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Determining the effects of ammonia on fathead minnow (*Pimephales promelas*) reproduction

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Abstract

Ammonia can cause adverse reproductive and mortality effects in individual fish by interacting with the central nervous system. The last published study that assessed the effects of ammonia on fathead minnow reproduction was a lifecycle study conducted in 1986. Our study's main goal was to re-evaluate ammonia toxicity on fathead minnow, *Pimephales promelas*, reproduction using a 20-day fecundity flow-through diluter method. Flow-through diluter systems have been used by regulatory agencies, such as the U.S. Environmental Protection Agency, in the past as an effective way to estimate acceptable levels of contaminants. This study used a 20 day flow-through diluter method to test the effects of environmentally relevant concentrations of ammonia on *P. promelas* reproduction. There was a significant difference in cumulative egg production among treatments (ANOVA; $F = 10.167$, $p = <0.01$, $df = 3$). All three concentrations of ammonia tested in this study significantly reduced fecundity after 20 days of exposure (Dunnett's, $p = <0.05$ for each treatment). The lowest un-ionized ammonia concentration (0.07 mg/L at a pH of 7.3 and temperature of 25.1 ºC) tested during this study resulted in a 29% decrease in cumulative fecundity. Because all tested ammonia concentrations caused an effect on *P. promelas* reproduction, the no effect concentration was estimated to be 0.022 mg/L un-ionized ammonia (1.99 mg/L total ammonia - nitrogen). This estimate was determined using a U.S. EPA program to calculate the 10% effect concentration of ammonia on *P. promelas* reproduction. This value is much lower than the previous reported no effect concentration on *P. promelas* reproduction (0.37 mg/L un-ionized ammonia or 6.43 mg/L total ammonia - nitrogen) as determined from the 1986 study, which was used to determine the ammonia water quality criteria by the U.S. Environmental Protection Agency. Our results should be considered in the next revision of water quality criteria.

Keywords:

Ammonia, Toxicology, Flow-through, Reproduction, Promelas

1.0 Introduction:

Ammonia is a common pollutant in aquatic systems and can be toxic to fish. Sewage effluent generally contains very low concentrations of ammonia; however during wastewater treatment plant (WWTP) malfunctions or in parts of the world that have nonexistent or ineffective treatment systems, total ammonia concentrations can occur at concentrations greater than 20 mg/L (Passell et al. 2007) in waterways. Ammonia can also enter waterways through farm animal operations, and the magnitude of ammonia pollution is entirely dependent on the size of the operation (Robbins et al. 1972). Other sources of ammonia include agricultural fertilizers and, during runoff and flooding events, nitrogen released through agriculture practices could enter nearby streams.

Ammonia exists in two forms, ionized (NH_4^+) and un-ionized (NH_3) (Mayes et al. 1986; Thurston et al. 1986). Un-ionized ammonia is the principle toxic form of ammonia in the environment (Constable et al. 2003). Environmental conditions, such as pH and temperature, alter relative concentrations of un-ionized and ionized ammonia in the water. Although total ammonia concentration (NH_4^+ & NH_3) is typically measured in WWTP effluent across the United States, NH_3 concentrations generally are not (Passell et al. 2007). NH_3 is considered more toxic to fish than NH_4^+ (Delos and Erickson 1999; Mayes et al. 1986; Thurston et al. 1986). As a neutral molecule, NH_3 is able to easily diffuse across the epithelial membranes of aquatic organisms (Delos and Erickson 1999). The toxicity of ammonia is highly dependent on temperature and pH of the water because environmental variables influence the form and the bioavailability of ammonia (Delos and Erickson 1999). As the temperature and pH of water increase, the toxicity of ammonia to fathead minnows, *Pimephales promelas,* increases as well (Delos and Erickson 1999). Most ammonia criteria and permit limits are expressed in terms of

129 total ammonia – nitrogen (TAN) (Delos and Erickson 1999). Therefore, we will report both NH_3
130 and TAN values from this study.

131 Typical NH_3 concentrations in untreated wastewater are between 12 and 45 mg/L (Carey
132 and Migliaccio 2009). In secondary wastewater treatment plants using activated sludge, NH_3
133 concentrations typically range between 1 and 10 mg/L (Carey and Migliaccio 2009). In advanced
134 secondary treatments, NH_3 concentrations range between 1 and 3 mg/L (Carey and Migliaccio
135 2009). In tertiary, NH_3 concentrations are usually below 0.1 mg/L (Carey and Migliaccio 2009).
136 Previously, the U.S. Environmental Protection Agency (EPA) National Exposure Research
137 Laboratory (NERL) in Cincinnati, OH conducted a survey of 50 wastewater treatment plants
138 throughout the United States (2004). The average concentration of NH_3 in water samples from
139 these plants was 0.1 mg/L, with a maximum of 0.76 mg/L NH_3 (Lazorchak and Smith 2004).

