DETERMINING THE EFFECTS OF MULTIPLE STRESSORS ON FATHEAD MINNOWS (*PIMEPHALES PROMELAS*) USING A FLOW-THROUGH DILUTER SYSTEM

By

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ABSTRACT

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Aquatic organisms are exposed to a multitude of contaminants and to fully understand the impact of multiple stressors on fish populations, we must first understand the mechanism of action for each toxicant and how the combined effects manifest at the level of the individual. 17α -ethinylestradiol has been known to cause adverse reproductive effects including reduced fecundity and fertility, intersex and sex change in fish by mimicking naturally produced estrogen at low concentrations. Ammonia has been known to cause adverse reproductive and mortality effects in individual fish through effects or damage to the central nervous system. A flow-through diluter system was used to test the individual effects of environmentally relevant concentrations of these two contaminants as well as their mixture on fathead minnow reproduction. Significant findings from this study suggest that U.S. EPA water quality criterion for ammonia in waters where nonsalmonids are present (8.4mg/L) is above the lowest observable effect concentration for fathead minnow reproduction. The lowest un-ionized ammonia concentration (5mg/L) tested during this study resulted in a 29% decrease in cumulative fecundity. The mixture of both 17α -ethinylestradiol and ammonia at their respective environmentally relevant concentration resulted in increased mortality, but did not to show adverse effects on reproduction. This study demonstrated the need for toxicity testing with multiple stressor scenarios and the importance of re-evaluating current U.S. EPA water quality criteria.

Copyright by BRANDON MICHAEL ARMSTRONG 2011 To my wife, Kristen, without whom I would most certainly be lost.

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CHAPTER ONE: INTRODUCTION

Concern over the harmful effects of trace chemicals found in wastewater treatment plant (WWTP) effluent has risen over the past two decades with the discovery that such chemicals often act as endocrine disruptors (Parrott & Blunt 2004, Jensen et al. 2000, Lange et al. 2000). Wastewater treatment plant effluent is often comprised of a mixture of chemicals from household and industrial sources and little is known about the effects of individual endocrine disrupting chemicals (EDCs) on fish and wildlife. Perhaps what is more alarming is that there is a paucity of information on how multiple EDCs interact within individual organisms. Endocrine disrupting compounds are defined as exogenous agents that interfere with the production, release, transport, metabolism, binding, action, or elimination of endogenous hormones responsible for the maintenance of homeostasis and regulation of developmental processes (Parks et al. 1999). A mixture of chemicals can have unexplained interactive effects on the endocrine system of fish and other aquatic organisms (Jobling & Tyler 2003). This study's aim was to outline a protocol for testing multiple stressors and to determine how two chemicals with different modes of action interact within a fish to affect reproduction.

The effects of multiple stressor interactions on fish and wildlife are notoriously difficult to assess because of several reasons. First, time lags between initial cause and expression of cumulative effects can be long, thus making it difficult to determine a cause-effect relationship (Adams 2005). Second, it is difficult to generate a hypothesis to test because we often do not know if the combined effects of two chemicals will be additive, synergistic or antagonistic. Synergistic effects occur when two chemicals are mixed and the effects are more than the sum of their individual effects. Antagonistic

effects occur when the individual effects of two contaminants counteract one another when mixed, and result in a much lower effect than predicted if the effects were additive. Finally, several mechanisms may be involved during multiple stressor interactions (Bonga 1997).

Information about the responses of animals to multiple natural and anthropogenic stressors is, at the present time, insufficient for researchers to predict their combined effects (Jenssen 2006); often the effects of just single stressors are not well-delineated. However, the reality is that fish are exposed to multiple stressors in their natural settings and there exists a need to understand how different chemicals interact within an organism to assess population risk. Multiple stressors can interact with both the immune and endocrine systems simultaneously and such interactions occur in many environments where fish are threatened with pollutants, parasites, and other environmental stressors (Jobling & Tyler 2003).

Endocrine disrupting compounds, a common component of WWTP effluent, are found in many water systems throughout the United States and around the world. These chemicals have been linked to high levels of intersex, sex change and skewed sex ratios in fish populations (Parrott & Blunt 2005, Lange et al. 2001. Jensen et al. 2001). Sources of EDCs have been cited as pesticides, oral contraceptives, dioxins, and other industrial compounds (Parrott & Blunt 2005, Lange et al. 2001). The majority of these chemicals are released into the aquatic environment via sewage systems and very few WWTPs have proper mechanisms in place to completely remove these contaminants (Parrott & Blunt 2005). Of particular interest are chemicals that mimic naturally produced estrogen; such chemicals have the potential to disrupt the endocrine system during the critical sexual development period (Lange et al. 2001). These estrogen imitators have been shown to induce feminization in male fathead minnows, *Pimephales promelas* (FHM), by stimulating the production of eggs and plasma vitellogenin, a lipo-phospho-glycoprotein precursor to egg yolk that is normally produced in females (Parks et al. 1999). Estrogen mimics also disrupt egg production in female FHM (Parrott & Blunt 2005). The process of vitellogenesis is sensitive to EDCs (Murphy et al. 2005), and perturbations in this process can have ecological consequences, because vitellogenesis is directly related to fecundity and egg quality of individual fish. This study examined the effects of 17α -ethinylestradiol (EE2) which is a synthetic estrogen and the most commonly used active ingredient in oral contraceptives (Lange et al. 2001) and a known EDC (Ankley et al. 2001, Lange et al. 2001, & Parrott & Blunt 2005).

Ammonia is another common pollutant in aquatic systems and has been found to be extremely toxic to fish. Effluent generally contains very low concentrations of ammonia; however during WWTP malfunctions or in parts of the world that have nonexistent or ineffective treatment systems, ammonia concentrations in waterways can be greater than 20mg/L (Passell et al. 2007). Farm animal operations are another source of water pollution including ammonia and the extent of pollution is entirely dependent on the size of the production (Robbins et al. 1972). Fertilizers used during agricultural operations also contain a significant amount of nitrogen. During runoff and flooding events, nitrogen produced by these types of operations could be released into nearby streams. Ammonia exists in two forms, ionized (NH4⁺) and un-ionized (NH3) (Thurston et al. 1986, Mayes et al. 1986). While total ammonia concentration is heavily monitored in WWTP effluent across the United States, NH3 concentrations generally are not (Passell et al. 2007) even though un-ionized ammonia is the larger contributor of ammonia toxicity to fish (Thurston et al. 1986, Mayes et al. 1986, U.S. EPA 1999). As a neutral molecule it is able to easily diffuse across the epithelial membranes of aquatic organisms (U.S. EPA 1999). The toxicity of un-ionized ammonia is highly dependent on temperature and pH of the water (U.S. EPA 1999). As the temperature and pH of water increase, the toxicity of un-ionized ammonia to FHM increases as well (U.S. EPA 1999).

Increased ammonia levels within the body result in both chronic and acute effects in fish. Ammonia targets the central nervous system of vertebrates and at high concentrations can result in acute toxicity (Randall & Tsui 2002). Convulsions followed by death are likely the result of this acute toxicity during exposures of high NH3 concentrations. The mechanism of action for this toxicity is believed to be from an increased activation of the glutamate receptors for the amino acid, N-methyl-D-aspartic acid (NMDA) (Randall & Tsui 2002). These receptors are responsible for controlling synaptic plasticity and memory function. This increased activation of these receptors leads to a depolarization of neurons and results in subsequent cell death within the central nervous system.

