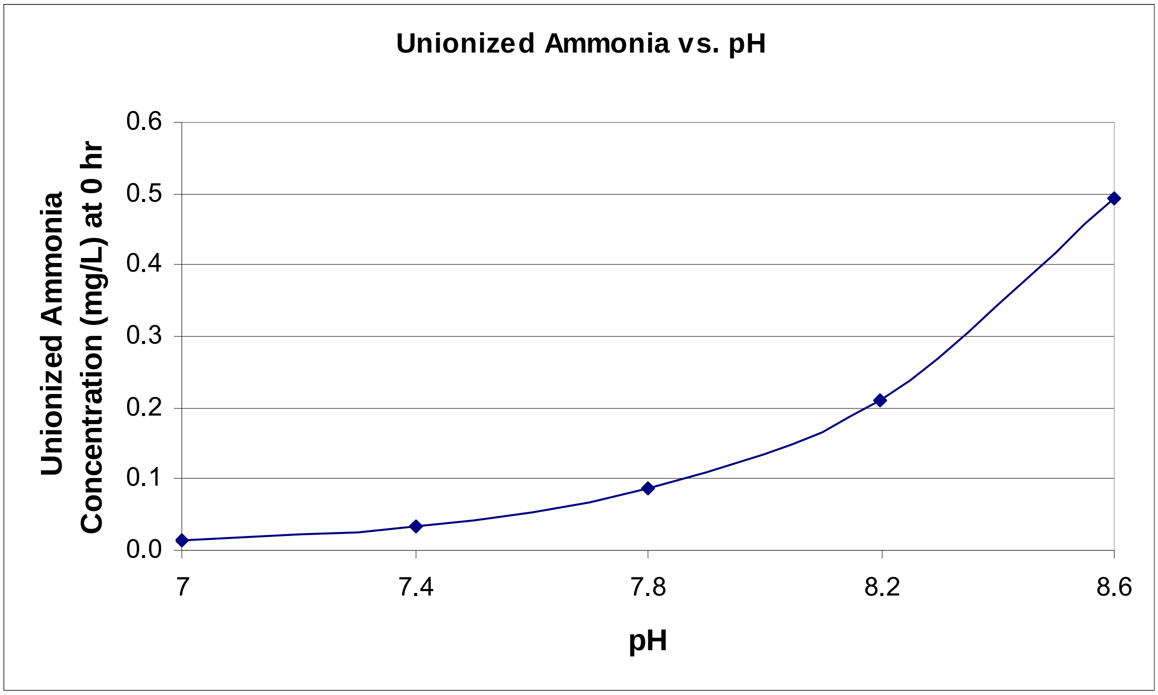


Figure 4 shows increase mean measured UIA concentrations at pH 7, 7.4, 7.8, 8.2, and 8.6 at a constant TAN concentration.

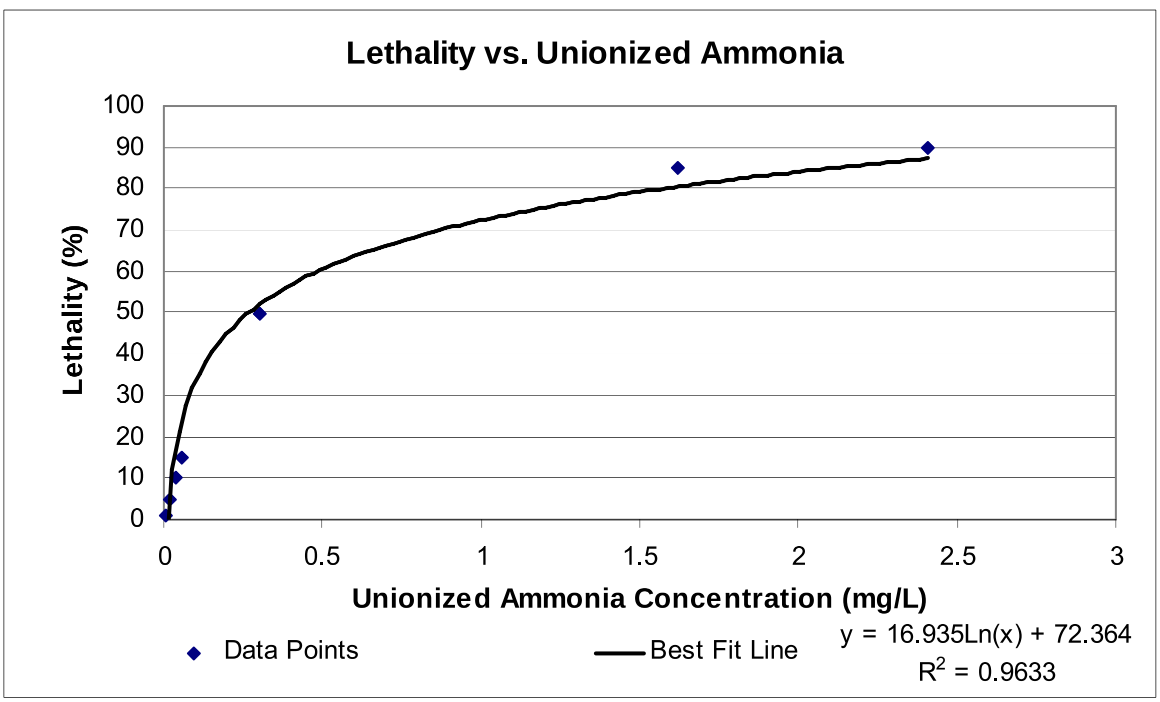


Figure 5 Lethal UIA of *P forbesi* exposed to different pH levels at 20ºC. The UIA concentrations for LC 5, 10, and 50 are 0.021, 0.038, and 0.303 mg/L respectively. Note that lethality at 85% (1.618 mg/L) and 90% (2.406 mg/L) were calculated from EPA Probit analysis program

Subtask 3-3: Full life-cycle static-renewal bioassay (31-d) to estimate the chronic effects of total ammonia on growth and survival of P. forbesi.

The purpose of this task is to determine chronic effect of ammonia on growth and survival of *P. forbesi*. On May 14, 2010, a 31-d life-cycle study was initiated with 4 replicates of 3 gravid female *P. forbesi* per replicate concentration and 5 TAN treatment concentrations (0, 0.50, 1.00, 2.00, and 4.00 mg/L) at 2.06 ± 0.01 ppt salinity, pH= 7.90 ± 0.01, Alkalinity (88.0±2.3mg/L), Conductivity (3243.5±25.4µHMOS), Hardness (436.0±6.1mg/L), Dissolved oxygen (>8 mg/L), and temperature 20°C. 80% of the test waters were replaced every 2 to 3 days and ammonia concentrations in each treatment were analyzed with an ion selective electrode (ISE) and meter (**Table 10**). Raw data of the number of nauplii, juvenile, and adult *P. forbesi* produced per 2-3 days per beaker (n= 3 females per beaker for 4 replicate beakers) is shown in **Appendix I**. **Appendix II** shows the average number of nauplii, juvenile, and adult *P. forbesi* produced in each treatment concentration over time. From **Appendix I**, the sum total number of nauplii, juvenile, and adult produced by each female during the entire course of 31 days of TAN exposure is summarized in **Table 11.** Note: the total number of nauplii, juvenile, and adult produced per female in **Table 11** were derived by dividing the total number of organism produced during the entire course of the 31 days in all beakers by 12 females (3 females per replicate beaker for 4 replicate beakers) (see **Appendix III** for the sum total of nauplii, juvenile, and adult *P. forbesi.* The means with 95% confidence interval (CI) of total number of nauplii, juvenile, and adult *P. forbesi* produced at 31 days of exposure to TAN are presented in **Figure 6A-6C**.

Results indicated a dose-response relationship of TAN exposures and *P. Forbesi* survival and reproduction (Table 11 and Figure 6). At the end of 31-d, the average ratios of new adults produced per initial gravid female as a function of ammonia was: Control (3.9), 0.36 mg TAN/L (2.3), 0.79mg TAN/L (1.9), 1.62mg TAN/L (2.0), and 3.23 mg TAN/L (1.7). The 31-d TAN exposure affected copepods survival and population recruitment where significantly lower numbers of nauplii and juvenile in gravid females exposed to 0.79, 1.62, and 3.36 mg TAN/L when compared to control and 0.36 mg TAN/L. No significant differences in number of nauplii and juvenile were observed between control and 0.36 mg/L treatment groups (**Figures 6A-6B**). At the end of 31-d, significant differences in mean total number of adult *P. forbesi* production were observed between 0.36, 0.79, 1.62, and 3.36 mg TAN/L treatment group and control ($P<0.05$; **Figure 6C**). Both one-way ANOVA and Kruskal-Wallis test indicated that ammonia significantly affects the population of *P. forbesi* (p-values 0.0004, 0.0075 respectively). The ANOVA analysis of differences in population decline between control and 0.36 mg TAN/L TAN treatment group showed significant difference at p-value = 0.0256 while the Kruskal-Wallis test yielded marginal significance (p-value = 0.059). Therefore, we estimated the Lowest Observable Effective Level (LOEL) of TAN to be at 0.36 mg TAN/L. A No Observed Effect Level (NOEL) was impossible to calculate but must be less than 0.36 mg TAN/l.

Table 10 Mean (mg/L) ± standard error of nominal and measured concentrations of Total Ammonia Nitrogen (TAN)

Nominal TAN	Measured TAN		
	0 hr	48 or 72 hrs	Average from 0 to 48 or 72 hr
Control	<0.1	<0.1	<0.1
0.5	0.36 ± 0.01	0.49 ± 0.01	0.42 ± 0.01
1.0	0.79 ± 0.01	0.94 ± 0.02	0.86 ± 0.01
2.0	1.62 ± 0.02	1.72 ± 0.02	1.65 ± 0.01
4.0	3.23 ± 0.03	3.36 ± 0.02	3.29 ± 0.02

Table 11 Mean number of nauplii, juvenile, and adult *P. forbesi* produced per female at the end of 31 days of exposure to total ammonia nitrogen (TAN)

Measured mg TAN/L	# of nauplii	# of juvenile	# of Adult
0	27.417	17.333	3.917
0.36	23.000	17.167	2.333
0.79	15.083	9.833	1.917
1.62	14.917	3.167	2.000
3.23	10.500	2.750	1.667

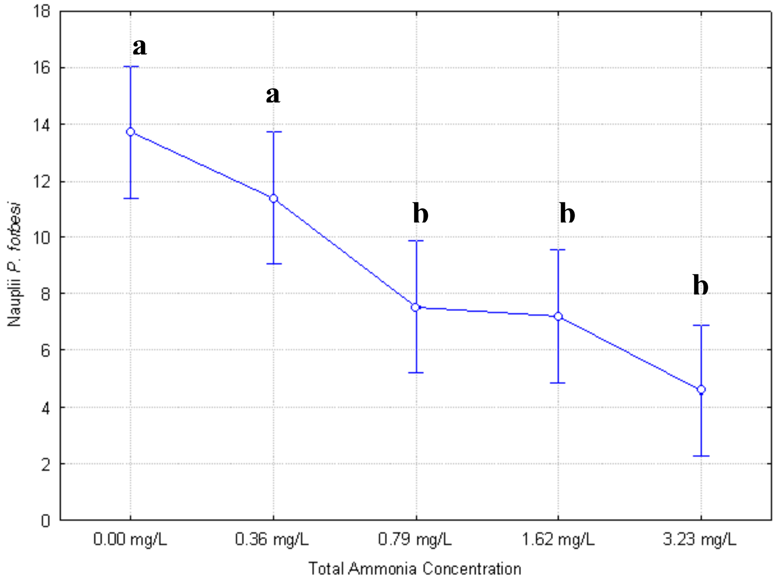


Figure 6A Mean total number of *P. forbesi* nauplii produced per beaker during the 31 days of exposure to total ammonia. Different letters indicate significant difference (P<0.05) among ammonia treatments. (Bars = 0.95 confidence intervals).