140 Increased internal concentrations of ammonia in fish can also lead to both acute and
141 chronic toxicity. Ammonia targets the central nervous system of vertebrates via the cerebral
142 energy metabolism pathway by activating the glutamate receptors for the amino acid, N-methyl-
143 D-aspartic acid (NMDA) (Randall and Tsui 2002). These receptors are responsible for
144 controlling synaptic plasticity and memory function. Increased activation of these receptors leads
145 to an instant depolarization of neurons and results in cell death within the central nervous system.
146 At high concentrations, symptoms of acute ammonia toxicity in fish include convulsions
147 followed by death (Randall and Tsui 2002). Thurston et al. (1983) reported a linear relationship
148 between the *P. promelas* 96 hr LC50 for NH_3 and temperature: $LC50 = 0.4304 + 0.1225 * temperature$. At 25°C, the anticipated temperature of our control and dilution water, we expected
149
150 the 96 hr LC50 to be 3.49 mg/L NH_3.

Chronic effects of ammonia toxicity on *P. promelas* include damage to the gills and respiratory apparatus and at concentrations higher than 0.9 mg/L NH_3 (14.5 mg/L TAN) can induce mortality (Thurston et al. 1986). Ammonia can reduce growth and inhibit reproductive success in *P. promelas* (Thurston et al. 1986). The no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) of NH_3 on *P. promelas* egg production and viability were reported as low as 0.37 mg/L (6.43 TAN) and 0.91 mg/L, respectively (14.5 TAN) (Thurston et al. 1986). Brain lesions in *P. promelas* were observed at a 0.21 mg/L (3.5 mg/L TAN) but were not found at 0.11 mg/L NH_3 (1.68 mg/L TAN) (Thurston et al. 1986).

Because the mechanism by which ammonia impacts fish reproduction has not clearly been defined (Person Le Ruyet et al. 1998), we chose to investigate the effects of ammonia on vitellogenin production in fathead minnows. Vitellogenin is a lipo-phospho-glycoprotein precursor to egg yolk that is normally produced in females (Parks et al. 1999). Perturbations in vitellogenin production can have ecological consequences, because vitellogenesis is related to fecundity and egg quality of individual fish (Murphy et al. 2005). We were also interested in how ammonia affects the development of secondary sexual characteristics. *P. promelas* males develop a dorsal fatpad upon maturity that assists them in preparing the spawning site and taking care of the eggs post spawn (Smith and Murphy 1974). Breeding tubercles also form on the snout of mature *P. promelas* males and Heming et al. (2001) reported that these secondary sexual characteristics were reduced when *P. promelas* males were exposed to effluent concentrations.

The *P. promelas* used in this study were cultured in-house at the U.S. EPA Cincinnati-AWBERC location using established methods (NERL 2002). *P. promelas* have been used in laboratory studies as a model species in toxicological research for decades. They are a representative of the ecologically important Cyprinidae family (Ankley and Villeneuve 2006;

Jensen et al. 2001), have a broad distribution in both lentic and lotic environments across North America (Jensen et al. 2001) and are an opportunistic omnivore tolerant of a wide range of water types (Ankley and Villeneuve 2006). They have been used extensively in chronic life stage and early life stage survival and development tests (Ankley et al. 2001) partly because culturing *P. promelas* in a laboratory setting is relatively easy (NERL 2002; Ankley et al. 2001). *P. promelas* are a good model organism because of their life-history: they are fractional spawners; produce clutches of 50 to 100 eggs every 3 to 5 days and have a rapid life cycle reaching maturity within 4 to 5 months (Ankley et al. 2001). Controlling their reproductive life cycle is readily achieved through alterations in temperature and photoperiod (NERL 2002). Extrapolating data from the laboratory to the field is a challenging task for ecotoxicologists. However, previous studies also suggest that *P. promelas* response to chemicals is comparable to responses observed in a variety of threatened and endangered fish (Ankley et al. 2001). Laboratory *P. promelas* have also played a key role in predicting the bioavailability of inorganics and other contaminants through the use of modeling and caged deployments (Ankley and Villeneuve 2006) and have been used as a model organism to make inferences on wild populations.