Human urine contains a high amount of ammonia and can contain trace amounts of EE2 (Parrott & Blunt 2005). It is possible that fish could be exposed to mixtures of both EE2 and ammonia during release of WWTP effluent because of prevalence of birth control usage and presence in urine, as well as the inability of WWTP to completely remove these chemicals from effluent. In this study I tested the effects of both ammonia and EE2 on fathead minnow survival and reproduction. Specifically, the ultimate goal of this project was to determine whether ammonia will impact the exposure of FHM to EE2 and which endpoints are most sensitive to use in detecting changes in exposure and effects. The hypotheses of this study were:

H1: Current published lowest observable effect concentrations for EE2 effects on FHM reproduction will be reduced when coupled with the multiple stressor effect of ammonia because having an added environmental stressor present will increase the fish's ventilation rate and therefore increase the uptake of EE2.

H2: Ammonia concentrations at or near U.S. EPA Water Quality Guidelines will result in reduced vitellogenin expression in FHM males and females because stress has been shown to decrease vitellogenin production in fish.

This thesis is split up in to separate chapters which focus on addressing these hypotheses. Chapter two investigates how NH3 affects FHM reproduction during a 21 day flow-through diluter study. This experiment was needed because of the lack of background information on the effects of NH3 on FHM reproduction; the most recent study prior to this experiment was conducted in 1986 (Thurston et al. 1986). Specifically, for this study, I needed to determine the NOEC of NH3 on FHM reproduction using the 21 day flow-through diluter method so that I could test the effects of both EE2 and NH3. Chapter three investigates the mixture effects of NH3 and EE2, each at their NOEC, on FHM reproduction and addresses both hypothesis (H1 and H2).

CHAPTER TWO: DETERMINING THE NO OBSERVABLE EFFECT CONCENTRATION OF NH3 ON FATHEAD MINNOW REPRODUCTION

Introduction:

Ammonia is a common pollutant in aquatic environments and sources include agricultural run-off, biological waste decomposition and WWTP effluent (Randall & Tsui 2002, U.S. EPA 1999). The effects of ammonia on aquatic systems have been documented world-wide, most commonly in regions with a high human population and/or large animal farms (Randal & Tsui 2002). In fish, ammonia toxicity has been reported to cause chronic effects such as reduced growth, reproduction and fertilization rates as well as acute effects including convulsions and comas which result in subsequent death (Randall & Tsui 2002, Thurston et al. 1986).

Fathead minnows were chosen as the model organism for this experiment. This species is a part of the ecologically relevant Cyprinidae family and its reproductive life cycle can be altered in a laboratory setting to produce clutches of 50-100 eggs every 3-5 days (Appendix A). FHM have a broad distribution across North America in both lentic and lotic environments (Ankley and Villeneuve 2006) and are tolerant of a wide range of water types (Ankley and Villeneuve 2006).

Ammonia toxicity has chronic effects on fish such as damage to the gills and respiratory impairment and will result in acute effects such as mortality at sufficiently high concentrations. Ammonia also has been known to reduce growth and inhibit reproductive success in FHM (Thurston et al. 1986). The no observable effect concentration (NOEC) and lowest observable effect concentration (LOEC) of NH3 on FHM egg production and viability were reported as 0.37mg/L and 0.91mg/L, respectively (Thurston et al. 1986). NOEC is the greatest concentration or amount of a substance as

determined by an experiment or observation, that causes no alterations of morphology, functional capacity, growth, development, or life span of target organisms distinguishable from those observed in control organisms of the same species and strain under the same defined conditions of exposure (McNaught & Wilkinson 1997). LOEC is the lowest observable concentration or amount of a substance as determined by an experiment or observation, that does not cause any alteration of morphology, functional capacity, growth, development or life span of target organisms of the same species and strain under the same defined conditions of the exposure (McNaught & Wilkinson 1997). FHM survival NOEC and LOEC were 0.44mg/L and 0.91mg/L, respectively. Another metric, the LC50 which is a statistically derived concentration of a substance in an environmental medium expected to kill 50% of organisms in a given population under a defined set of conditions (McNaught & Wilkinson 1997), was estimated to be 1.50mg/L for FHM (Alexander et al. 1986). Brain lesions were noted at a concentration of 0.21mg/L but not found at a concentration of 0.11mg/L NH3 (Thurston et al. 1986).

The purpose of this experiment was to test environmentally relevant concentrations of NH3 and to determine the NOEC of NH3 on fish reproduction that would be used during the mixture experiment portion of this study (Chapter 3). There was a need to determine a NOEC on FHM reproduction using the 21 day flow-through diluter method. Prior to this study, the most recent study reporting FHM reproductive effects after an NH3 exposure was Thurston et al. (1986). This study was a life-cycle study, therefore the published NOECs needed to be tested in order to determine if they were true NOECs using my methodology.

I determined the NOEC on FHM reproductive endpoints by using a flow-through diluter system to continually dose adult male and female FHM to three concentrations of NH3. The endpoints of this experiment included fecundity, fertility, male secondary sexual characteristics, mortality and vitellogenin expression in both plasma and liver. Fecundity, fertility and mortality were monitored on a daily basis while vitellogenin expression and male secondary sexual characteristic endpoints were measured at the end of the 21 day study.

Methods:

This study used a flow-through diluter system to dose FHM to three concentrations of ammonia and a control. The setup and design of the flow-through diluter system is an established U.S. EPA protocol for conducting 21 day toxicity tests and allows for a continuous toxicant dosing at predetermined concentrations throughout the entire study (Appendix A). Total ammonia concentrations tested in this study were 5.0mg/L (0.06mg/L NH3), 15.0mg/L (0.18mg/L NH3), and 30.0mg/L (0.42mg/L NH3). The U.S. EPA lab in Cincinnati, OH conducted a survey of 50 wastewater treatment plants from all over the United States in 2004. The average concentration of NH3 for these samples was 0.1mg/L and was as high as 0.76mg/L NH3 in an individual sample (Lazorchak & Smith 2004). Thurston et al. 1986 reported the NH3 NOEC on FHM reproduction was 0.37mg/L. Therefore, my NH3 baseline experiment tested concentrations similar to those tested before by Thurston et al. (1986) and also bracketed the average WWTP effluent NH3 concentration. Ammonium chloride (NH₄Cl) was chosen as the source for ammonia in this study because it did not produce any precipitants at the pH and temperature of the control/dilution water in the presence of fish. In a previous pilot study, I tried to use ammonium phosphate dibasic. 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, I wanted to eliminate any potentially confounding variables. Therefore, ammonium chloride was chosen as the NH3 source for this study.

Water chemistries were monitored several times each week throughout the study to ensure proper NH3 exposure. Total ammonia concentrations in individual testing chambers were measured once each week. Also, total ammonia concentration was measured three times each week from a composite of all 7 replicates within a treatment group. Because temperature and pH can affect total ammonia concentrations, temperature and pH readings for each testing chamber were recorded soon after a total ammonia measurement was performed. I estimated NH3 concentrations by using an equation provided by the Michigan Department of Environmental Quality: $NH3 = Total Ammonia/(1 + 10^{(0.09018 + (2729.92/(Temp^{\circ}C + 273.15)) - pH)}).$

The diluter system required daily maintenance to ensure proper dosing during the 21 day exposure period. Superstocks were mixed fresh daily to provide the diluter system with a constant source of toxicant. The 30mg/L total ammonia (0.42mg/L NH3) superstock was made by mixing 48g of NH₄Cl into 18L of laboratory line water. The 15mg/L total ammonia (0.18mg/L NH3) superstock was made up by mixing 24g of NH₄Cl into 18L of laboratory line water. The 5mg/L (0.06mg/L NH3) total ammonia superstock was made by mixing 8g of NH₄Cl into 18L of laboratory line water. The pumps were calibrated three times each week to ensure proper mixing of toxicant and

target concentration delivery to the testing chambers. The tubes for the peristaltic pumps were replaced weekly to prevent any breakage or tube malfunctioning.