Cell No.	Duncan test; variable Nauplii *P. forbesi* Total Ammonia Concentration	{1} 13.708	{2} 11.375	{3} 7.5417	{4} 7.2083	{5} 4.5833
1	0.00 mg/L		0.162817	0.000513	0.000327	0.000029
2	0.36 mg/L	0.162817		0.022871	0.018088	0.000191
3	0.79 mg/L	0.000513	0.022871		0.841388	0.094742
4	1.62 mg/L	0.000327	0.018088	0.841388		0.116830
5	3.23 mg/L	0.000029	0.000191	0.094742	0.116830	

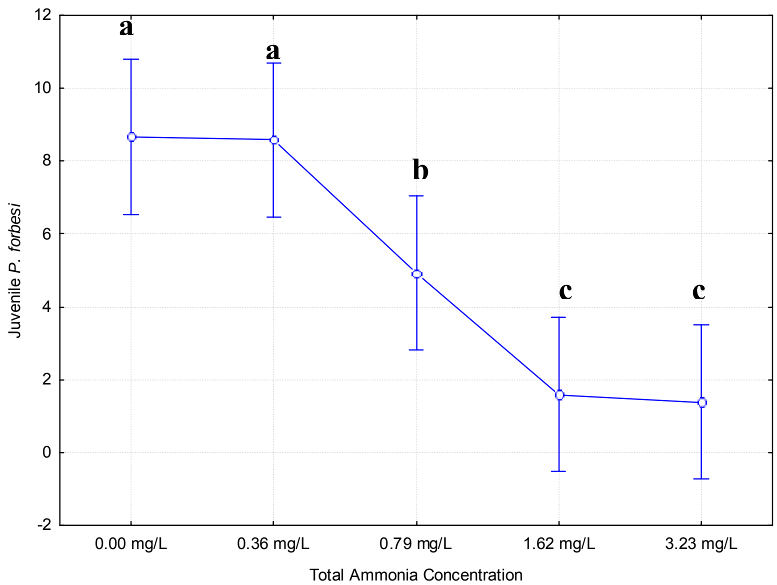


Figure 6B Mean total number of juvenile *P. forbesi* produced per beaker during the 31 days of exposure to total ammonia. Different letters indicate significant difference (P<0.05) among ammonia treatments. (Bars = 0.95 confidence intervals)

Cell No.	Duncan test; variable Juvenile*P. forbesi* Total Ammonia Concentration	{1} 8.6667	{2} 8.5833	{3} 4.9167	{4} 1.5833	{5} 1.3750
1	0.00 mg/L		0.956220	0.019417	0.000058	0.000038
2	0.36 mg/L	0.956220		0.016911	0.000069	0.000055
3	0.79 mg/L	0.019417	0.016911		0.029498	0.027373
4	1.62 mg/L	0.000058	0.000069	0.029498		0.890708
5	3.23 mg/L	0.000038	0.000055	0.027373	0.890708	

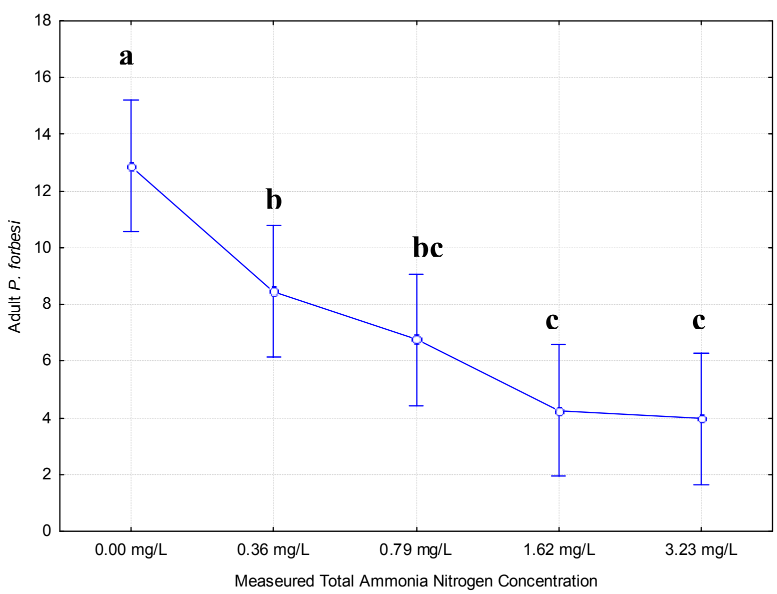


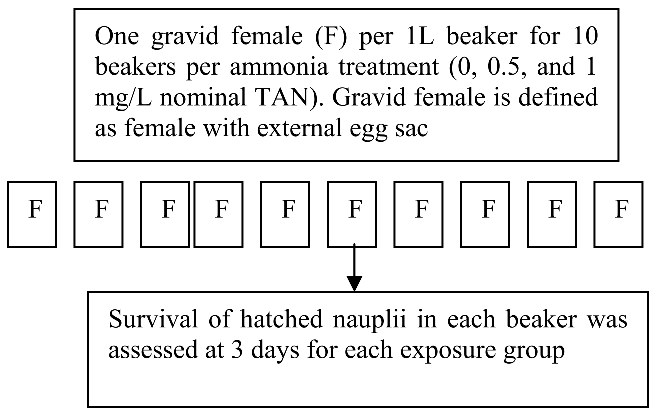
Figure 6C Mean total number of adult *P. forbesi* produced per beaker during the 31 days of exposure to total ammonia. Different letters indicate significant difference (P<0.05) among ammonia treatments. (Bars = 0.95 confidence intervals).

Cell No.	Duncan test; variable Adult *P. forbesi* Total Ammonia Concentration	{1} 12.875	{2} 8.4688	{3} 6.7500	{4} 4.2500	{5} 3.9688
1	0.00 mg/L		0.008099	0.000346	0.000003	0.000004
2	0.36 mg/L	0.008099		0.301567	0.015206	0.011641
3	0.79 mg/L	0.000346	0.301567		0.132934	0.114624
4	1.62 mg/L	0.000003	0.015206	0.132934		0.865771
5	3.23 mg/L	0.000004	0.011641	0.114624	0.865771	

Subtask3-4-1: Reproductive fitness of adult female P. forbesi exposed to 0, 0.5, and 1.0 mg/L of total ammonia at pH 7.8 and 20ºC

The purpose of this subtask was to follow up on the 31-d full life cycle test and reassess the fecundity and fertility of adult females exposed to ammonia and the survivability of nauplii as the full life cycle testing suggested that reproduction and/or survival of the early nauplii was the most sensitive life stage. **Figure 7** shows the experimental design for Subtask3-4-1 initiated on August 20, 2010. Briefly, we exposed one gravid female per replicate using 1L-beaker for each of 10 replicates per treatment group for three treatment concentrations (0, 0.5, and 1.0 mg/L) of total ammonia. A total of 30 beakers for 2 days were used. The number of nauplii generated from each gravid female in the 10 beakers was counted at day-3 to assess the survival of newly hatched nauplii.

Figure 7 shows the experimental design for Subtask 3-4-1



Result of Task3-4-1 is summarized in **Table 12**. **Table 13** shows the ammonia concentrations in each treatment analyzed with an ion selective electrode (ISE) and meter. The average number of offspring in the Control, 0.38, and 0.79 mg/L TAN treatments was 7.6, 5.5, and 5.4 nauplii per female, respectively. Analysis of Variance and Dunnett's Multiple Comparison Test demonstrated that the number of nauplii present at day three were less in the 0.38 mg/ L treatment than in the control (**Appendix IV**). On average the 0.38 mg/L TAN treatment had 27.6% less young than the control. The LOEL was 0.38±0.011 mg/L measured TAN. It is impossible to calculate a NOEL from these data. These results confirm the earlier findings of the full life cycle test that population level effects occur at concentrations less than 0.36 to 0.38 mg TAN/L.

Table 12 Number of nauplii present per beaker on day3 in each treatment for subtask 3-4-1

	Control									
	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10
Day0	1GF	1GF	1GF	1GF	1GF	1GF	1GF	1GF	1GF	1GF
Day3	9	7	8	8	9	6	10	6	8	5
	0.5 mg/L									
	0.5-1	0.5-2	0.5-3	0.5-4	0.5-5	0.5-6	0.5-7	0.5-8	0.5-9	0.5-10
Day0	1GF	1GF	1GF	1GF	1GF	1GF	1GF	1GF	1GF	1GF
Day3	10	3	4	6	6	3	10	5	3	5
	1.0 mg/L									
	1.0-1	1.0-2	1.0-3	1.0-4	1.0-5	1.0-6	1.0-7	1.0-8	1.0-9	1.0-10
Day0	1GF	1GF	1GF	1GF	1GF	1GF	1GF	1GF	1GF	1GF
Day3	7	5	7	6	6	5	3	3	9	3

GF= Gravid female, i.e., with external egg sac

Table 13 Nominal and measured (mean ± standard error) TAN concentrations

Nominal mg TAN/L	Temp (°C)	Alkalinity (mg/L)	Conductivity µHMOS	DO mg/L	Hardness mg/L	pH	Salinity ppt	Measured mgTAN/L
0	20	80	3000	>8	440	7.8	2	<0.1
0.5	20	80	3000	>8	440	7.8	2	0.38±0.011
1.0	20	80	3000	>8	440	7.8	2	0.79±0.003

Subtask3-4-2: Survival of 3-d old of P. forbesi nauplii exposed to TAN at pH 7.8 and 20ºC

The purpose of this subtask was to investigate the effects of ammonia exposure to nauplii to determine the mean lethal concentration. On October 5, 2010, groups of 3 day old *P. forbesi* (N = 15 per replicate; 4 replicates per concentration) were exposed separately to ammonia for 4 days at 20ºC and pH 7.8.

Results of Subtask3-4-2 are summarized in **Table 14**. **Table 15** shows the nominal and measured concentrations of TAN analyzed with an ion selective electrode (ISE) and meter. U.S. Environmental Protection Agency Probit Analysis Program results revealed the measured lethal concentrations (LC) of total ammonia for *P forbesi* nauplii at day 4 to be: LC5 = 0.591 mg TAN/L; LC10 = 0.731 mg TAN/L; LC50 = 1.547 mg TAN/L. Using Fisher Exact/Bonferroni-Holm Test, the EC5 and EC10 with 0.95 confidential intervals were 0.1773 (0.1373, 0.7134) and 0.62 (0.1758, 0.9667) mg TAN/L and the NOEL and LOEL were 0.62 mg/L and 0.95 mg TAN/L respectively for *P. forbesi* nauplii (**Appendix V**). Results indicated the nauplii stages (4d-LC50 = 1.547 mg/L) are more sensitive to TAN than the juvenile stages (6d-LC50 = 6.014 mg/L) but less sensitive than newly hatched nauplii. The newly hatched nauplii had an LOEL of 0.38 mg TAN/L while a similar effect level for 3 day old individuals was 0.95 mg TAN/L, respectively. These results emphasize the importance of testing with as young a copepod life stage as possible.