The purpose of this experiment was to test environmentally relevant concentrations of NH_3 and to determine the 20 day NOEC of NH_3 on fish reproduction using a flow-through diluter system. Prior to this study, the most recent study reporting *P. promelas* reproductive effects after an NH_3 exposure was Thurston et al. (1986). Thurston et al. (1986) was a full life-cycle study, therefore the published NOECs needed to be tested in order to determine if they were accurate NOECs using our study's methodology. We were also concerned over the results of the study conducted by Thurston et al. (1986). Thurston et al. (1986) reported a very low

196 control reproduction suggesting that a reexamination of the effects of NH_3 on *P. promelas*
197 reproduction was warranted.

198 **2.0 Material and Methods:**

199 **2.1 Determination NH_3 Concentrations:**

200 This study used a flow-through diluter system to determine the effects of ammonia on *P.*
201 *promelas* reproduction. The setup and design of the flow-through diluter system follows an
202 established U.S. EPA protocol (NHERL 2002) for conducting 21 day toxicity tests and allows for
203 a continuous toxicant exposure at predetermined concentrations throughout the entire study. The
204 concentrations of ammonia tested in this study are similar to previous studies and span realistic
205 concentrations observed in natural systems. However our exposure conditions were quite
206 different. Three expected TAN concentrations were tested in this study including: 5.0 mg/L, 15.0
207 mg/L, and 30 mg/L TAN. These concentrations were chosen based on average concentrations
208 found in effluents of tertiary, advanced secondary and secondary wastewater treatment plants.
209 Expected NH_3 concentrations were converted from the predicted TAN measurements using the
210 equations:

211 $Total\ ammonia = TAN * (\frac{17}{14})$ and $NH3 = Total\ Ammonia/(1 + 10\text{^}(0.09018 + (2729.92/$
212 $(\text{Temp°C} + 273.15)) - \text{pH}))$ (Delos and Erickson 1999). We expected to test NH_3 concentrations
213 of 0.06, 0.17 and 0.34 mg/L using dilution water with pH of 7.3 and temperature of 25°C.
214 Thurston et al. (1986) reported a NH_3 NOEC on *P. promelas* reproduction at 0.37 mg/L (6.43
215 mg/L TAN). Therefore, this study tested concentrations similar to those tested by Thurston et al.
216 (1986) and also bracketed the average U.S. WWTP effluent NH_3 concentration of 0.1mg/L
217 (Lazorchak and Smith 2004). Ammonium chloride (NH_4Cl) was chosen as the source for
218 ammonia in this study because it did not produce any precipitates at the pH and temperature of

the control/dilution water used in this study while in the presence of fish. In a previous pilot study, ammonium phosphate dibasic was used as the ammonia source. We found that when fish were introduced to the system and fed, the pH of the tanks increased to the point where the ammonium phosphate dibasic reacted with the control water and produced a calcium phosphate precipitate. Although this precipitate was probably not toxic, we wished to avoid any potentially confounding variables.

2.2 Experimental Setup:

We implemented an established U.S. EPA protocol (NHERL 2002) for conducting our flow-through diluter experiments to explore how NH_3 affects *P. promelas* reproduction. We used lab-line water for our experiments, which is the U.S. EPA (Cincinnati) in-house culture water with a hardness of 180 mg/L as $CaCO_3$. The water was made by passing tempered tap water through a set of activated carbon filters, for the removal of chlorine and organics. Liquid calcium chloride was then added to the water to supplement the hardness. Following treatment, the water moved through four 1892.7 L fiberglass tanks for conditioning. After conditioning, the water was pumped into the water delivery system and then fed to the diluter system. The diluter system was designed to provide a continuous flow of toxicant at three treatment concentrations plus a control to the glass aquarium testing chambers.

The diluter system was designed to test 28 testing chambers and two blank tanks. The blank tanks did not contain any fish and were needed to measure background water chemistry in case high mortality was observed in the control chambers. The temperature within all the chambers was monitored weekly and heated using a water bath to 25.0 ± 1.0 °C. The 28 testing chambers allowed for seven replicates of each of the three testing concentrations and control. Each replicate consisted of 2 adult male and 4 female *P. promelas* along with three spawning

242 tiles that were made of halved sections of 3" inside diameter PVC pipe at a length of 4". Three
243 tiles in each chamber ensured that there were enough territories for both males and a refuge for
244 females.