This experiment consisted of a 14 day acclimation period and a 21 day exposure period. A total of 168 six month old FHM were used during each experiment study. Standard lengths and wet weights of all males and females were collected prior to the study to ensure each individual met the testing criteria (Appendix B). Fish were 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. Any mortality occurrences during the acclimation period were recorded and the sex of the individual was noted; deceased fish were replaced only 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 ensure no significant mortality was occurring. If any statistically significant differences in reproduction or high mortality occurred, the study was terminated and a new batch of fish chosen. 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 21 consecutive days during which survival, fecundity, fertility and mortality data was recorded daily. Any fish that died during the 21 day exposure period was not replaced. Bacteria convert ammonia to nitrite (NO2) and this can be detrimental to a study (Thurston et al. 1986), therefore testing chambers were cleaned three times each week to reduce the amount of bacteria buildup. Extreme caution was taken during tank cleaning to prevent unnecessary stress to the fish. Tanks were cleaned using a sponge to scrape down the sides of each tank. A sink siphon was then used to remove excess food and debris. Daily fecundity data, by tank, was recorded by counting the number of eggs laid on each spawning tile. Tiles containing eggs were marked with respective tank number and egg count data and then placed in an aerated egg bath of $25^{\circ}C \pm 1^{\circ}C$. Seventy-two hours after collection, fertilized eggs in the eyed stage were counted.

During the study, fish were fed on a regular schedule in order to maximize reproduction. Each testing chamber was fed three times each day. Between 8:00 and 10:00am EST, the fish were fed 20ml of concentrated newly hatched (<24H old) brine shrimp, *Artemia salina*. Between 12:00 and 1:00pm, the fish were then fed with 1.0ml of frozen adult brine shrimp (San Francisco Bay Co.). The final feeding occurred between 4:00 and 5:00 with 20ml of concentrated newly hatched brine shrimp supplied to each chamber. The feeding regimen adopted here was found to maximize reproduction and provide the healthiest fish during a 21 day control pilot study conducted by the U.S. EPA in Cincinnati, OH.

The NOEC ammonia study was terminated after 21 days of exposure and the fish were transferred for necropsy. Endpoint data collected were male secondary sexual characteristics, fecundity, fertility, gonadosomatic index (GSI), fatpad index (FPI), liver vitellogenin concentration and plasma steroid concentration. Prior to necropsy, the fish were anesthetized in a 200mg/L concentration of tricaine methanesulfonate (MS-222) and standard length and wet weights were measured. The caudal tail was severed to allow for collections of blood from the caudal artery via a heparinized capillary tube. Following collection, the heparinized capillary tube was centrifuged at 3000rpm for 1 minute to

separate the plasma from the red blood cells. After centrifugation the plasma was transferred into a labeled vial and then snap-frozen in liquid nitrogen. Immediately following plasma collection, the fish was euthanized by severing the spine just behind the nape of the fish. Secondary sexual characteristics of males were then recorded. Each tubercle was counted and fatpad size was scored using an U.S. EPA scoring system (U.S. EPA 2002), summarized in Table 1.

Table 1. Fatpad Scoring System used by the U.S. EPA.	
Rating	Description
None	No fatpad visible
Small	Small fatpad evident
Medium	Fatpad is prominent, clearly above the body surface but not overhanging
Large	Fatpad is very prominent and is starting to overhang the body surface

Each fatpad was separated using a scalpel and then weighed. This weight was then divided by the respective fish's body weight to calculate a fatpad index (FPI). Gonads and livers from males and females were removed and a wet tissue weight was taken. These weights were then divided by the body weight of the fish to calculate a gonadosomatic index (GSI) and hepatosomatic index (HSI) for each fish.

Vitellogenin in the liver samples was measured using "real time" quantitative polymerase chain reactions (QPCR) via a method already described (U.S. EPA 2009). Total RNA from the liver samples was isolated using TriReagent[®] (U.S. EPA 2005). Relative concentrations of the total RNA were then measured using an ultraviolet spectrophotometer. The isolated RNA was then converted into complementary DNA (cDNA) using a reverse transcriptase. A diluted sample of cDNA was then used for the PCR reactions along with a Hot Start DyNAmo[™] SYBR[®] green master-mix. Amplification of normalizing gene (18S) sequences was performed using universal 18S

primer pairs. Cycling was carried out using a thermocycler in cycles of 94°C, 60°C and 70°C. The fluorescent intensity of vitellogenin response from each sample was then calculated as a ratio of vitellogenin:18S and compared against the controls. An Enzyme-linked immuno-sorbent assay, ELISA, was used for the detection and quantification of vitellogenin in the plasma samples. These samples were sent to the U.S. EPA laboratory in Duluth, Minnesota for analysis.

Results:

FHM were sensitive to ammonia in both egg production and egg fertilization after 20 days of exposure. The analysis only includes the first 20 days of exposure as the control/dilution water pump failed on exposure day 21. The pump failure significantly increased ammonia concentrations and lead to mortality in each of the treatments. Therefore, day 21's egg production was removed from the analysis. The NH3 baseline experiment met EPA's mortality criteria in the controls (≥90% survival) as total mortality was 2.4% (97.6% survival) over the 20 day study. The only treatments that experienced mortality in the 20 day exposure were the control and 5mg/L NH3 treatment. These treatments each had a single male mortality; no female mortality was experienced during the 20 day exposure period.

Over the course of the 20 day exposure period the average eggs per female per day in the control, 5.0 mg/L (0.06 mg/L NH3), 15 mg/L (0.18 mg/L NH3) and 30 mg/L total ammonia (0.42 mg/L NH3) were 16.17 ± 2.69 , 11.61 ± 2.53 , 8.2 ± 1.58 and 5.85 ± 1.47 eggs per female per day, respectively (Figure 1).



This data set was not normally distributed so a non-parametric test, Kruskal-Wallis, was performed. The Kruskal-Wallis test determined that the treatments were significantly different from one another (K = 10.20, p = 0.017, df = 3). A Kruskal-Wallis Post hoc test (kruskalmc) was then performed in Program R and determined that the two highest concentrations (0.18 and 0.42mg/L NH3) were significantly different from the control (p = 0.05). Because female mortality did not occur during the 20 day exposure period, I could analyze cumulative egg production. When male mortality occurred, it was usually only a single male from a replicate and therefore I did not have to adjust the collected data.

After day 1 of exposure, all treatments produced fewer cumulative number of eggs than the control indicating a rapid response of egg production to ammonia exposure

(Figure 2). Cumulative egg production at the end of the experiment was normally distributed and verified by a non-significant Shapiro-Wilks test for normality (W=0.9846,



Variances amongst treatments were similar as verified by a non-significant Levene's test for homogeneity of variance (F=0.2436, p = 0.865, df = 3). An analysis of variance (ANOVA) was performed on cumulative egg production and found to be significantly different among treatments (F=10.167, p = <0.01, df = 3). A Tukey Honestly Significant Difference (Tukey HSD) test found that all three concentrations were significantly different from the control in terms of cumulative egg production (p = <0.05 for each treatment).