Table 14 Acute ammonia toxicity testing of 3-day-old nauplii (N=15 per replicate beaker and a total of 60 nauplii per treatment concentration)

Treatments	Number Survivor	Total survivors	% Survival
control - A	12	50	83.33
control - B	15		
control - C	11		
control - D	12		
0.4 ppm - A	9	45	75.00
0.4 ppm - B	13		
0.4 ppm - C	15		
0.4 ppm - D	8		
0.8 ppm - A	12	45	75.00
0.8 ppm - B	13		
0.8 ppm - C	8		
0.8 ppm - D	12		
1.2 ppm - A	11	38	63.33
1.2 ppm - B	6		
1.2 ppm - C	11		
1.2 ppm - D	10		
1.6 ppm - A	9	31	51.67
1.6 ppm - B	7		
1.6 ppm - C	8		
1.6 ppm - D	7		

Table 15 Nominal and measured (mean ± standard error) of total ammonia concentrations

Nominal mgTAN/L	Temp (°C)	Alkalinity (mg/L)	conductivity (μHMOS)	DO mg/L	Hardness mg/L	pH	Salinity ppt	Measured mgTANmg/L
0	20	95±9	3238±79	>8	458±27	7.8	2	<0.1
0.4	20	95±9	3238±79	>8	458±27	7.8	2	0.26±0.01
0.8	20	95±9	3238±79	>8	458±27	7.8	2	0.62±0.01
1.2	20	95±9	3238±79	>8	458±27	7.8	2	0.95±0.02
1.6	20	95±9	3238±79	>8	458±27	7.8	2	1.23±0.02

Discussion and Conclusions:

The overall objectives of this study are to 1) investigate the acute and chronic toxic effects of ammonia on the estuarine copepod *P. forbesi*, and 2) determine whether environmentally relevant concentrations of ammonia in the SFE potentially contribute to the decline in the abundance of *P. forbesi*.

Eurytemora affinis and *Pseudodiaptomus forbesi* are the principal food source of endangered larval and pelagic fish in the SFE. The abundance of pelagic organisms including fish, zooplankton (copepods) and nutritious phytoplankton have declined to record low numbers in this estuarine ecosystem (Sommer et al., 2007). The 20 mm survey by CDFG indicated that *P. forbesi* were abundant in June 2008 at the Cache Slough region that provide important spawning habitats to pelagic fishes in the upper SFE. In addition, based on SWRCB's field water quality data collected in the North delta, *P. forbesi* populations were observed in these sites at pH ranges of 7.4 to 7.8 and water temperature of 20°C. These pH and temperature levels were therefore selected in our laboratory studies to match the conditions of the copepod habitats in the field. It should be noted however, that other water quality parameters such as alkalinity, electric conductivity, salinity, and water hardness may have potentially affected the toxicity of total ammonia to *P. forbesi*. In this context, additional investigations are warranted on the impacts of these parameters on the growth, reproduction, and survival of the copepods.

Acute Toxicity of Ammonia and effects of pH on ammonia toxicity

Two sets of results demonstrate that *P. forbesi* is more acutely sensitive to TAN at low pH values. At 8 mg TAN/L the time to 50% mortality of *P. forbesi* was approximately 1.5 times faster at pH 7.4 (76 hr) than at pH 7.8 (135 hr) (**Figures 8A** and **8B**). In addition, 50% mortality was also observed in *P. forbesi* exposed to 4 (83hr) and 6 (93hr) mg TAN/L at pH 7.4. This result was not observed for *P. forbesi* at pH 7.8 even with extended periods of exposure time (**Figures 8a** and **8B**). Finally, in **Figure 9**, the 4d-LC50 at pH 7.4 was 2.96 mg TAN/L while the 6 day LC50 at pH 7.8 was 6.014 mg TAN/L. These results clearly demonstrate that *P. forbesi* is more sensitive to ammonia at lower pH and that the copepod is more sensitive to IA than to UIA**.**

To determine a threshold concentration of UIA that is toxic to *P. forbesi*, an additional ammonia toxicity assay was initiated in Task3-2. Assuming *P. forbesi* is not sensitive to changes in pH alone, results indicate a decrease *P. forbesi* survival at relatively constant IA but a higher UIA concentration. Time to 50% mortality of *P. forbesi* was faster at pH 8.6 (66 hr) when compared to pH 8.2 (87 hr) at 3.71 mg/L TAN (**Figure 10**). The 4d-LC50 was 0.303 mg UIA/L**.** This demonstrates that both the UIA and IA fractions contribute to the overall toxic response of the organism.

The equilibrium equation of TAN based on the presence of IA and UIA forms are outlined below:

:

$$\text{LOW PH} \leftarrow \qquad \rightarrow \text{HIGH PH}$$
$$NH_4^+ \text{(IA)} + H_20 \leftrightarrow NH_3 \text{(UIA)} + H_30^+$$

$$\text{TAN} = \text{IA} + \text{UIA}$$

Taken together, these results demonstrate that *P. forbesi,* unlike fish, is more sensitive to TAN at lower pH values. One of the potential factors affecting variability to pH sensitivities are attributed to the physiologic and mechanistic differences in ammonia excretion between fish and copepods. Because fish excrete UIA as waste, fish are more sensitive to higher environmental pH promoting higher concentrations of UIA. In contrast, copepods excrete IA as waste, this fraction increases with lower environmental pH levels. We hypothesize that osmoregulatory and excretory systems of less than 3 day old nauplii may not be sufficiently developed to pump intercellular IA concentrations against a high external IA gradient. Further, the majority of ammonia in the environment is in the IA form. Because *P. forbesi* is more sensitive to IA as we have demonstrated in our studies, exposure to the dominant ionized form in the environment may render relatively more adverse effects to copepods than to fish. For further information on the potential differences between fish and copepods on ammonia susceptibility, the readers are directed to refer to review articles by Wilkie, 1997 and Jawed, 1973 on the mechanisms of ammonia excretion in fish and zooplankton. Finally, others investigators have observed increasing invertebrate sensitivity to TAN with decreases in pH (Armstrong et al 1978; Borgmann, 1994; Ankley et al 1995). Our results are consistent with their observations that copepod like amphipod may be responding more to IA than to UIA.

Since environmentally relevant concentrations of total ammonia and pH are rarely observed at concentrations >1 mg/L and >pH 8.2 in the SFE, toxic effects of UIA which is usually < 1-2% of TAN depends on pH and temperature is unlikely the major factor affecting the survival of *P. forbesi* in this study. It has been demonstrated in these studies that sufficiently high concentrations of IA in water of low pH is lethal to *P. forbesi*, even though the UIA concentration may not be. It is probably an over-simplification to attribute the toxicity of ammonia only to UIA at high pH and to IA at low pH. There may be a contribution from each ionized and unionized fraction at a TAN concentration found to be toxic. It should be noted that our result does not suggest that copepods are not sensitive to UIA, but that more UIA is needed to decreased copepod survival compared to less IA at lower pH. Accordingly, we suggest caution in the interpretation of results on ammonia-induced responses of different test organisms to consider the contribution from both UIA and IA fractions. Because 99.02% (2.931 of 2.960 mg/L) of the TAN is in IA form and a small fraction of 0.98% (0.029 of 2.960 mg/L) is UIA form, and IA was shown to be the most toxic fraction to *P. forbesi* in current study, for simplicity, we recommend that future copepod studies on ammonia will be expressed in terms of TAN. The intention is to suggest using TAN instead of IA or UIA alone despite the sensitivity differences in ammonia between copepods and fish.

The acute toxicity of nauplii to TAN was also evaluated in Task3-4-2. Results indicated that the nauplii stages (4d-LC50 = 1.547 mg/L @ pH7.8) was more sensitive to TAN than the juvenile stages (6d-LC50 = 6.014 mg/L @ pH7.8 or 4d-LC50 = 2.96 mg/L @ pH7.4). These results further support the hypothesis that early life stages are more sensitive to TAN than juvenile and adult stages.

Chronic Toxicity of Ammonia

There is a dose-response relationship of ammonia exposure and the number of nauplii, juvenile, and adult *P. forbesi* production (**Table 11, Appendix II and III**). A decline in the abundance of appropriate-sized prey can have detrimental effects on larval fish (Houde 1987). For example, reduced larval growth may lead to long periods of vulnerability to predation and starvation that may reduce available reserved energy, and reduced swimming speed to evade predators (Moyle 1992, Houde 1987, Nobriga 2002). Most larval fish in the SFE begin their feeding regimen on zooplankton such as *E. affinis* and *P. forbesi* during exogenous feeding. The abundance of these copepods at all life stages of development in important spawning habitats of pelagic fishes such as the Cache Slough region is therefore very critical to the survival and growth of larval fishes (Sommer et al., 2007). Missed opportunities for larval fishes to predate on nauplii and juvenile copepods, and for juvenile and adult fishes to graze on adult copepods, can render serious consequences in the growth and reproduction of native fishes in California.

Results in Task3-3 indicated significantly lower numbers of nauplii and juvenile when gravid females were exposed to 0.79, 1.62, and 3.36 mg/L compared to control and 0.36 mg/L TAN (**Figures 6A-6B**). No significant differences in the number of nauplii and juveniles were observed between control and 0.36 mg/L treatment groups (**Figures 6A-6B**). However, at the end of 31-d, significant differences in mean total number of adult *P. forbesi* production were observed between 0.36, 0.79, 1.62, and 3.36 mg/L treatment group and control ($P<0.05$; **Figure 6C**). These results are supported in Task3-4 showing significantly lower number of newborn nauplii surviving to 3-day old when exposed to 0.38 mg/L TAN. In addition, **Appendix III** shows the survivals of nauplii to juvenile stage were 63.2% (208/329) for 0 mg/L, 74.6% (206/276) for 0.36 mg/L, and 65.2% (118/181) for 0.79 mg/L of TAN while the survival of juvenile to adult stage were 22.6% (47/208), 13.6% (28/206), and 19.5% (23/118), respectively. These results indicated the overall lower recruitment rate of juvenile to new adult *P. forbesi* despite initial higher number of nauplii production by female exposed to 0.36 mg TAN/L and suggested that chronically exposure to TAN can affect *P. forbesi* survival. Based on these results, we postulated that chronic exposure of *P. forbesi* to environmentally relevant concentrations of 0.3-0.8 mg TAN/L may have significant implications on adult copepod recruitment hence the abundance of *P. forbesi* in the SFE.

In conclusion, results of the acute toxicity testing of *P. forbesi* nauplii and juveniles suggested that the environmentally relevant concentrations of TAN measured in water samples collected from Sacramento River at Hood and Cache Slough region between April and July 2009 do not affect the survival of nauplii and juvenile *P. forbesi*. However, the chronic 31-d life-cycle and newborn nauplii toxicity studies confirmed lower recruitment of new adult *P. forbesi* in gravid females chronically exposed to 0.36 and 0.79 mg TAN/L and newborn nauplii acutely exposed to 0.38 mg TAN/L when compare to the control. Our research demonstrates that concentrations of TAN in the Sacramento River at and downstream of Hood are potentially at toxic levels to *P. forbesi* (**Figure 11).** In contrast, it is impossible to determine whether TAN concentrations in the Cache Slough complex are toxic or not. The LOEL for *P. forbesi* at environmentally realistic River and Slough pH and temperatures values is between 0.36 and 0.38 mg TAN/L. Average TAN concentrations in summer at Hood were 0.46 with one value as high at 0.65 mg/L (**Table 1A**). Concentrations in the Sacramento River as far downstream as Isleton periodically reached 0.40 mg/L during the summer of 2009 (**Figure 11** and Foe et al., 2010). Isleton is 30 river miles below Hood. In contrast, average TAN concentrations during the same time period in the Cache Slough complex were between 0.01 and 0.1 mg/L. The highest TAN value measured in the Slough was 0.23 mg TAN/L on 27 April in the lower Ship Channel at the entrance to Cache Slough. This value is about 60 percent of the acute LOEL value for 3-day old nauplii. The present study did not determine a NOEL for this life stage. So, it is impossible to ascertain whether toxic episodes of TAN extend to the Cache Slough complex or not.