245 Water chemistries in the testing chambers were monitored several times each week
246 throughout the study to ensure proper NH_3 exposure. Total ammonia – nitrogen concentrations in
247 individual testing chambers were measured once each week. These measurements were made
248 using an expandable Ion Analyzer (Orion 9400, Orion, Beverly, MA, USA) equipped with an
249 ammonia ion-selective electrode (Orion 9512, Orion, Beverly, MA, USA) as described in
250 American Public Health Association (1995). Also, TAN concentrations were measured three
251 times each week from a composite of all 7 replicates within a treatment group. Because
252 temperature and pH can affect NH_3 concentrations, temperature and pH readings for each testing
253 chamber were recorded soon after a TAN measurement was performed. NH_3 concentrations were
254 adjusted to account for pH and temperature using the equation derived from the U.S. EPA's
255 water quality criteria document (Delos and Erickson 1999). The blank tanks were required to
256 monitor the water quality of the control and dilution water without the presence of fish; if
257 mortality in the control tanks had occurred, the blank tank water quality data could be analyzed
258 to determine if water quality was an issue.

259 Our experiment consisted of a 14 day control water acclimation period and a 20 day
260 exposure period. A total of 168 six month old *P. promelas* were used during this study. Standard
261 lengths and wet weights of all males and females were collected prior to the study to ensure each
262 individual met the weight criteria (males: 3.5 ± 0.5 g, females: 1.0± 0.5 g). These criteria were
263 chosen based on the average size fish raised at the U.S. EPA (AWBERC) culture facility in
264 Cincinnati, OH and it was chosen to reduce biological variance in vitellogenin response. Fish

were then randomly placed into the testing chambers immediately following weight and length data collection. Because handling induces stress and ultimately the production of cortisol, the 14 day acclimation period allowed these individuals to acclimate to the testing environment prior to the exposure period. No mortality was observed during the acclimation period. Reproduction was monitored during the acclimation period to ensure each treatment was producing similar numbers of eggs prior to exposure and to monitor the tanks for significant mortality. On day 14 of the acclimation period, the toxicant pumps were turned on and dosing began for the exposure period.

The exposure period for each experiment ran for 20 consecutive days during which fecundity, fertility and mortality data were recorded daily. Any fish that died during the 20 day exposure period was not replaced. Daily, tanks were monitored for fecundity by counting the number of eggs laid on each spawning tile. The tiles with attached eggs were marked for their tank number, eggs were counted and then the tile was placed in an aerated egg bath of 25.0 ˚C ± 1.0 ˚C. Seventy-two hours after collection, fertilized eggs in the eyed stage were counted. Three times a week during testing, the testing chambers were cleaned using a sponge to scrape down the sides of the tank to reduce the amount of bacteria buildup. Every caution was taken during tank cleaning to prevent unnecessary stress to the fish. A sink siphon was then used to remove excess food and debris upon settling.

During the study, fish were fed on a regular schedule in order to maximize reproduction. Each testing chamber was fed three times each day. Once, between 8:00 and 10:00 am EST, the fish were fed 20 ml of concentrated newly hatched (< 24 H old) brine shrimp, *Artemia salina* via GSL® (Ogden, UT). Between 12:00 and 1:00 pm, the fish were fed with 1.0 ml of frozen San Francisco Bay Brand, Incorporated® (Newark, CA) adult brine shrimp. The final feeding occurred between 4:00 and 5:00 pm and consisted of 20 ml of concentrated newly hatched brine

288 shrimp. This feeding regimen was previously developed during a 21 day control pilot study
289 conducted by the U.S. EPA in Cincinnati, OH to maximize reproduction and maintain the health
290 of the fish.

291 The ammonia study was terminated after 20 days of exposure and the fish were
292 transferred to stations for necropsy to measure reproductive endpoints. The specific endpoint
293 data collected were male secondary sexual characteristics, fecundity, fertility, gonadosomatic
294 index (GSI), fatpad index (FPI), and liver vitellogenin. All procedures followed approved
295 Institutional Animal Care and Use Committee (IACUC) protocols. Prior to necropsy, the fish
296 were anesthetized in a 200 mg/L concentration of tricaine methanesulfonate (MS - 222) and
297 standard length and wet weights were measured. The tail was severed to collect blood from the
298 caudal artery via a heparinized capillary tube. Following blood collection, the heparinized
299 capillary tube was centrifuged at 5900 g for 1 minute to separate the plasma from the red blood
300 cells. After centrifugation the plasma was transferred into a labeled vial and then snap-frozen in
301 liquid nitrogen. Immediately following plasma collection, the fish was euthanized by severing
302 the spine just behind the nape of the fish. Secondary sexual characteristics of males were then
303 recorded. Each tubercle was counted and fatpad size was scored using an U.S. EPA scoring
304 system (Ankley et al. 2001). Each fatpad was separated using a scalpel and then weighed to
305 calculate a fatpad index (FPI) comparative to the fish body weight. Gonads and livers from males
306 and females were removed and a wet tissue weight was taken to calculate a gonadosomatic index
307 (GSI) and hepatosomatic index (HSI) when compared against the fish's body weight.