This experiment also met the aforementioned fecundity and fertility rate criteria (Ankley et al. 2001). Fertility rates during the 21 day exposure period in the control, 5.0mg/L, 15mg/L and 30mg/L total ammonia were 89.66%, 94.39%, 92.99%, and 80.32%, respectively (Figure 3).



The fertility data were not normally distributed amongst treatments, however each individual treatment was normally distributed as verified by individual Shapiro-Wilks tests (W = 0.9744 p = 0.9283 for control, W = 0.8908 p = 0.2789 for 5mg/L NH3, W = 0.9398 p = 0.6369 for 15mg/L NH3 and W = 0.9927 p = 0.9948 for 30mg/L NH3. The ANOVA that tested for differences in egg fertilization between treatments was significant (F = 6.141, p = .003, df = 3). The 30mg/L total ammonia group was statistically different from the control as verified by a Tukey HSD test (p = 0.046).

Ten ammonia sampling events occurred throughout the 21 day study in each of the treatments. The control and blank tanks did not have significant concentrations of ammonia (Figure 4). The blank tanks were required to monitor the water quality of the control and dilution water without the presence of fish. Therefore if mortality in the



control tanks had occurred, I could check the blank tank water quality data to determine if water quality was an issue. The final ammonia concentration in the tanks matched their desired concentrations. The 5mg/L total ammonia treatment tanks averaged 4.0mg/L total ammonia (range: 2.74 to 4.74mg/L). The 15mg/L total ammonia treatment tanks averaged 12.1mg/L total ammonia (range: 10.40 to 14.22mg/L). The 30mg/L total ammonia treatment tanks averaged 26.8mg/L total ammonia (range: 24.65 to 31.2mg/L). Mean measured concentrations throughout the study in the treatment tanks appear sufficiently similar to expected concentrations, however the coefficient of variance (CV) was well outside acceptable parameters (<20%). 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 replicates. Removal of data collected on this last day resulted in CV's of ammonia concentrations that were within an acceptable range (<20%). I adjusted the un-ionized

ammonia concentration for temperature and pH using the Michigan Department of Environmental Quality's formula (Figure 5).



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 between treatments. Conductivity was significantly different between treatments (ANOVA, F = 19.109, p = < 0.01, df = 3) which was to be expected considering ammonium chloride was being added to each NH3 treatment.

Liver vitellogenin concentrations were analyzed on both male and female FHM. Exposure to ammonia did not induce vitellogenin production in male fish (Figure 6). While the control male livers exhibited a slight vitellogenin response, this was considered non-significant as the females within the tank were most likely releasing some natural estrogens which may have caused the males to display low levels of vitellogenin.



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). Concentration of vitellogenin in male livers suggests a reduction in vitellogenin, however the reduction was not significant (F = 1.34, p = 0.27, df = 3). Female vitellogenin expression also was not significantly different from the controls (K = 2.44, p = 0.49, df = 3) (Figure 7). The data was not normally distributed so therefore the Kruskal-Wallis non-parametric test was used in the analysis. A non-parametric test was conducted on the female liver vitellogenin concentration after several transformations failed to produce a normalized data set.



Fatpad, gonadosomatic and hepatosomatic index data were collected during this experiment, however the results were extremely variable and bias was introduced depending on which technician was removing the tissues. For example, one fatpad was marked as "Large" but weighed less than a fatpad that was marked "Small". I felt that these differences were sufficient means to disregard these data in my analysis. Similarly, because the evaluation of other secondary sex characteristics was subjective and variable, I decided to disregard tubercle count data. For future studies, it is advised that one technician concentrate solely on one tissue throughout the entire necropsy to reduce the variability in tissue collection and tubercle count data.

Discussion:

The goal of my study was to determine a NOEC for NH3 on fish reproduction and my results were surprising when compared to what has been published previously. The lowest NH3 concentration that I tested was 5mg/L total ammonia (0.06mg/L NH3),

which caused a significant fecundity effect (29% reduction) in cumulative eggs after a 20 day exposure. This concentration is far below any NOEC reported previously. Thurston et al. (1986) reported 0.37mg/L NH3 as the no effect level on FHM egg production. The observed reproductive effect at such very low concentrations is significant as the current average NH3 concentration discharging from a US WWTP effluent is estimated to be 0.1mg/L (Lazorchak & Smith 2004), twice that of the 0.06mg/L NH3 concentration found to cause an effect on fecundity during this study.

The differences in results between this study and those reported by Thurston et al. (1986) were likely due to differences in methodology. Thurston et al. (1986) implemented a life cycle study where three to five day old FHM larvae were tested in concentrations of NH3 beyond the age of maturation. My study was only a 21 day study that exposed adult FHM to similar concentrations of NH3. Prior to my study, I speculated that the 21 day study would result in much higher NOECs than the life cycle study as the length of NH3 exposure was much shorter. Since my study ended up determining considerably lower NOECs for FHM reproduction, the differences in methodology needs to be documented.

The control reproduction was extremely low in the study conducted by Thurston et al. (1986). The tanks averaged only 1.29 eggs per female-day. This low reproduction did not meet the 15 eggs per female per day criteria used during my study (Ankley et al. 2001). The controls also produced fewer eggs than any NH3 concentration except for the 0.91mg/L NH3 concentration that induced 100% mortality. It is unknown as to why the controls produced so few eggs during the life cycle study of Thurston et al. (1986) but

could be related to having a different male to female ratio than the one implemented during my 21 day exposure.

Thurston et al. (1986) also used a 30L tank volume compared to my 9.5L volume. The difference in tank volume was due to differences in the amount of fish being exposed in each replicate. I wanted to maximize the number of replicates for each tank concentration; therefore I tested 7 replicates per concentration each with a 2 male to 4 female ratio. This ratio was known to optimize control reproduction from an earlier 21 day flow through diluter pilot study. Thurston et al. (1986) only tested one replicate per treatment, each containing 50 three to five day old larvae. After 60 days of exposure the 50 fish within each tank were randomly thinned to 15 fish each containing no more than 4 FHM males. My 21 day test tested a more stringent male to female ratio than Thurston et al. (1986). The number of males in each treatment ranged from 3 to 5 and females ranged from 3 to 11 in Thurston et al. (1986). The differences in the number of males and females between treatments could have impacted reproduction during the life cycle study reported by Thurston et al. (1986).

Flow rates to the tanks were also considerably different between studies; Thurston et al. (1986) implemented a 1L flow every 4-5 minutes where my study used a 40ml/min flow. This difference was unlikely to influence reproduction as there were no reports of water quality issues for either study. Temperature and dissolved oxygen concentrations in the control tanks were similar between each study; Thurston et al. (1986) used a control water with a temperature at 24.0°C (+/- 0.08°C) and dissolved oxygen content of 6.49mg/L (+/- 0.41) where my study used a control water at 25.1°C (+/- 0.30°C) and dissolved oxygen content of 6.26mg/L (+/- 0.84). The pH of the control water was

different between each study; Thurston et al. (1986) control water had a pH of 8.05 (+/-0.04) and in my study was 7.43 (+/- 0.06). It is unknown as to whether or not the difference in pH resulted in differences in reproduction between the two studies. Thurston et al. (1986) also tested a fluctuating photoperiod where the amount of daylight shortened and lengthened throughout the life cycle study (10.0 – 15.75 hours of daylight) and my study kept the photoperiod constant (16 hours of daylight).