Figure 8A Time to 50% mortality of *P. forbesi* at pH 7.8

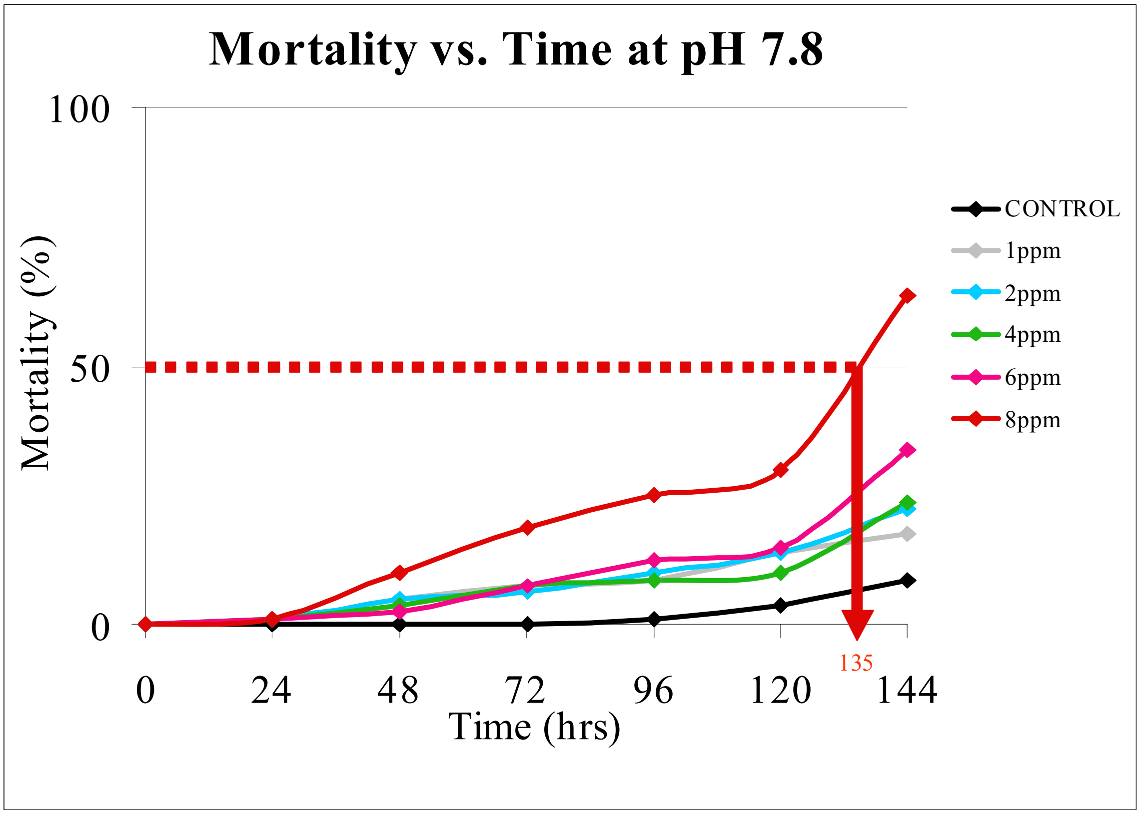


Figure 8B Time to 50% mortality of *P. forbesi* at pH 7.4

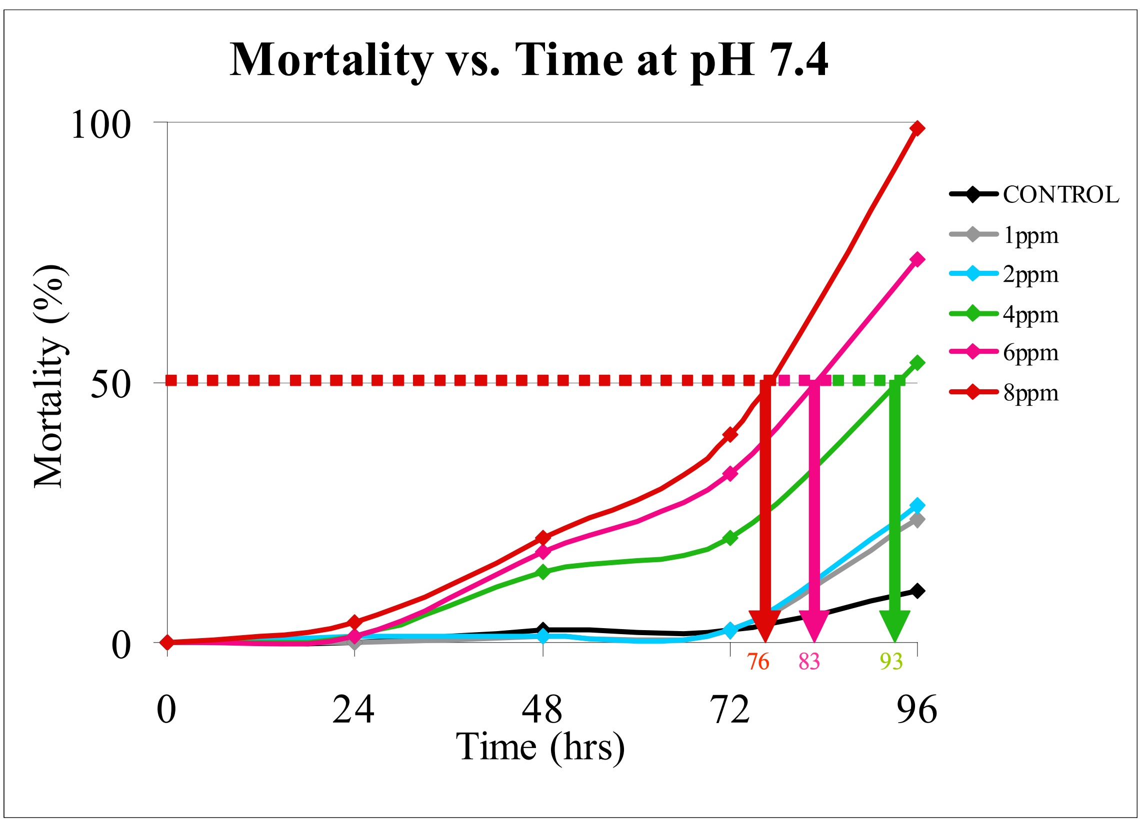


Figure 9 LC50 of *P. forbesi* at pH7.4 and 7.8

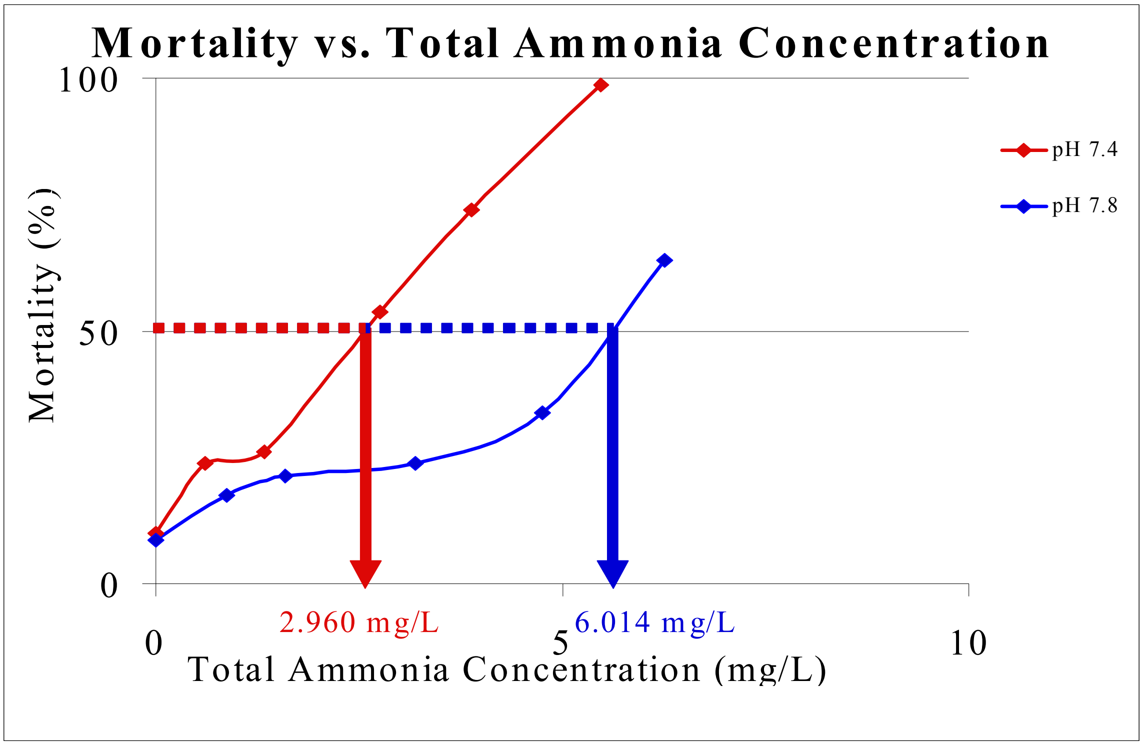


Figure 10 Time to 50% mortality of *P. forbesi* at different pH levels

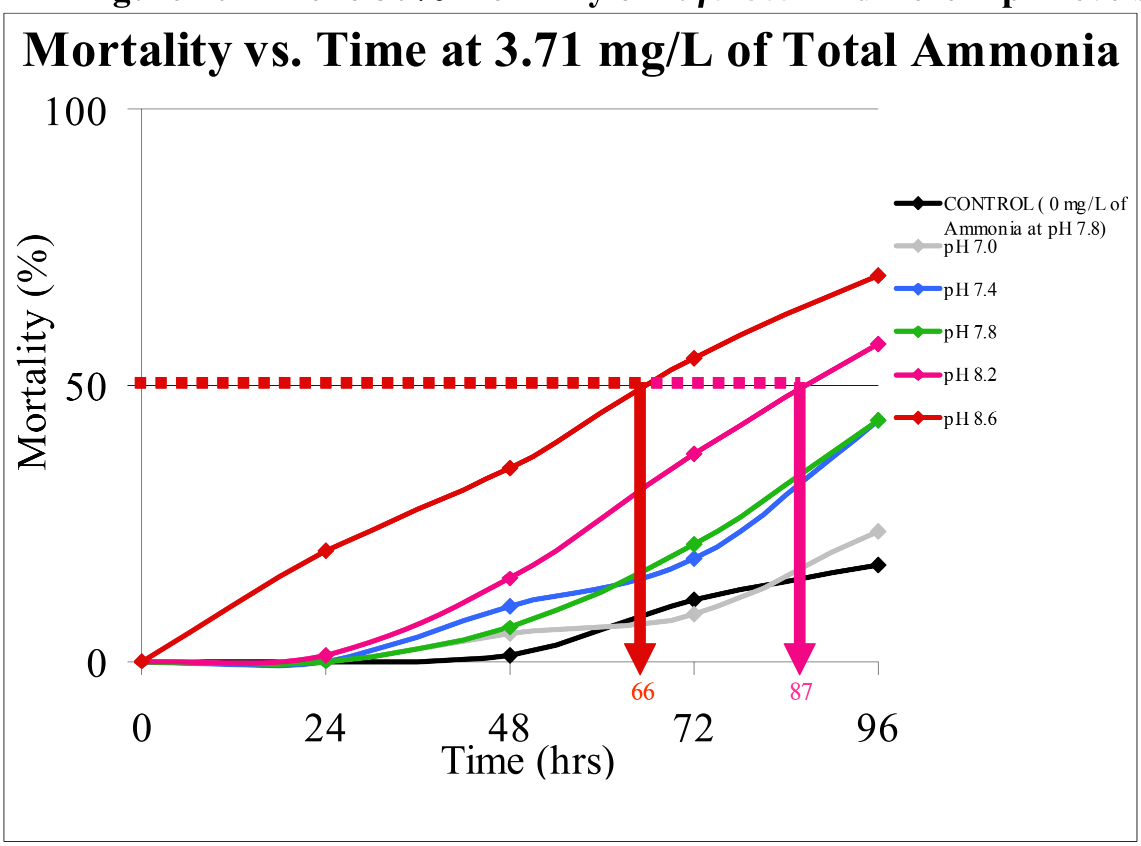
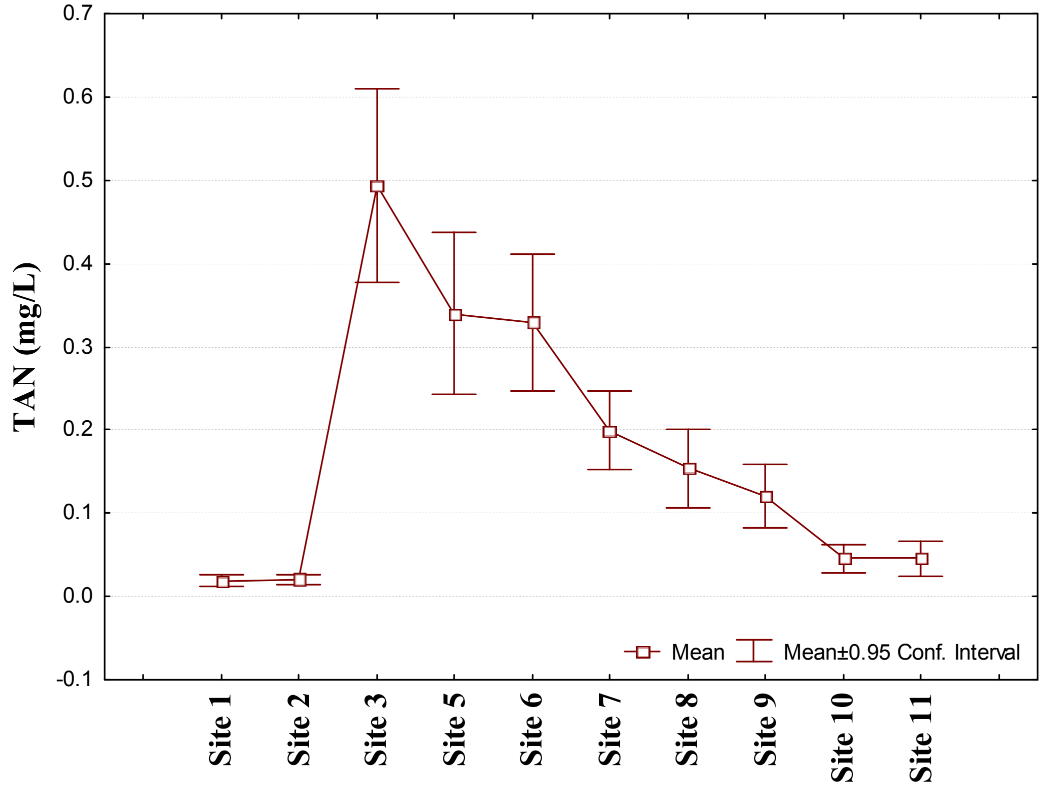
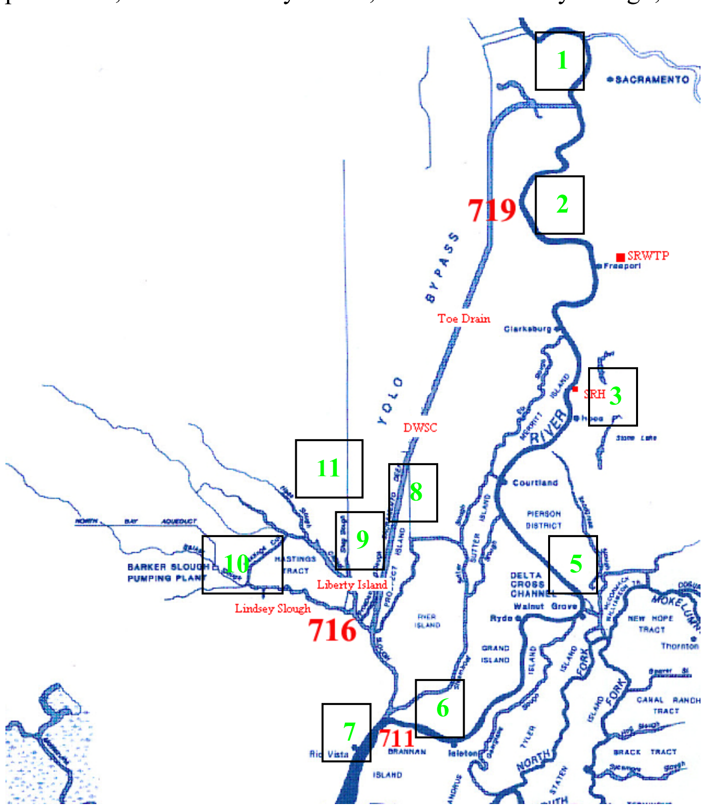


Figure 11. TAN concentrations in the North Delta collected by the SWRCB (Foe et al., 2010) between March 16 and December 8 2009



Site 1 = Sacramento River at Tower Bridge; Site 2 = Sacramento River at Garcia Bend; Site 3 = Sacramento River at Hood; Site 5 = Sacramento River at Walnut Grove; Site 6 = Sacramento River at Isleton; Site 7= Sacramento River at Rio Vista; Site 8 = Deep water Ship Channel; Site 9 = Liberty Island; Site 10 = Lindsey Slough; and Site 11 = Toe Drain.



References

Armstrong, D.A., D. Chippendale, A.W. Knight, and J.E. Colt. 1978. Interaction of ionized and un-ionized ammonia on short-term survival and growth of prawn larvae, *Macrobrachiurn rosenbergii*. Biol. Bull. (Woods Hole, Mass.), 154:15-34.

Ankley, GT, MK Schubauer-Berigan, and PD Monson. 1995. Influence of pH and Hardness on Toxicity of Ammonia to the Amphipod *Hyalella azteca*. Can. J. Fish. Aquat. Sci. 52:2078-2083

Augspurger T, Keller AE, Black MC, Cope G, Dwyer FJ. 2003. Derivation of water quality guidance for protection of freshwater mussels (Unionidae) from ammonia exposure. Environ Toxicol Chem 22:2569–2575.

Borgmann, U. 1994. Chronic toxicity of ammonia to the amphipod *Hyallella azteca*: importance of ammonium ion and water hardness. Environ. Pollut. 86: 329-335.

Foe C., Ballard, A, Fong S, 2010. Nutrient concentrations and biological effects in the Sacramento-San Joaquin Delta. Technical report. 90 pg.

Ger KA., Teh SJ, Goldman CR, 2009a. Microcystin-LR toxicity on dominant copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* on the upper San Francisco Estuary. Science of the Total Environment 407:3854-4857.

Ger KA, Teh SJ, Baxa DV, Lesmeister S, Goldman CR, 2009b. The effects of dietary *Microcystis aeruginosa* and microcystin on the copepods of the upper San Francisco Estuary. Freshwater Biology, 55, 1548–1559

Ger, K.A., Arneson, P., Goldman, C.R, and Teh, S.J. 2010. Species specific differences in the ingestion of Microcystis cells by the calanoid copepods Eurytemora affinis and Pseudodiaptomus forbesi J. Plankton Research, 32 (10):1479-1484

Houde, ED. 1987. Fish early life dynamics and recruitment variability. American Fisheries Society Symposium 2:17-29.