308 Vitellogenin was measured from the liver samples using "real time" quantitative
309 polymerase chain reactions (QPCR) via a method already described (Biales et al. 2007). Total
310 RNA from the liver samples was isolated using TriReagent® (Chomczynski and Mackey 1995).

311 Relative concentrations of the total RNA were measured using an ultraviolet spectrophotometer.
312 The isolated RNA was converted into complementary DNA (cDNA) using a reverse
313 transcriptase. A diluted sample of cDNA was then used for the PCR reactions along with a Hot
314 Start DyNAmo™ SYBR® green master-mix. Amplification of normalizing gene (18S) sequences
315 was performed using universal 18S primer pairs. Cycling was carried out using a thermocycler in
316 cycles of 94 °C, 60 °C and 70 °C. The fluorescent intensity of vitellogenin response from each
317 sample was calculated as a ratio of vitellogenin:18S and compared against the controls. An
318 Enzyme-linked immuno-sorbent assay, ELISA, was used for the detection and quantification of
319 vitellogenin in the plasma samples (Jensen and Ankley 2006). These samples were sent to the
320 U.S. EPA laboratory in Duluth, Minnesota for analysis.

321 **2.3 Statistical Analysis**

322 All statistical analyses were conducted using a free online statistical package, Program R,
323 found at http://www.r-project.org. All data were checked for both normal distribution using a
324 Shapiro-Wilks test for normality and homogeneity of variance via a Levene's test. In cases
325 where the data were both normally distributed and had a homogeneous variance, an analysis of
326 variance (ANOVA) was conducted. This occurred for all the water chemistry data as well as the
327 cumulative egg production data set. If the ANOVA was found to be significant, a Dunnett's test
328 was performed to determine significant difference between groups and the control. Data that
329 were not normally distributed were transformed prior to ANOVA analysis. If reasonable
330 transformations did not normally distribute the data, a non-parametric analysis was performed
331 using a package within Program R (npmc) to determine differences between each group and the
332 control. This program provided simultaneous rank test procedures for a one-way layout without
333 presuming a certain distribution.

The determination of a NOEC was difficult because all NH_3 concentrations caused a significant fecundity effect. In this case an online program provided by the U.S. EPA, the Toxicity Relationship Analysis Program (TRAP), was used to calculate EC10 values. We believe the EC10 value is a reasonable estimate of the NOEC. A nonlinear regression was conducted using log-transformed data and sigmoid threshold as the curve shape during the TRAP estimate.

3.0 Results:

3.1: Water Chemistry Analysis

Water chemistry analysis is summarized in Table 1. Ammonia sampling, conducted 10 times at two day intervals, suggested that the testing chambers were receiving appropriate doses of ammonia throughout the experiment except for day 21. The dilution water pump failed on the last day of the study and as a result the ammonia concentrations spiked significantly on the last day because only the toxicant pump was dosing the undiluted superstock to the tank replicate. Therefore, all data collected after day 20 were discarded. The control tanks did not contain significant concentrations of ammonia. The 20 day average ammonia concentration in the tanks matched their desired concentrations. Mean measured concentrations throughout the study in the treatment tanks appear sufficiently similar to expected concentrations and all CV's of ammonia concentrations that were within an acceptable range (< 20 %). Other chemical parameters including dissolved oxygen (ANOVA, $F = 0.003$, $p = 1.00$, $df = 3$), pH (ANOVA, $F = 2.940$, $p = 0.065$, $df = 3$) and temperature (ANOVA, $F = 0.144$, $p = 0.932$, $df = 3$) were determined for the test solutions and showed no differences among treatments. There were no differences found in pH measurements among treatments. Conductivity was significantly different among treatments (ANOVA, $F = 19.109$, $p = < 0.01$, $df = 3$) which was to be expected considering ammonium chloride was being added to each NH_3 treatment.

357 **3.2 Reproductive Endpoints:**

358 *P. promelas* were sensitive to ammonia in both egg production and egg fertilization after
359 20 days of exposure. The NH_3 experiment met the U.S. EPA's mortality criteria in the control
360 treatment (≥ 90 % survival) because total mortality was 2.4 % (97.6 % survival) over the 20 day
361 study. The only fish that experienced mortality in the 20 day exposure were those in the control
362 and 5 mg/L NH_3 treatment and these treatments only had a single male and no female mortality
363 and as a result, had no effect on female fecundity.