The fish in my 21 day study were fed only newly hatched brine shrimp and frozen adult brine shrimp. Thurston et al. (1986) fed both newly hatched brine shrimp and commercial trout food (Silvercup salmon). I decided not to use trout food in my study because it builds up easily and fouls the water; I wished to reduce the frequency of tank cleanings to minimize disturbance. Differences in food quality could have impacted reproduction, however this is unlikely as the fish tested in my study and those tested by Thurston et al. (1986) were not reported to show signs of emaciation. In light of all these differences, I would recommend that the study conducted by Thurston et al. (1986) be repeated by the U.S. EPA in Cincinnati, Ohio in order to determine if the results documented in that study can be replicated. I also recommend that the study be repeated and controlled for consistent photoperiod and male to female ratio in each exposed chamber and also implemented using a minimum control reproduction criteria specified by the U.S. EPA that is similar to the 15 eggs per female per day criteria used in my study.

The cumulative egg production appeared to be the most sensitive endpoint, but other endpoints indicated potential for quantifying NH3 exposure and effect. During the last week of exposure, days 14 to 21, FHM in all tested NH3 concentrations appeared to have a reduction in reproduction. The two highest concentrations, 0.18mg/L and 0.42mg/L NH3, produced very few eggs during the last five days of exposure. If this study were to be carried out for an additional length of time, I think changes in the average eggs produced per female per day after exposure to NH3 endpoint would have shown similar reductions as cumulative egg production as the treatments appeared to produce fewer eggs over time in comparison to the control.

The determination of a NOEC was difficult because all NH3 concentrations caused a significant fecundity effect. Therefore, I assumed that 50% of the LOEC (0.06mg/L NH3) was a good estimate of NOEC (0.03mg/L NH3) on FHM fecundity. This assumption is justified because the 0.06mg/L NH3 concentration caused a significant effect in only one of the two fecundity endpoints.

The lowest concentration tested in this study, 0.06mg/L NH3, is lower than U.S. EPA water quality guidelines for adult fish exposed to NH3. These current water quality criteria for fish are based on toxicity tests using rested or non-stressed fish (Randall & 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 & Tsui 2002) 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 been known to increase ammonia toxicity in some fish species (Randall & 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 obtain a full understanding of how ammonia

toxicity relates to cortisol production in FHM and at which rate the induction of vitellogenin is reduced.

Currently, the U.S. EPA (1999) has developed its recommended water quality criteria based on whether or not salmon or early life stage fish are present within a body of water. There are two criterions that a freshwater source must meet: an acute criteria concentration (CMC) and a chronic criteria concentration (CCC). The literature states that freshwater aquatic organisms will not be affected adversely if the one hour average concentration of total ammonia does not exceed, more than once every three years on the average, the CMC. In addition, they will not be affected unacceptably if the 30 day average of total ammonia does not exceed, more than once every three years on the average, the CCC. These criteria are temperature and pH based, so for these purposes I standardized all NH3 values to a pH and temperature similar to the dilution water tested in this study, 7.3 and 25°C, respectively. If salmon are present, the CMC is 5.62mg/L total ammonia and if they are not present the CMC is 8.4mg/L (0.063 mg/L and 0.094 mg/L NH3, respectively). If early life stage fish are present the CCC is 1.23mg/L and if they are not present the CCC is 1.8 mg/L total ammonia (0.014 mg/L and 0.02 mg/L NH3).

The reduction in reproductive endpoints in terms of both fecundity and fertilization success can be detrimental to fish populations. The current CMC guidelines for adult, non-salmonid fish such as the fathead minnow is currently set at 0.094 mg/L NH3. This concentration is well above my observed LOEC concentration of 0.06 mg/L NH3. This tested concentration considerably lowered reproductive output (29%

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reduction) when compared to the control. The production of fewer eggs could result in decreased population abundance for fish exposed to these levels of NH3.

In this study I found a few disadvantages of using a flow-through dilutor system. Such disadvantages include the challenge of maintaining a constant rate of NH3, the dependence on functional and operational equipment, and a reliance on chemistry data for nominal concentrations. In a natural environment, NH3 concentrations change due to rain events and fluctuations in WWTP effluent concentrations. However, because this test was only looking at the effects of NH3, I did not need to mimic rain events or fluctuations in toxicant concentrations. Pump failure could have resulted in an alteration of tested concentrations and indeed, pump failure occurred at the end of the NH3 baseline experiment on Day 21. The laboratory line control and dilution water pump failed which meant that only the superstock was dispensed into the testing chambers. This caused a significant spike in NH3 concentrations, therefore only the first 20 days of the study were used to analyze reproductive endpoints. Despite issues with the flow-through diluter system, this study still produced several significant results.

Since the fish were housed in relatively small aquaria, it is possible that artifacts and unknown stressors could have influenced the results of this study. Territorial defense, mate selection, foraging for food and overall male dominance were probable throughout the study. However, much consideration was taken to limit any competition stress during the study. Three spawning tiles were placed in to each tank in order to provide each male a spawning site to court female; the remaining spawning tile was to serve as a hiding place for the remaining females. Each day the replicates were fed at three different times in order to reduce the aggressiveness of the fish over food availability. Other stressors including tank cleaning and technician presence were also minimized to reduce influencing endpoint data. Cleaning was conducted in order to remove excess food and fish waste. Since the fish biomass within each tank differed by less than 10% (12.89g +/-0.6g) the amount of waste being produced by the fish was consistent between replicates. Therefore the amount of time it took to clean each tank was approximately the same between replicates and this stressor should have had minimal influence on reproduction. Also, when the fish were not being fed or tanks being cleaned, a black liner was pulled down over the diluter system to minimize the presence of technicians walking around the room. Therefore it these experimental artifacts were not likely to strongly influence the results of this study.

Fish can be exposed to elevated NH3 concentrations both internally and externally and both exposures can have effects on reproduction. Increased exposure to environmental NH3 reduces the ability of the fish to reduce its internal NH3 concentration. Many fish species have the ability to detoxify internal ammonia by converting it to glutamine, glutamate or urea (Miller 2001). As the exposure to external ammonia is prolonged, this detoxification mechanism is weakened and ultimately the fish experiences ammonia toxicity.

Ammonia concentration in the water is likely to be directly proportionate to the distance downstream of a WWTP discharge. River water most certainly will dilute the concentration of ammonia in the effluent and as the distance downstream from the release point increases, ammonia will become far more dilute. This study showed that very low concentrations of ammonia, 0.06ppm under very specific environmental conditions known to optimize reproduction (light intensity, temperature, dissolved oxygen content,

etc.) can cause adverse reproductive effects in fish as an individual contaminant The adverse reproductive effects could increase if other environmental stressors were present.

Mobility of FHM within streams has been described and it is generally quite restricted and only experienced during periods of high discharge (Schlosser 1995). FHM movement tends to be relatively short in duration (less than 2 days) which suggests that FHM dwelling near WWTP effluent will not have the ability to escape its exposure. Seasonal effects may be observed as well because during warmer months, WWTP effluent concentrations in the river will increase due to lower groundwater inputs and reduced rain events. This increase in concentrations could be critical to species such as the FHM as it occurs during their spawning season. FHM spawn when temperatures reach above 16°C, extending from May and into the middle of August (Starrett 1951). Hodges (2007) found that during periods of low flow and stream drying, movement of minnow species into deep pools was observed. These pools create a refuge for small fish; however can lead to exposure to a variety of environmental stressors. In the real world, fish are exposed not only to ammonia, but also to mixtures of other chemicals and a variety of environmental stressors such as fluctuating temperatures and episodes of low dissolved oxygen. It is unknown how these naturally occurring environmental stressors will interact with additional inputs of ammonia by WWTP effluent.