http://www.science.calwater.ca.gov/events/workshops/workshop_ammonia.html

http://science.calwater.ca.gov/pdf/workshops/workshop_ammonia_bckgrnd_paper_nh4-nh3_030209.pdf

http://www.dfg.ca.gov/delta/projects.asp?ProjectID=20mm

Jawed, M. 1973. Ammonia excretion by zooplankton and its significance to primary productivity during summer. Marine biology, 23: 2,115-120

Johnson, MW. 1948. The postembryonic development of the copepod *Pseudodiaptomus euryhalinus Johnson*, and its phylogenetic significance. Transactions of the American Microscopical Society 67, 319-330.

Kimmerer W. 2004. Open Water Processes of the San Francisco Estuary: From Physical Forcing to Biological Responses. SAN FRANCISCO ESTUARY AND WATERSHED SCIENCE, 2(1): Article 1

Kormanigk A and Camerojn N. 1981. Ammonia excretion in animals that breathe water: A review. Marine Biology Letters 2: I 1-23.

Losordo, T. M., M. P. Masser, and Racocy J. 1992. Recirculating aquaculture tank production systems. An overview of critical conservations. Publication no. 451.

Southern Regional Aquaculture Center, Stoneville, Mississippi, USA.

Lopez CB, Cloern JE, Schraga TS, Little AJ, Lucas LV, Thompson JK, Burau JR. 2006 Ecological Values of Shallow Water Habitats: Implications for the restoration of distrurbed ecosystems. ECOSYSTEMS, 9: 422-440.

Masser, M. P., J. Rakocy, and T. M. Losordo. 1992. Recirculating aquaculture tank production system. Management of recirculating systems. Publication no. 452. Southern Regional Aquaculture Center, Stoneville, Mississippi, USA

Moyle, PB, Herbold, B, Stevens, DE, and LW Miller. 1992. Life history and status of delta smelt in the Sacramento-San Joaquin Estuary, California. Transactions of the American Fisheries Society 121:67-77.

Nobriga, ML 2002. Larval Delta Smelt Diet Composition and Feeding Incidence: Environmenatl and Ontogenetic Influences. California Fish and Game 88(4):149-164.

Pequeuax A and Gilles R 1981. Na+ fluxes across isolated perfueed gills of the Chinese crab *Eriocheir sinensis*. Journal of Experimental Biology 3:73-186.

Regnault M. 1987. Nitrogen Excretion in Marine and Fresh-Water Crustacea. Biol. Rev. 62:1-24.

Sampaio, L. A., W. Wasielesky, and K. Campos Miranda-Filho. 2002. Effect of salinity on acute toxicity of ammonia and nitrite to juvenile *Mugil platanus*. Bulletin of Environmental Contamination and Toxicology 68:668–674.

Sommer T., Armor C., Baxter R., Breuer R., Brown L., Chotkowski M., Culberson S., Feyrer F., Gingras M., Herbold B., Kimmerer W., Mueller-Solger A., Nobriga M., and K. Souza 2007.The collapse of pelagic fishes in the upper San Francisco Estuary. Fisheries, 32, 270–277.