364 Over the course of the 20 day exposure period the average number of eggs produced per
365 female per day in the control, 0.07 mg/L NH_3 (4.33 mg/L TAN), 0.21 mg/L NH_3 (12.54 mg/L
366 TAN) and 0.47 mg/L NH_3 (26.93 mg/L TAN) were 16.17 ± 2.69, 11.61 ± 2.53, 8.2 ± 1.58 and
367 5.85 ± 1.47 eggs per female per day, respectively (Figure 1). Results of the nonparametric test
368 determined that there was a significant difference among treatments and that the two highest
369 concentrations (0.21 and 0.47 mg/L NH_3) were significantly different from the control ($p < 0.05$).

370 After the first day of exposure, fewer eggs were produced in all treatments, compared to
371 the control, which indicated a rapid response of egg production to ammonia exposure (Figure 2).
372 Cumulative egg production at the end of the experiment was normally distributed ($W = 0.9846$, p
373 $= 0.9426$), variances amongst treatments were similar ($F = 0.2436$, $p = 0.865$, $df = 3$) and results
374 from the ANOVA suggested significant differences in cumulative egg production among
375 treatments ($F = 10.167$, $p = <0.01$, $df = 3$), and all three concentrations of NH_3 significantly
376 reduced cumulative egg production compared to the control (Dunnett's, $p = <0.05$ for each
377 treatment).

378 The control treatment from this experiment met the fecundity (greater than 15 eggs
379 produced per female per day) and fertility rate criteria (> 85%) (Ankley et al. 2001). Fertility

rates during the 20 day exposure period in the control, 0.07 mg/L, 0.21 mg/L and 0.47 mg/L NH_3 were 89.66%, 94.39%, 92.99%, and 80.32%, respectively (Figure 3). The fertility data were not normally distributed among treatments, however each individual treatment was normally distributed ($W = 0.9744$ $p = 0.9283$ for control, $W = 0.8908$ $p = 0.2789$ for 0.07 mg/L NH_3, $W = 0.9398$ $p = 0.6369$ for 0.21 mg/L NH_3 and $W = 0.9927$ $p = 0.9948$ for 0.47 mg/L NH_3). The ANOVA that tested for differences in egg fertilization among treatments found a significant difference ($F = 6.141$, $p = .003$, $df = 3$) and the 0.47 mg/L NH_3 treatment was significantly less than the control (Dunnett's, $p < 0.05$).

Fatpad, gonadosomatic and hepatosomatic index, and evaluation of other secondary sexual characteristic data were collected during this experiment. However, closer examination revealed inconsistencies in collection procedures and the results were discarded from the analysis of this experiment.

3.3 Molecular Endpoints:

Liver vitellogenin concentrations, which were collected as an alternative measure of fecundity, showed no differences among treatments and the control for both male and female *P. promelas*. Exposure to ammonia did not induce vitellogenin production in male fish (Figure 4). Although livers from the control male fish lightly expressed vitellogenin, this expression was negligible and was likely induced by low-levels of naturally occurring estrogens that were released by the females in the tank. A log transformation was conducted on the data to account for non-normal distributions. A Shapiro-Wilks test for normality was conducted on the log transformed data ($W=0.9612$, $p = 0.1196$). Variances amongst treatments were similar as verified by a non-significant Levene's test for homogeneity of variance ($F=2.76$, $p = 0.0537$, $df = 3$). Male liver vitellogenin concentrations suggest a reduction of vitellogenin production when *P.*

403 *promelas* are exposed to ammonia, however this reduction was not significant ($F = 1.34$, $p = 0.27$, $df = 3$). Female vitellogenin expression (Figure 5) also was not significantly different from
405 the controls as determined by the Program R npmc package.

406 **4.0 Discussion:**

407 **4.1 No Observable Effect Concentration (NOEC):**

408 The goal of this study was to determine a NOEC for NH_3 on fish reproduction and the
409 results differed from previously published data. The lowest NH_3 concentration tested in this
410 study was 0.07 mg/L (4.33 mg/L TAN), which caused a significant fecundity effect (29 %
411 reduction) in cumulative egg production after a 20 day exposure. This concentration is far below
412 any prior reported NOEC. Thurston et al. (1986) reported 0.37 mg/L NH_3 (6.43 mg/L TAN) as
413 the no effect level on *P. promelas* egg production.

414 There have been recent criticisms regarding NOECs which suggest they are not a very
415 conservative measurement and that their values tend to be located at concentrations where
416 significant effects occur (Hoekstra and van Ewijk 1993). We chose to report more conservative
417 values in addition to the NOEC (EC10 values calculated using the U.S. EPA's TRAP program).
418 Using the observed TAN values for each of the test treatments including control, the program
419 estimated the EC10 for cumulative fecundity at 1.99 mg/L TAN (0.022 mg/L NH_3 at a pH of 7.3
420 and temperature of 25°C).