This study required extensive time and resources to complete and to fully explore the effects of toxicants on fish reproduction; such methodology should be complemented by less labor intensive computational methods. As an example, to conduct this study a total of 35 testing days were needed to fully understand the toxicity of NH3 on FHM reproduction. This study also involved many resources, including expensive equipment, coordination with multiple agencies, and the assistance of more than 10 technicians during the 6 hour long necropsy. Following each necropsy, several hours were required to analyze samples for vitellogenin concentration. In addition, prior to the initiation of this experiment, several pilot studies were run using the flow-through diluter system. These pilot studies were aimed at maximizing control reproduction and consisted of different feeding regimens, types of food, flow rates, male to female ratios, and light intensity. This project was extremely cost intensive and therefore it is recommended that a computational modeling approach should be applied to help predict the effects of multiple stressors.

Computational models may play a significant role in relating the findings of studies like these to impacts on wild fish populations. Miller et al. (2007) suggested that data derived from laboratory studies which analyzed reproductive and survival endpoints on an individual could be related to the population level as long as a clear biological linkage could be established. Mechanistic studies regarding ammonia toxicity have not been thoroughly researched (Wood 2001). In order for computational models to use fecundity data derived from this study, future studies need to focus on determining a clear mode of action for NH3 on fish reproduction. The mode of action of NH3 is most likely linked to increased stress of the fish which leads to increased ventilation, plasma concentrations of glucose and cortisol, and oxygen consumption (Wood 2001). Miller et al. (2007) found that when FHM were exposed to various androgens, fecundity was directly proportional to vitellogenin concentration. As female FHM vitellogenin was reduced, the fecundity was also reduced. The reduction in vitellogenin was then used to

predict population trajectories. This research suggests that models can be used to relate results of laboratory studies, such as the one conducted here, to populations level effects.

In conclusion, the results of this study suggest that U.S. EPA water quality criteria be re-evaluated to include recent research focused on both individual contaminants as well as multiple stressor interactions. Future research should focus on exposing fish to environmentally relevant concentrations of NH3 while the fish are being exposed to other environmental stressors in order to determine if a similar reproductive NOEC exists. Once these questions have been answered, computational models could then be created to predict population trajectories in order to determine if wild fish populations are at risk.

CHAPTER THREE: DETERMINING THE MIXTURE EFFECTS OF NH3 AND EE2 ON FATHEAD MINNOW REPRODUCTION

Introduction:

Very few studies have tested the effects of multiple stressors on fish reproduction; most research has been focused on the effects of single contaminants. The U.S. EPA has used these single contaminant exposure studies to set current water quality criteria. However, in real world situations, fish are thought to be exposed to a variety of contaminants; the interactive effects of these contaminants on reproduction are unknown. For example, WWTP effluent is known to contain multiple contaminants, such as NH3 and EE2 (Nash et al. 2004, Parrott & Blunt 2004, Lange et al. 2000 & U.S. EPA 1999). Both NH3 and EE2 have been known to reduce FHM reproduction (Chapter 2, Nash et al. 2004, Parrott & Blunt 2004, Pawlowski et al. 2003, Lange et al. 2000 & Thurston et al. 1986), but there is little information on how these two chemicals interact within an individual fish. This study was designed to determine the simultaneous effects of both NH3 and EE2 on fish reproduction. As more data become available on multiple stressor relationships, it is my view that the U.S. EPA water quality criteria will need to be readjusted to incorporate multiple stressor scenarios.

To date, there are no records of any veterinary use of EE2, which suggests that this chemical is exclusive to human oral contraceptives and hormone replacement therapy (King County 2007). EE2 is excreted through urine as free EE2 or is conjugated to increase solubility (e.g. EE2-glucuronide) and undergoes limited degradation in WWTP (Parrott & Blunt 2005). Bacteria from WWTP metabolize EE2-glucuronide and then release free EE2 into the water body, thereby, increasing the concentration of free EE2 released from a sewage treatment plant (Parrott & Blunt 2005). EE2 concentrations may also increase over time, causing concerns because of its non-degrading property (Wolfand 2007).

Surveys of WWTP around the world have detected EE2 concentrations greater than lng/L (Parrott & Blunt 2005) which is significant because previous studies suggest that an EE2 dose of less than or equal to 1ng/L can decrease FHM male sexual characteristics, reduce egg fertilization and also reduce reproductive success. Fertilization success and male secondary sexual characteristics were significantly different in EE2exposed males (<1ng/L) from the controls during a life cycle exposure (Parrott & Blunt 2005). Pawlowski et al. (2004) also found that male secondary sexual characteristics were significantly different from the controls at 1ng/L EE2 in a 3 week exposure. The mean number of spawned eggs per breeding pair had a lowest observable effect concentration (LOEC) of 0.1ng/L EE2. A LOEC is the lowest concentration found by an experiment or observation which causes an alteration of morphology, functional capacity, growth, development, or life span of a target organism distinguishable from control organisms of the same species and strain under defined conditions of the exposure (McNaught & Wilkinson 1997). Nash et al. (2004) demonstrated that when male and female zebra fish, Danio rerio, were exposed to concentrations of 0.5ng/L EE2 over a 40 day period, males showed a significant increase in vitellogenin expression, but there were no observed differences in female vitellogenin expression. Additional studies report that vitellogenin expression in male FHM was significantly greater than that of control in fish exposed to concentrations <1.0ng/L of EE2 (Pawlowski et al. 2004).

When exposed to EE2 concentrations higher than 1ng/L, male FHM experienced alterations of the testis and experienced intersex development. No testicular tissue was

found in male FHM exposed to concentrations of EE2 higher than 4ng/L after exposure for 289 days (Grist et al. 2003). The decrease in the testicular tissue of male fish may be due to a decrease of plasma sex steroids (Parrott & Blunt 2005) which also occurs when exposed to EE2. A LOEC for the gonadosomatic index (GSI), which is a ratio of the gonad weight to the fish's total weight, for male and female *P. promelas* was 10 and 100ng/L EE2, respectively (Pawlowski et al. 2004). Parrott & Blunt (2005) reported female FHM experienced significantly different GSI when exposed to EE2 concentrations between 3.5 and 23ng/L. Kidd et al. (2007) conducted a whole lake experiment and found when male FHM were exposed to 5-6ng/L EE2 concentrations they produced significant amounts of vitellogenin and showed signs of intersex through oocyte development in the testis. After 7 years of exposure, the population of FHM within the lake was near extinction, thus implying that estrogenic exposures can impact the sustainability of wild populations.

The uptake mechanism of EE2 of fish is still being studied, but some pathways have been identified and once in the water column, EE2 is readily absorbed via branchial absorption (i.e. through the gills). The absorption rate is entirely dependent on the fish's gill ventilation volume (Skillman 2006). If a fish is stressed, such as in the case of poor water quality, the presence of ammonia, or other factors, the ventilation rate of the gills generally increases leading to a higher absorption rate of EE2. EE2 and other xenobiotics are diffused across the gill epithelium through lipid membranes (Di Giulio and Hinton 2008). EE2 has an octanol-water partition coefficient (K_{ow}) value of 3.67, meaning that it has a relatively high sorption potential (King County 2007). K_{ow} is defined as the ratio of a chemical substance concentration in the octanol phase to its concentration in the

aqueous phase. Xenobiotic uptake via branchial absorption is relatively low for toxicants with a K_{ow} value less than 1. Uptake rates increase fourfold between K_{ow} value 1.0 and 3.0.