Szumski DS, Barton DA, Putnam HD, Polta RC. 1982. Evaluation of EPA Un-Ionized Ammonia Toxicity Criteria. Journal (Water Pollution Control Federation), Vol. 54, No. 3, Part I (Mar., 1982), pp. 281-291.

Teh SJ, Lu, M, Teh, FC.Lesmeister S, Werner, I, Krause, J and Deanovic L. 2008. Toxic effects of surface water in the upper San Francisco Estuary on *Eurytemora Affinis.* Final report to San Luis and Delta-Mendota Water Authority.

Teh SJ, Lesmeister S, Flores I, Kawaguchi, M and Teh, FC. 2009. *Acute Toxicity of Ammonia, Copper, and Pesticides to Eurytemora affinis and Pseudodiaptomus forbesi* http://www.swrcb.ca.gov/rwqcb5/water_issues/delta_water_quality/ambient_ammonia_concentrations/index.shtml

United States Environmental Protection Agency. 1985. Ambient water quality criteria for ammonia - 1984. EPA-440/5-85-001. National Technical Information Service, Springfield, VA.

United States Environmental Protection Agency 1999. 1999 Update of ambient water quality criteria for ammonia. EPA-822-R-99-014. National Technical Information Service, Springfield, VA.

United States Environmental Protection Agency. 2002. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fifth Edition. October 2002 EPA-821-R-02-012.

United States Environmental Protection Agency. 2002. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Fourth Edition. October. EPA-821-R-02-013.

United States Environmental Protection Agency 2009. Draft 2009 update aquatic life ambient water quality criteria for ammonia – Freshwater. December EPA-822-D-09-001.

Wilkie, M. 1997. Mechanisms of ammonia excretion across fish gills. Comp. Biochem. Physiol. 118A: 1, 39–50.

Emerson, K., R.C. Russo, R.E. Lund and R.V. Thurston. 1975. Aqueous ammonia equilibrium calculations: Effect of pH and temperature. J. Fish. Res. Bd. Can. 32:2379-2383.

Wang N, Ingersoll CG, Greer IE, Hardesty DK, Ivey CD, Kunz JL, Dwyer FJ, Roberts AD, Augspurger T, Kane CM, Neves RJ, Barnhart MC. 2007. Chronic toxicity of copper and ammonia to juvenile freshwater mussels (Unionidae). Environ Toxicol Chem 26:2048–2056.

Werner, I., Markiewicz, D., Deanovic, L., Connon, R., Beggel, S., Teh, S., Stillway, M., and Reece, C. 2010. Pelagic Organism Decline (POD): Acute and Chronic Invertebrate and Fish Toxicity Testing in the Sacramento-San Joaquin Delta 2008-2010. Final Report to the California Department of Water Resources, Sacramento, CA.

Wood, C.M. 1993. Ammonia and urea metabolism and excretion. In: The physiology of fishes. Evans, D.H. (Ed.). CRC Press, Ann Arbor, MI. pp 379-424.

Appendix I. Raw data of 31 days *P. forbesi* full life-cycle static renewal bioassay

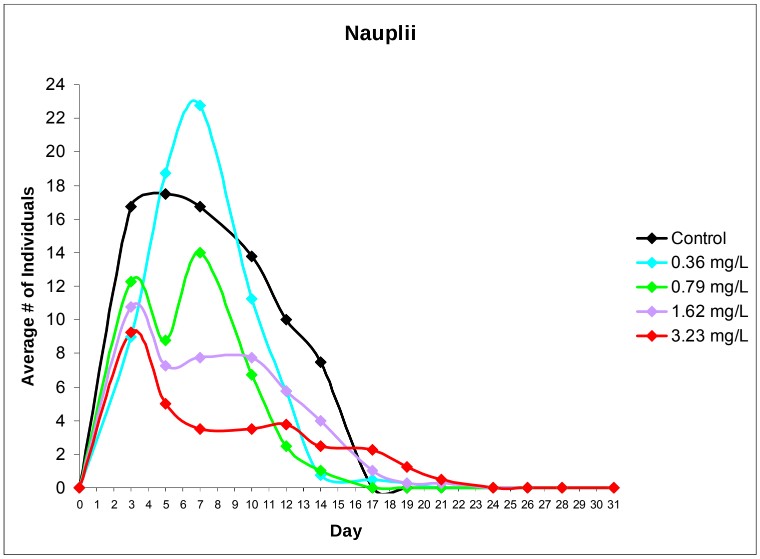
NAUPLII														
Treatment	DAY0	DAY3	DAY5	DAY7	DAY10	DAY12	DAY14	DAY17	DAY19	DAY21	DAY24	DAY26	DAY28	DAY31
0-A	0	17	20	17	15	10	7	0	0	0	0	0	0	0
0-B	0	20	20	20	20	10	10	0	0	0	0	0	0	0
0-C	0	15	15	15	10	10	3	0	0	0	0	0	0	0
0-D	0	15	15	15	10	10	10	0	0	0	0	0	0	0
SUM	**0**	**67**	**70**	**67**	**55**	**40**	**30**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
MEAN	**0**	**16.75**	**17.50**	**16.75**	**13.75**	**10.00**	**7.50**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
JUVENILE														
0-A	0	0	0	2	8	10	10	7	1	0	0	0	0	0
0-B	0	0	0	0	10	10	20	15	9	9	0	0	0	0
0-C	0	0	0	0	10	10	15	9	1	0	0	0	0	0
0-D	0	0	0	0	10	10	15	15	2	0	0	0	0	0
SUM	**0**	**0**	**0**	**2**	**38**	**40**	**60**	**46**	**13**	**9**	**0**	**0**	**0**	**0**
MEAN	**0.00**	**0.00**	**0.00**	**0.50**	**9.50**	**10.00**	**15.00**	**11.50**	**3.25**	**2.25**	**0.00**	**0.00**	**0.00**	**0.00**
ADULT														
0-A	0	0	0	0	0	0	0	10	16	17	16	12	11	11
0-B	0	0	0	0	0	0	10	14	18	24	26	30	30	26
0-C	0	0	0	0	0	0	0	13	20	15	12	12	10	7
0-D	0	0	0	0	0	0	0	4	12	9	8	8	8	3
SUM	**0**	**0**	**0**	**0**	**0**	**0**	**10**	**41**	**66**	**65**	**62**	**62**	**59**	**47**
MEAN	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**2.50**	**10.25**	**16.50**	**16.25**	**15.50**	**15.50**	**14.75**	**11.75**
NAUPLII														
Treatment	DAY0	DAY3	DAY5	DAY7	DAY10	DAY12	DAY14	DAY17	DAY19	DAY21	DAY24	DAY26	DAY28	DAY31
0.36-A	0	9	15	21	10	5	0	0	0	0	0	0	0	0
0.36-B	0	7	25	20	5	5	0	0	0	0	0	0	0	0
0.36-C	0	10	25	25	10	10	0	2	1	0	0	0	0	0
0.36-D	0	10	10	25	20	3	3	0	0	0	0	0	0	0
SUM	**0**	**36**	**75**	**91**	**45**	**23**	**3**	**2**	**1**	**0**	**0**	**0**	**0**	**0**
MEAN	**0.00**	**9.00**	**18.75**	**22.75**	**11.25**	**5.75**	**0.75**	**0.50**	**0.25**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
JUVENILE														
0.36-A	0	0	0	0	5	7	15	0	0	0	0	0	0	0
0.36-B	0	0	0	0	20	20	8	7	2	0	0	0	0	0
0.36-C	0	0	0	1	20	30	5	20	3	0	0	0	0	0
0.36-D	0	0	0	0	10	20	4	2	7	0	0	0	0	0
SUM	**0**	**0**	**0**	**1**	**55**	**77**	**32**	**29**	**12**	**0**	**0**	**0**	**0**	**0**
MEAN	**0.00**	**0.00**	**0.00**	**0.25**	**13.75**	**19.25**	**8.00**	**7.25**	**3.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
ADULT														
0.36-A	0	0	0	0	0	0	0	1	0	0	0	0	0	0
0.36-B	0	0	0	0	0	0	5	4	4	8	4	4	4	3
0.36-C	0	0	0	0	0	5	10	10	21	23	30	30	20	18
0.36-D	0	0	0	0	0	5	15	16	8	8	6	6	6	7
SUM	**0**	**0**	**0**	**0**	**0**	**10**	**30**	**31**	**33**	**39**	**40**	**40**	**30**	**28**
MEAN	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**2.50**	**7.50**	**7.75**	**8.25**	**9.75**	**10.00**	**10.00**	**7.50**	**7.00**

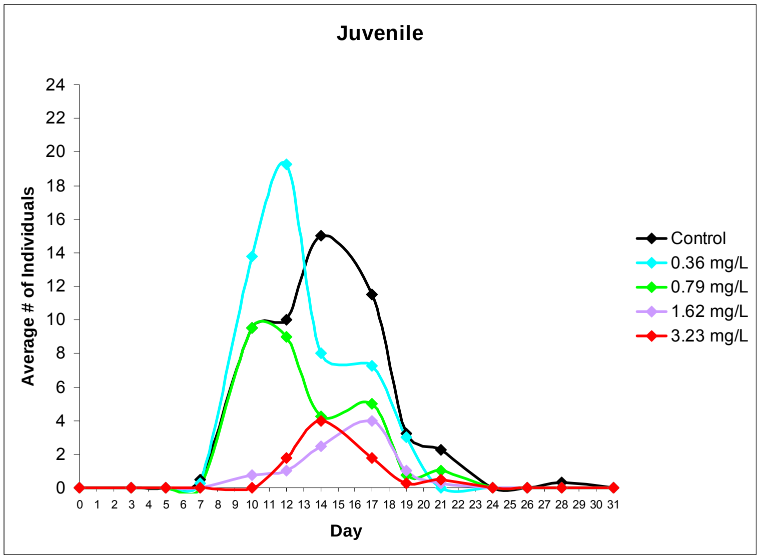
NAUPLII														
Treatment	**DAY0**	**DAY3**	**DAY5**	**DAY7**	**DAY10**	**DAY12**	**DAY14**	**DAY17**	**DAY19**	**DAY21**	**DAY24**	**DAY26**	**DAY28**	**DAY31**
0.79-A	0	1	5	15	3	0	0	0	0	0	0	0	0	0
0.79-B	0	20	10	10	15	5	4	0	0	0	0	0	0	0
0.79-C	0	8	8	16	8	1	0	0	0	0	0	0	0	0
0.79-D	0	20	12	15	1	4	0	0	0	0	0	0	0	0
SUM	**0**	**49**	**35**	**56**	**27**	**10**	**4**	**0**	**0**	**0**	**0**	**0**	**0**	**0**
MEAN	**0.00**	**12.25**	**8.75**	**14.00**	**6.75**	**2.50**	**1.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**
JUVENILE														
0.79-A	0	0	0	0	15	15	5	7	2	4	0	0	0	0
0.79-B	0	0	0	0	7	10	5	8	1	0	0	0	0	0
0.79-C	0	0	0	0	6	6	5	0	0	0	0	0	0	0
0.79-D	0	0	0	0	10	5	2	5	0	0	0	0	0	0
SUM	**0**	**0**	**0**	**0**	**38**	**36**	**17**	**20**	**3**	**4**	**0**	**0**	**0**	**0**
MEAN	**0.00**	**0.00**	**0.00**	**0.00**	**9.50**	**9.00**	**4.25**	**5.00**	**0.75**	**1.00**	**0.00**	**0.00**	**0.00**	**0.00**
ADULT														
0.79-A	0	0	0	0	0	0	7	10	10	10	10	10	10	10
0.79-B	0	0	0	0	0	0	12	2	2	9	10	3	3	4
0.79-C	0	0	0	0	0	0	0	6	3	3	2	1	1	1
0.79-D	0	0	0	0	0	0	9	12	15	9	8	8	8	8
SUM	**0**	**0**	**0**	**0**	**0**	**0**	**28**	**30**	**30**	**31**	**30**	**22**	**22**	**23**
MEAN	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**7.00**	**7.50**	**7.50**	**7.75**	**7.50**	**5.50**	**5.50**	**5.75**