421 **4.2 New Findings:**

422 Many previous studies have focused on non-reproductive endpoints in regards to
423 ammonia exposure (Spencer et al. 2008). The reason for this is likely due to the longer life-
424 cycles and hatching periods of other species of fish (trout, bass, etc.) which have been used in
425 previous ammonia research (Spencer et al. 2008). Thurston et al. (1986) reported significant

426 differences in fathead minnow reproduction at exposures to higher NH3 levels than the findings
427 of our study. We think that he differences in results between our study and those reported by
428 Thurston et al. (1986) could be due to differences in methodology. Thurston et al. (1986)
429 implemented a life cycle study where three to five day old *P. promelas* larvae were tested in
430 concentrations of NH_3 beyond the age of maturation. Our study was only a 20 day study that
431 exposed adult *P. promelas* to similar concentrations of NH_3. Prior to this study, it was speculated
432 that the 20 day study would result in much higher NOECs than the life cycle study as the length
433 of NH_3 exposure was much shorter and did not include a larval and juvenile life-stage exposure.
434 However, because this study suggests considerably lower NOECs for *P. promelas* reproduction,
435 the differences in methodology needed to be documented.

436 Control reproduction was much lower in the Thurston et al. (1986) study. The control
437 tanks averaged only 1.29 eggs per female-day compared to our 16.17 eggs per female per day.
438 This low reproduction did not meet the 15 eggs per female per day criteria established by the
439 U.S. EPA for 21 day studies (NHERL 2002). The control fish in the Thurston et al (1986) study
440 also produced fewer eggs than any NH_3 treatment, except for the 0.91 mg/L NH_3 (14.5 mg/L
441 TAN) concentration that induced 100% mortality. It is unknown as to why the controls produced
442 so few eggs during the life cycle study of Thurston et al. (1986) but could be related to having a
443 different male to female ratio than the one maintained in our 20 day exposure study. We suggest
444 using a minimum control reproduction criteria specified by the U.S. EPA for future reproduction
445 studies that is similar to the 15 eggs per female per day criteria used in our study.

446 Differences between our study and the Thurston et al. (1986) study could also be related
447 to tank volume and male/female ratios. The tanks used by Thurston et al. (1986) held 30 L
448 compared to our 9.5 L volume chambers, and each volume held a different number of fish per

449 replicate. Our study maximized the number of replicates for each tank concentration; therefore 7
450 replicates were tested per concentration each with a 2 male to 4 female ratio. This ratio
451 optimized control reproduction from an earlier 21 day flow through diluter pilot study. In
452 comparison, Thurston et al. (1986) only tested one replicate per treatment and each replicate
453 consisted of 50 three to five day old larvae. In the Thurston et al. (1986) study, 50 fish within
454 each tank were randomly thinned to 15 fish each containing no more than four *P. promelas*
455 males after 60 days of exposure. The male to female ratio ranged from 3:11 to 4:5 between
456 replicates which could potentially explain discrepancies in reproductive output.

457 The cumulative egg production appeared to be the most sensitive endpoint, but other
458 endpoints indicated potential for quantifying NH_3 exposure and effect. During the last week of
459 exposure, days 14 to 21, *P. promelas* in all tested NH_3 concentrations appeared to have a
460 reduction in reproduction. The fish exposed in the two highest concentrations, 0.21 mg/L and
461 0.47 mg/L NH_3, produced very few eggs during the last five days of exposure and as the
462 experiment progressed the egg production per female per day decreased. If this study were to be
463 carried out for an additional length of time, the average eggs produced per female per day after
464 exposure to NH_3 endpoint may have shown similar reductions on cumulative egg production as
465 the treatments appeared to produce fewer eggs over time in comparison to the control.

466 Currently, the U.S. EPA (1999) has developed its recommended water quality criteria
467 based on whether or not salmon or early life stage fish are present within a body of water. The
468 U.S. EPA ammonia water quality criteria were set using both acute and chronic toxicity studies
469 conducted between 1984 and 1999, including Thurston et al. (1986). These studies were
470 conducted using a wide range of methodologies. Our results suggest that the U.S. EPA water
471 quality criterion for ammonia be revisited and consider whether the 21 day method results will

improve the current criteria. We believe that the low control reproduction in Thurston et al. (1986) should be considered when revising the U.S. EPA ammonia criteria.