EE2, a hydrophobic compound, is relatively insoluble in water and can easily be absorbed by fatty tissues or membranes. EE2 persists in the organism longer than native hormones and in turn can activate more hormone receptor sites leading to a higher overall potency than native hormones. Once it enters the fish via the gills, the EE2 is distributed into the blood supply where it binds to plasma proteins. The bloodstream then distributes the protein bound EE2 to the several organs such as the liver, kidney, testes/ovaries, and brain. These tissues all contain estrogen receptors. Fish have three distinct estrogen receptors, ER α , ER β and ER γ (Hawkins et al. 2000). ER α is the principle estrogen receptor in the liver and is the primary control of estrogen-responsive genes in fish (Skillman et al. 2006). This estrogen receptor is also expressed in several other tissues including the gonads (ovaries or testes) and brain (Islinger et al. 2003). EE2 will bind to these receptor sites and mimic the functions of the naturally produced estrogen. Once the site is activated, the proliferation of certain cell types (i.e. production of vitellogenin) begins (King County 2007, Osborne et al. 2007).

Once the hormone EE2 enters the fish via the gills, the bloodstream distributes the toxicant to the kidney and liver. Weber et al. (2004) reported that kidney tubule cells were undergoing necrosis leading to nephrotoxicity when fish were exposed to EE2. The glomeruli within the kidney were enlarged and undergoing a significant extent of cell death during the EE2 exposure. Damage to the kidney could result in poor excretory and osmoregulatory functioning (Weber et al. 2004) leading to a lower elimination rate of

xenobiotics. It was unknown as to whether or not the nephrotoxicity was a direct endpoint of exposure to EE2 or an elevated concentration of vitellogenin. Another finding by Weber et al. (2004) was that the fish were also undergoing hepatotoxicity, i.e. damage to the liver via necrosis. Since hepatocytes have the ability to regenerate, the constant replacement of these cells could lead to liver carcinogenesis and a lowered energy supply for reproduction (Weber et al. 2004).

In addition to disrupting the functions of the kidney and liver, the gonads of fish are also affected by exposure to levels of EE2. The production of and plasma level concentration of testosterone and its metabolite, 11-ketotestosterone, is reduced by exposure to EE2 (Wolfand 2007, Filby et al. 2007). 11-ketotestosterone is associated with the development of secondary sexual characteristics and essential reproductive behaviors (Wolfand 2007). EE2 inhibits the genes responsible for coding the enzymes (CYP11A1, CYP17, 3 β -HSD, and cytochrome P450 11B) involved in the production of androgens (Filby et al. 2007). The ratio of androgens to estrogens produces a 'male' versus a 'female' hormonal environment and if disrupted can lead to altered sex ratios (Filby et al. 2007).

Another effect of EE2 exposure in fish is the induction of vitellogenin in male fish. Vitellogenin is normally synthesized in the liver of female fish (Jobling & Tyler 2003) and transferred to the ovaries by circulation to eventually form yolk (Jobling & Tyler 2003, Islinger et al. 2003). Vitellogenin production is regulated by the native steroid 17 β -estradiol, E2. After production, vitellogenin is biotransformed by phosphorylation, glycosylation, and lipidation and the metabolites are secreted into the bloodstream (Parks et al. 1999). Once in the bloodstream, the vitellogenin metabolites are transported to the oocyte surface where they are seized by receptor-mediated endocytosis. Enzymes then break down the vitellogenin metabolites into egg yolk proteins (phosvitin and vitellin) and lipids as food for the developing embryo (Parks et al. 1999). When exposed to EE2, male fish will produce vitellogenin (Jobling & Tyler 2003). Therefore, a good indication of exposure to EE2 is concentrations of vitellogenin in the blood of male fish (Jobling & Tyler 2003). Another advantage of using vitellogenin and other biomarker data is that responses at lower levels of organization can be much more specific to modes or mechanisms of action (MOA) (Miller et al. 2007).

For several fish species, glucuronidation is the major pathway for inactivating and eliminating substances via the bile (Skillman et al. 2006). EE2 undergoes glucuronide conjugation and secretion into bile, which is similar to EE2 metabolism in mammals. The highest concentration of EE2 is found in the bile as glucuronide conjugates. Glucuronidation does not appear to significantly facilitate excretion of EE2 (Skillman et al. 2006). EE2 undergoes enterohepatic recirculation in some fish species such as trout, where the EE2-glucuronide conjugates in the gall balder are released into the gut and reabsorbed into the intestinal tract. This process increases the half-life of EE2 within the organism (Schultz et al. 2001) and allows for a re-dosing of the organism. After reabsorption into the gastrointestinal tract, the EE2-glucuronide conjugates then undergo deconjugation and are reabsorbed into the bloodstream or excreted via feces (Schultz et al. 2001). This recirculation allows the EE2 to become concentrated in the blood plasma, up to nearly 780 fold relative to the exposure water (Skillman et al. 2006) Extensive enterohepatic recirculation and mass balance studies suggest that most of the excretion of EE2 is the unconjugated form (Skillman et al. 2006).

The NH3 baseline experiment (Chapter 2) revealed a couple of significant findings regarding NH3 toxicity on FHM reproduction. All three concentrations tested in this experiment (0.06, 0.18 and 0.42mg/L NH3) significantly reduced fecundity (Chapter 2). The most important finding from this study was that the estimated 0.03mg/L NH3 NOEC for FHM fecundity is far below previously reported values. Thurston et al. (1986) reported the CETC for FHM egg production as 0.58mg/L. The LOEC determined by this study (0.06mg/L NH3) is also below current U.S. EPA water quality criteria. NH3 also appeared to reduce the induction of vitellogenin in males exposed to natural estrogens being produced by female fish.

One mechanism by which EE2 and NH3 can interact synergistically is by decreasing egg production in FHM. Individually, these chemicals both inhibit reproduction in fish (Thurston et al. 1986, Parrott & Blunt 2005, Lange et al. 2001, Jensen et al. 2001, Chapter 2). A fish's stress response includes an increased ventilation rate in order to increase the oxygen uptake rate of the gills (Bonga 1997). Additional stressors such as ammonia may increase the uptake of EE2 due to this increased ventilation rate; thus these two contaminants may act synergistically by increasing a fish's sensitivity to the other. Lower concentrations of EE2 may have a higher absorption rate due to increased ventilation when in the presence of NH3.

In addition to acting synergistically with one another, NH3 and EE2 may act antagonistically on other endpoints such as vitellogenin production. Stress can also induce the formation of cortisol, which can decrease the production of vitellogenin in fish (Giesy et al. 2003) and potentially act antagonistically from EE2 effects. High concentrations of toxic substances such as ammonia have been shown to impair vitellogenesis in fish (Ma et al. 2005). Due to this antagonistic relationship, vitellogenin production in a male fish may not be initiated during an EE2 exposure when in the presence of NH3.

The main hypothesis of this experiment was that the effects of EE2 on FHM reproduction will experience a synergistic relationship when in the presence of ammonia. I tested this hypothesis using the flow-through diluter system (Appendix A) by testing the individual reproductive effects of both NH3 and EE2 at their respective estimated NOEC as well as the mixture of the two contaminants. A follow up hypothesis is that EE2 and NH3 will act antagonistically on vitellogenin production in FHM males, and I tested this hypothesis by analyzing both male and female FHM plasma and liver vitellogenin concentrations from the diluter system experiment.

Data from a previous EE2 baseline study conducted in 2009 at the U.S. EPA facility located in Cincinnati, Ohio was used to determine the testing concentration of EE2 to *P. promelas* (Figure 8).