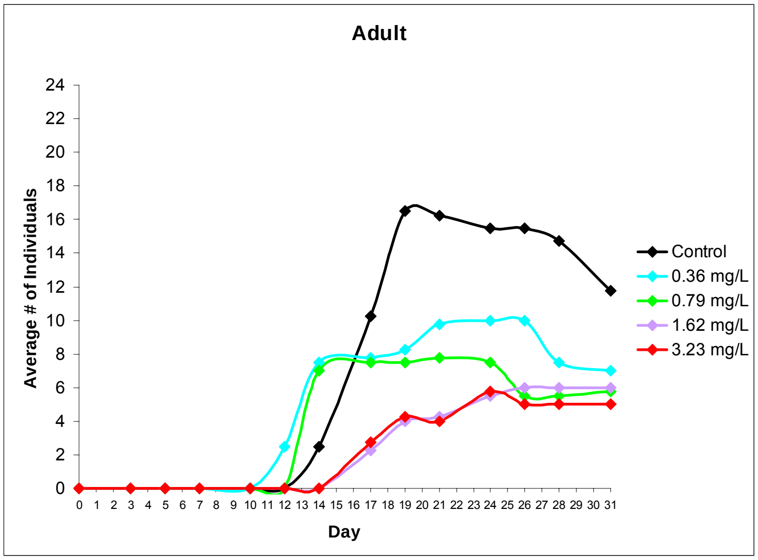
NAUPLII														
Treatment	**DAY0**	**DAY3**	**DAY5**	**DAY7**	**DAY10**	**DAY12**	**DAY14**	**DAY17**	**DAY19**	**DAY21**	**DAY24**	**DAY26**	**DAY28**	**DAY31**
1.62-A	0	15	10	10	10	1	0	1	0	0	0	0	0	0
1.62-B	0	10	6	10	1	4	1	0	0	0	0	0	0	0
1.62-C	0	9	6	5	10	8	7	0	0	1	0	0	0	0
1.62-D	0	9	7	6	10	10	8	3	1	0	0	0	0	0
SUM	**0**	**43**	**29**	**31**	**31**	**23**	**16**	**4**	**1**	**1**	**0**	**0**	**0**	**0**
MEAN	**0.00**	**10.75**	**7.25**	**7.75**	**7.75**	**5.75**	**4.00**	**1.00**	**0.25**	**0.25**	**0.00**	**0.00**	**0.00**	**0.00**
JUVENILE														
1.62-A	0	0	0	0	0	0	0	1	0	0	0	0	0	0
1.62-B	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.62-C	0	0	0	0	2	2	4	6	0	0	0	0	0	0
1.62-D	0	0	0	0	1	2	6	9	4	1	0	0	0	0
SUM	**0**	**0**	**0**	**0**	**3**	**4**	**10**	**16**	**4**	**1**	**0**	**0**	**0**	**0**
MEAN	**0.00**	**0.00**	**0.00**	**0.00**	**0.75**	**1.00**	**2.50**	**4.00**	**1.00**	**0.25**	**0.00**	**0.00**	**0.00**	**0.00**
ADULT														
1.62-A	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.62-B	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1.62-C	0	0	0	0	0	0	0	0	3	5	5	5	5	5
1.62-D	0	0	0	0	0	0	0	9	13	12	17	19	19	19
SUM	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**9**	**16**	**17**	**22**	**24**	**24**	**24**
MEAN	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**2.25**	**4.00**	**4.25**	**5.50**	**6.00**	**6.00**	**6.00**

NAUPLII														
Treatment	**DAY0**	**DAY3**	**DAY5**	**DAY7**	**DAY10**	**DAY12**	**DAY14**	**DAY17**	**DAY19**	**DAY21**	**DAY24**	**DAY26**	**DAY28**	**DAY31**
3.23-A	0	12	0	0	0	0	1	2	0	0	0	0	0	0
3.23-B	0	7	5	2	0	5	6	7	5	2	0	0	0	0
3.23-C	0	8	10	7	9	5	3	0	0	0	0	0	0	0
3.23-D	0	10	5	5	5	5	0	0	0	0	0	0	0	0
SUM	**0**	**37**	**20**	**14**	**14**	**15**	**10**	**9**	**5**	**2**	**0**	**0**	**0**	**0**
MEAN	**0.00**	**9.25**	**5.00**	**3.50**	**3.50**	**3.75**	**2.50**	**2.25**	**1.25**	**0.50**	**0.00**	**0.00**	**0.00**	**0.00**
JUVENILE														
3.23-A	0	0	0	0	0	0	0	0	0	1	0	0	0	0
3.23-B	0	0	0	0	0	0	0	1	0	0	0	0	0	0
3.23-C	0	0	0	0	0	5	10	2	1	0	0	0	0	0
3.23-D	0	0	0	0	0	2	6	4	0	1	0	0	0	0
SUM	**0**	**0**	**0**	**0**	**0**	**7**	**16**	**7**	**1**	**2**	**0**	**0**	**0**	**0**
MEAN	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**1.75**	**4.00**	**1.75**	**0.25**	**0.50**	**0.00**	**0.00**	**0.00**	**0.00**
ADULT														
3.23-A	0	0	0	0	0	0	0	0	0	0	1	1	1	1
3.23-B	0	0	0	0	0	0	0	0	1	1	1	1	1	1
3.23-C	0	0	0	0	0	0	0	9	11	11	13	13	13	13
3.23-D	0	0	0	0	0	0	0	2	5	4	8	5	5	5
SUM	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**11**	**17**	**16**	**23**	**20**	**20**	**20**
MEAN	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**2.75**	**4.25**	**4.00**	**5.75**	**5.00**	**5.00**	**5.00**

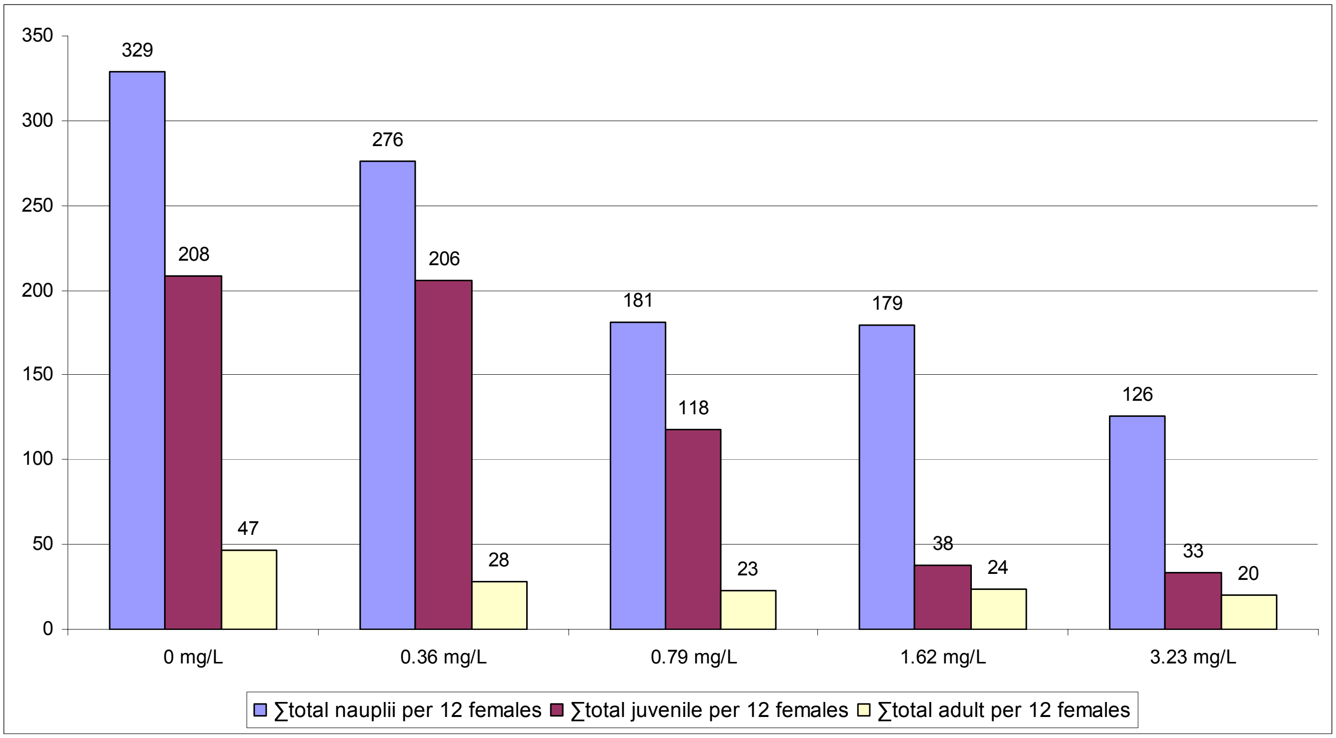
Appendix II. Average number of nauplii, juvenile, and adult *P. forbesi* produced per 2-3 days during the 31-d full life cycle study







Appendix III The sum total number of nauplii, juvenile, and adult *P. forbesi* produced during the 31-d full life cycle study



APPENDIX IV

CETIS Summary Report - 1b

Report Date: 07 Nov-10 13:43 (p 1 of 1)
Test Code: 07-3679-3553/2BEA93D1

Ceriodaphnia 7-d Survival and Reproduction Test — **UC Davis Aquatic Toxicology Laboratory**

Batch ID:	16-6449-0360	**Test Type:**	Reproduction-Survival (7d)	**Analyst:**	
Start Date:		**Protocol:**	EPA/821/R-02-013 (2002)	**Diluent:**	
Ending Date:	**Species**		Pseudodiaptomus forbesii	**Brine:**	
Duration:		**Source:**	In-House Culture	**Age:**	

Sample ID:	20-3194-2571	**Code:**	791CFBAB	**Client:**	Chris Foe
Sample Date:	05 Oct-10 01:00	**Material:**	Ammonium Chloride	**Project:**	Ammonia Study
Receive Date:		**Source:**	In House Water		
Sample Age:	26d 23h	**Station:**			

Batch Note: P. forbesii reproduction ammonia sensitivity test

Sample Note: Ammonia - Nominal

Comparison Summary

Analysis ID	Endpoint	NOEL	LOEL	TOEL	PMSD	TU	Method
13-2924-3024	Reproduction	<0.5	0.5	N/A	24.91%		Dunnett's Multiple Comparison Test

Point Estimate Summary

Analysis ID	Endpoint	Level	mg/L	95% LCL	95% UCL	TU	Method
08-2641-8475	Reproduction	IC1	0.01478	0.009506	0.5105		Linear Interpolation (ICPIN)
		IC5	0.07613	0.04844	0.553		
		IC10	0.1581	0.09923	0.6605		
		IC15	0.2462	0.1525	0.7918		
		IC20	0.3411	0.2083	N/A		
		IC25	0.4432	0.2668	N/A		
		IC40	>1	N/A	N/A		
		IC50	>1	N/A	N/A		
		IC60	>1	N/A	N/A		
		IC75	>1	N/A	N/A		
		IC80	>1	N/A	N/A		
		IC85	>1	N/A	N/A		
		IC90	>1	N/A	N/A		
		IC95	>1	N/A	N/A		