While it was not the intent of this study to review U.S. EPA water quality guidelines, the lowest concentration tested in this study, 0.07 mg/L NH_3 (4.33 mg/L TAN), is lower than U.S. EPA water quality guidelines of 0.094 mg/L NH_3 (8.4 mg/L TAN) for adult, non-salmonid fish exposed to NH_3. These current water quality criteria for fish are based on toxicity tests using rested or non-stressed fish (Randall and Tsui 2002). These criteria may be overestimating the NOEC because the criteria do not account for swimming fish which generally have elevated internal ammonia levels compared to resting (Randall and Tsui 2002) fish nor stressed fish which have an increased level of cortisol. Cortisol is the primary steroid produced upon stimulation by an environmental stressor (Giesy et al. 2003). Stress, which can be induced from a variety of biological and chemical agents, and the induction of cortisol have increased ammonia toxicity in some fish species (Randall and Tsui 2002). Furthermore, the formation of cortisol can also decrease the production of vitellogenin in fish (Giesy et al. 2003). Additional studies are needed to understand how ammonia toxicity relates to cortisol production in *P. promelas* and at which rate the induction of vitellogenin is reduced.

4.3 Ecological Implications:

There exists a debate on ammonia's mechanism of action in freshwater fish (Person Le Ruyet et al. 1998). As a neurotoxin, exposure to ammonia can affect all biological functions of a fish. Fish can be exposed to elevated NH_3 concentrations both internally and externally and both exposures can have effects on reproduction (Person Le Ruyet et al. 1998). Increased exposure to environmental NH_3 reduces the ability of the fish to reduce its internal NH_3 concentration. Many fish species have the ability to detoxify internal ammonia by converting it to glutamine,

495 glutamate or urea (Miller et al. 2007). As the exposure to external ammonia is prolonged, this
496 detoxification mechanism is weakened and ultimately the fish experiences ammonia toxicity
497 (Miller et al. 2007).

498 In streams highly dominated by effluents, such as areas in the southwestern U.S.,
499 ammonia toxicity should be a concern. Many streams in this area of the U.S. receive a large
500 percentage of their base flow from the discharge of WWTPs (Monda et al. 1995). Studies have
501 reported high ammonia concentrations in streams with flow largely dominated by effluents.
502 These streams have reported un-ionized ammonia concentrations ranging from 0.21 to 0.75 mg/L
503 (Boyle and Fraleigh 2003; Schlosser 1995). As the distance downstream of the WWTP, NH_3
504 concentrations typically decrease, however even at long distances (i.e. >12 km) NH_3
505 concentrations during the summer months were still above concentrations that can cause fish
506 reproductive effects. These concentrations are much higher than the LOEC determined by this
507 study (0.07 mg/L) in which there was a 29 % reduction in cumulative fecundity. A reduction this
508 severe, in addition to other threats to minnow populations such as other contaminant exposures
509 and predation, has the potential to threaten population survival. The observed reproductive effect
510 at such very low concentration is significant as the current average NH_3 concentration
511 discharging from a U.S. WWTP effluent is 0.1 mg/L (Lazorchak and Smith 2001). This could
512 have severe impacts on fish assemblages in rivers that are highly dominated by WWTP effluent
513 or during summer months when WWTP effluents are a high percentage of the baseflow.

514 **4.4 Conclusion:**

515 This study determined that the NOEC of ammonia on fathead minnow reproduction after
516 20 days of exposure is estimated at 0.022 mg/L NH_3 (1.99 mg/L TAN). The results of this study
517 suggest that the next time the U.S. EPA water quality criteria are revised, current research with

518 more rigid control requirements should be considered. Future research should focus on exposing
519 fish to environmentally relevant concentrations of NH_3 while the fish are being exposed to other
520 environmental stressors in order to determine if a similar reproductive NOEC exists. Caged
521 deployments of *P. promelas* in situ have been a useful tool in assessing physiological responses
522 to emerging contaminant exposures (Kolok and Schoenfuss 2011). Information about the
523 responses of animals to multiple natural and anthropogenic stressors is, at the present time,
524 insufficient for researchers to predict their combined effects (Jenssen 2006); often the effects of
525 just single stressors are not well-delineated. However, the reality is that fish are exposed to
526 multiple stressors in their natural settings and there exists a need to understand how different
527 chemicals interact within an organism to assess population risk. Multiple stressors can interact
528 with both the immune and endocrine systems simultaneously and such interactions occur in
529 many environments where fish are threatened with pollutants, parasites, and other environmental
530 stressors (Jobling & Tyler 2003). Once these questions have been answered, computational
531 models could then be created to predict population trajectories in order to determine if wild fish
532 populations are at risk.

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