Reproduction Summary

Conc-mg/L	Control Type	Count	Mean	95% LCL	95% UCL	Min	Max	Std Err	Std Dev	CV%	Diff%
0	Dilution Water	10	7.6	7.011	8.189	5	10	0.288	1.578	20.76%	0.0%
0.5		10	5.5	4.516	6.484	3	10	0.4811	2.635	47.91%	27.63%
1		10	5.4	4.649	6.151	3	9	0.3672	2.011	37.24%	28.95%

Reproduction Detail

Conc-mg/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10
0	Dilution Water	9	7	8	8	9	6	10	6	8	5
0.5		10	3	4	6	6	3	10	5	3	5
1		7	5	7	6	6	5	3	3	9	3

APPENDIX V

CETIS Summary Report

Report Date: 08 Jul-11 12:52 (p 1 of 2)
Test Code: 12-1611-0831/487C60EF

Ceriodaphnia 7-d Survival and Reproduction Test — **UC Davis Aquatic Toxicology Laboratory**

Batch ID:	03-6525-3041	**Test Type:**	Reproduction-Survival (7d)	**Analyst:**	
Start Date:	01 Nov-10	**Protocol:**	EPA/821/R-02-013 (2002)	**Diluent:**	Laboratory Water
Ending Date:	08 Nov-10	**Species:**	Pseudodiaptomus forbesii	**Brine:**	Not Applicable
Duration:	7d 0h	**Source:**		**Age:**	

Sample ID:	02-5743-1783	**Code:**	F5818E7	**Client:**	Chris Foe
Sample Date:	01 Nov-10	**Material:**	Ammonium Chloride	**Project:**	Special Studies
Receive Date:		**Source:**	Unknown		
Sample Age:	N/A	**Station:**			

Batch Note: Ammonia Toxicity

Sample Note: Ammonia for Dr.Teh

Comparison Summary

Analysis ID	Endpoint	NOEL	LOEL	TOEL	PMSD	TU	Method
01-7171-6152	4d Survival Rate	0.62	0.95	0.7675	N/A		Fisher Exact/Bonferroni-Holm Test

Point Estimate Summary

Analysis ID	Endpoint	Level	mg/L	95% LCL	95% UCL	TU	Method
15-8199-3750	4d Survival Rate	EC1	0.115	0.1074	0.6343		Linear Interpolation (ICPIN)
		EC5	0.1773	0.1373	0.7134		
		EC10	0.62	0.1758	0.9667		
		EC15	0.7309	0.2157	1.026		
		EC20	0.8494	0.2569	1.084		
		EC25	0.9688	0.5912	N/A		
		EC40	>1.23	N/A	N/A		
		EC50	>1.23	N/A	N/A		
		EC60	>1.23	N/A	N/A		
		EC75	>1.23	N/A	N/A		
		EC80	>1.23	N/A	N/A		
		EC85	>1.23	N/A	N/A		
		EC90	>1.23	N/A	N/A		
		EC95	>1.23	N/A	N/A		

4d Survival Rate Summary

Conc-mg/L	Control Type	Count	Mean	95% LCL	95% UCL	Min	Max	Std Err	Std Dev	CV%	Diff%
0.1	Dilution Water	60	0.8333	0.693	0.9737	0	1	0.06862	0.3758	45.1%	0.0%
0.26		60	0.75	0.5869	0.9131	0	1	0.07972	0.4367	58.22%	10.0%
0.62		60	0.75	0.5869	0.9131	0	1	0.07972	0.4367	58.22%	10.0%
0.95		60	0.6333	0.4519	0.8148	0	1	0.08872	0.486	76.73%	24.0%
1.23		60	0.5167	0.3285	0.7048	0	1	0.09201	0.5039	97.54%	38.0%

Ceriodaphnia 7-d Survival and Reproduction Test — **UC Davis Aquatic Toxicology Laboratory**

4d Survival Rate Detail

Conc-mg/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10
0.1	Dilution Water	1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		0	0	0	0	0	0	0	0	0	0
0.26		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
0.62		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
0.95		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	0	0
		0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
1.23		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0

Ceriodaphnia 7-d Survival and Reproduction Test				UC Davis Aquatic Toxicology Laboratory	
Analysis ID:	15-8199-3750	**Endpoint:**	4d Survival Rate	**CETIS Version:**	CETISv1.7.0
Analyzed:	08 Jul-11 12:42	**Analysis:**	Linear Interpolation (ICPIN)	**Official Results:**	Yes

Batch ID:	03-6525-3041	**Test Type:**	Reproduction-Survival (7d)	**Analyst:**	
Start Date:	01 Nov-10	**Protocol:**	EPA/821/R-02-013 (2002)	**Diluent:**	Laboratory Water
Ending Date:	08 Nov-10	**Species:**	Pseudodiaptomus forbesii	**Brine:**	Not Applicable
Duration:	7d 0h	**Source:**		**Age:**	

Sample ID:	02-5743-1783	**Code:**	F5818E7	**Client:**	Chris Foe
Sample Date:	01 Nov-10	**Material:**	Ammonium Chloride	**Project:**	Special Studies
Receive Date:		**Source:**	Unknown		
Sample Age:	N/A	**Station:**			

Batch Note: Ammonia Toxicity

Sample Note: Ammonia for Dr.Teh

Linear Interpolation Options

X Transform	Y Transform	Seed	Resamples	Exp 95% CL	Method
Log(X+1)	Linear	57951	200	Yes	Two-Point Interpolation

Point Estimates

Level	mg/L	95% LCL	95% UCL
EC1	0.115	0.1074	0.6343
EC5	0.1773	0.1373	0.7134
EC10	0.62	0.1758	0.9667
EC15	0.7309	0.2157	1.026
EC20	0.8494	0.2569	1.084
EC25	0.9688	0.5912	N/A
EC40	>1.23	N/A	N/A
EC50	>1.23	N/A	N/A
EC60	>1.23	N/A	N/A
EC75	>1.23	N/A	N/A
EC80	>1.23	N/A	N/A
EC85	>1.23	N/A	N/A
EC90	>1.23	N/A	N/A
EC95	>1.23	N/A	N/A

4d Survival Rate Summary

			Calculated Variate(A/B)								
Conc-mg/L	Control Type	Count	Mean	Min	Max	Std Err	Std Dev	CV%	Diff%	A	B
0.1	Dilution Water	60	0.8333	0	1	0.06862	0.3758	45.1%	0.0%	50	60
0.26		60	0.75	0	1	0.07972	0.4367	58.22%	10.0%	45	60
0.62		60	0.75	0	1	0.07972	0.4367	58.22%	10.0%	45	60
0.95		60	0.6333	0	1	0.08872	0.486	76.73%	24.0%	38	60
1.23		60	0.5167	0	1	0.09201	0.5039	97.54%	38.0%	31	60

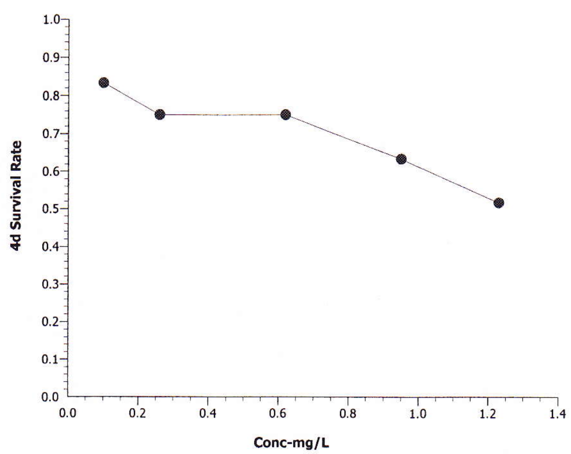
Ceriodaphnia 7-d Survival and Reproduction Test — **UC Davis Aquatic Toxicology Laboratory**

Analysis ID: 15-8199-3750	**Endpoint:** 4d Survival Rate	**CETIS Version:** CETISv1.7.0	
Analyzed: 08 Jul-11 12:42	**Analysis:** Linear Interpolation (ICPIN)	**Official Results:** Yes	

4d Survival Rate Detail

Conc-mg/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10
0.1	Dilution Water	1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		0	0	0	0	0	0	0	0	0	0
0.26		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
0.62		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
0.95		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	0	0
		0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
1.23		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0

Graphics



CETIS Analytical Report

Ceriodaphnia 7-d Survival and Reproduction Test				UC Davis Aquatic Toxicology Laboratory	
Analysis ID:	01-7171-6152	**Endpoint:**	4d Survival Rate	**CETIS Version:**	CETISv1.7.0
Analyzed:	08 Jul-11 12:42	**Analysis:**	STP 2x2 Contingency Tables	**Official Results:**	Yes

Batch ID:	03-6525-3041	**Test Type:**	Reproduction-Survival (7d)	**Analyst:**	
Start Date:	01 Nov-10	**Protocol:**	EPA/821/R-02-013 (2002)	**Diluent:**	Laboratory Water
Ending Date:	08 Nov-10	**Species:**	Pseudodiaptomus forbesii	**Brine:**	Not Applicable
Duration:	7d 0h	**Source:**		**Age:**	

Sample ID:	02-5743-1783	**Code:**	F5818E7	**Client:**	Chris Foe
Sample Date:	01 Nov-10	**Material:**	Ammonium Chloride	**Project:**	Special Studies
Receive Date:		**Source:**	Unknown		
Sample Age:	N/A	**Station:**			

Batch Note: Ammonia Toxicity

Sample Note: Ammonia for Dr.Teh

Data Transform	Zeta	Alt Hyp	Monte Carlo	NOEL	LOEL	TOEL	TU	PMSD
Untransformed		C > T	Not Run	0.62	0.95	0.7675		N/A

Fisher Exact/Bonferroni-Holm Test

Sample	vs	Sample	Test Stat	P-Value	Decision(0.05)
0.1		0.26	0.1844	0.3689	Non-Significant Effect
0.1		0.62	0.1844	0.3689	Non-Significant Effect
0.1		0.95	0.01116	0.0335	Significant Effect
0.1		1.23	0.0001894	0.0008	Significant Effect

Data Summary

Conc-mg/L	Control Type	No-Resp	Resp	Total
0.1	Dilution Water	50	10	60
0.26		45	15	60
0.62		45	15	60
0.95		38	22	60
1.23		31	29	60

CETIS Analytical Report

Report Date:	08 Jul-11 12:51 (p 2 of 2)
Test Code:	12-1611-0831/487C60EF

Ceriodaphnia 7-d Survival and Reproduction Test — **UC Davis Aquatic Toxicology Laboratory**

Analysis ID:	01-7171-6152	**Endpoint:**	4d Survival Rate	**CETIS Version:**	CETISv1.7.0
Analyzed:	08 Jul-11 12:42	**Analysis:**	STP 2x2 Contingency Tables	**Official Results:**	Yes

4d Survival Rate Detail

Conc-mg/L	Control Type	Rep 1	Rep 2	Rep 3	Rep 4	Rep 5	Rep 6	Rep 7	Rep 8	Rep 9	Rep 10
0.1	Dilution Water	1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		0	0	0	0	0	0	0	0	0	0
0.26		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
0.62		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
0.95		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	0	0
		0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
1.23		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	1	1	1	1	1	1	1	1	1
		1	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0
		0	0	0	0	0	0	0	0	0	0

Graphics