The EE2 study used the same diluter system and methodology as this project (Appendix A). A 0.33 dilution factor was used in determining the EE2 concentrations tested during the EE2 baseline study. This dilution factor was chosen because we needed to establish an EE2 NOEC for FHM egg production using this system. The low concentration, 0.5ng/L EE2, was chosen since it was the lowest concentration that could be analytically measured by the U.S. EPA's chemistry laboratory in Cincinnati, OH. Other flow-through diluter studies chose concentrations such that the lowest concentration was 10 times lower than the highest concentration. The 0.33 dilution factor satisfied both of these requirements. Three concentrations of EE2 were tested, 0.5ng/L, 1.5ng/L, and 4.5ng/L. Although recent studies have shown that concentrations as low as 0.1ng/L activate the production of vitellogenin in adult male FHM, this low concentration could not be tested because the U.S. EPA in Cincinnati, Ohio's chemistry methods require 0.2ng/L error.

Methods:

This experiment's purpose was to test the hypothesis that FHM will be more sensitive to EE2 when also exposed to NH3. Environmentally relevant concentrations of NH3 and EE2 that were at or near the NOEC for FHM reproduction as determined from the baseline studies were tested in order to mimic those concentrations found in an average WWTP effluent. By testing the two toxicants at their respective NOEC, any effect found in their mixture could be attributed to a multiple stressor relationship.

A control, 0.25ng/L EE2 concentration, 2.5mg/L total ammonia (0.03mg/L NH3), and a mixture of both the EE2 and NH3 concentrations were tested during this experiment. The 0.25ng/L EE2 concentration was chosen because it is near the average concentration found in WWTP effluent and is near the LOEC on FHM reproduction and also can stimulate the production of vitellogenin in male FHM. The EE2 baseline study found that after a 21 day exposure of FHM to 0.5ng/L EE2, reproduction was significantly reduced (Figure 8) and the induction of vitellogenin in EE2 exposed males was significantly greater than control males (Figure 9).



Figure 9: Average male vitellogenin liver concentrations. Sample Size (N) = 56. Conducted by U.S. EPA in Cincinnati, OH. Treatments included control, 0.5ng/L, 1.5ng/L and 4.5ng/L 17 α -ethinylestradiol (EE2). Error bars are \pm 1 standard error. Study duration was 21 days. A) Control versus 0.5, 1.5 and 4.5ng/L EE2. B) Control versus 0.5ng/L EE2.

The 0.03mg/L NH3 concentration was chosen because it was the estimated NOEC for NH3 on FHM reproduction per the NH3 baseline experiment results of this study (Chapter 2).

Previous laboratory studies that conducted exposures in static systems reported that EE2 breaks down over time resulting in nominal concentrations that are close to 0.0mg/L. Because the flow-through diluter system provided a constant renewal of testing solution, the breakdown of EE2 had a minimal influence on my nominal concentrations. This constant renewal also eliminated the need to use dimethyl sulfoxide, DMSO, as a carrier solvent. This was important because the potential adverse effects of DMSO on fish had not clearly been determined at the time of the study and could have resulted in false data interpretations after a 21 day exposure.

As performed in the NH3 experiment (Chapter 2), water chemistries were monitored several times each week throughout the study to ensure proper toxicant exposure. The NH3 baseline experiment determined that collecting individual tank ammonia measurements was unnecessary as all tank NH3 concentrations were similar to the composite concentration. Therefore only a total ammonia concentration was measured from a composite of all 7 replicates within a treatment group three times each week. Temperature and pH readings for each composite were recorded soon after a total ammonia measurement was performed. EE2 analysis was performed twice each week. One (1) liter samples from a composite of each treatment and their respective superstock were sent to the chemistry department at the U.S. EPA in Cincinnati, OH for analysis. The 0.25ng/L EE2 concentration was below detection limits using the U.S. EPA method; therefore two (2) liter samples of each testing chamber treatment were analyzed. The superstocks were measured to ensure that the flow-through diluter pumps were accurately and precisely mixing each treatment throughout the study.

The diluter system required daily maintenance to ensure proper dosing during the 21 day exposure period. Fresh superstocks were mixed daily in order to provide the diluter system with a constant source of toxicant. The 2.5mg/L total ammonia (0.03mg/L NH3) superstock was made by mixing 4g of NH₄Cl into 18L of laboratory line water. A 1.7mg/L EE2 dosing stock was provided by the U.S. EPA chemistry group for making the EE2 superstock. This superstock was made by adding 79.4 μ L of dosing stock to 18L

of laboratory line water. The pumps were calibrated three times each week to ensure proper mixing of toxicant and delivery to the testing chambers. The tubes for the peristaltic pumps were replaced weekly to prevent any breakage or tube malfunctioning.

Results:

This experiment met mortality criteria in the controls (Appendix B) as total mortality was 4.8% (95.2% survival) over the 21 day study. Male mortality was 1, 4, 3, and 6 for the control, 0.25ng/L EE2, 0.03mg/L NH3 and mixture treatments, respectively. Female mortality was 1, 1, 2 and 6 for each of the treatments, respectively. The mortality data were not normally distributed; therefore, a nonparametric Kruskal-Wallis test was performed. Total mortality was found to be significantly different amongst treatments (K = 7.972, p = 0.047, df = 3). The mixture (EE2 and NH3) treatment was significantly higher than the control for total mortality as determined by the Kruskal-Wallis multiple comparison Post hoc test (kruskalmc) in Program R (p = 0.05).

To account for female mortality in the treatments, egg production was standardized by taking the total number of eggs produced within each treatment and dividing by the total number of surviving females. Over the course of the 21 day exposure period the average eggs produced per female per day in the control, 0.25ng/L EE2, 2.5mg/L total ammonia and the mixture were 19.79 ± 2.82 , 23.49 ± 1.89 , 22.47 ± 2.48 and 21.02 ± 3.37 eggs per female per day, respectively (Figure 10).



Ammonia (NH3) and 17 α -ethinylestradiol (EE2) Interaction Experiment. Treatments included control, 0.25 ng/L EE2, 0.03mg/L NH3 and a mixture of both toxicants. Error bars are ± 1 standard error. Study duration was 21 days.

Because female mortality occurred during the 21 day exposure period, the egg production per female data set was chosen for analysis instead of cumulative egg production. Egg production per female per day was normally distributed and verified by a non-significant Shapiro-Wilks test for normality (W=0.9731 p = 0.076). Variances amongst treatments were similar as verified by a non-significant Levene's test for homogeneity of variance (F=2.2264, p = 0.09147, df = 3). There were no differences in egg production per female between treatments (ANOVA, F=0.363, p = 0.7799, df = 3).

Fertility rates during the 21 day exposure period in the control, 0.25ng/L EE2, 2.5mg/L total ammonia and the mixture were 89.08%, 88.87%, 88.14%, and 82.08%, respectively (Figure 11). Fertilization rates were normally distributed and verified via a Shapiro Wilks test for normality (W = 0.9414, p = 0.1202).



Figure 11: 21-Day Treatment Flow-through Diluter Concentration Fertilization Rate. Sample Size (N) = 7. Conducted 4/8/10. Unionized Ammonia (NH3) and 17 α ethinylestradiol (EE2) Interaction Experiment. Treatments included control, 0.25 ng/L EE2, 0.03mg/L NH3 and a mixture of both toxicants. Error bars are ± 1 standard error. Study duration was 21 days.

Differences in variances among treatments were negligible as verified by a nonsignificant Levene's test (F = 0.7773, p = 0.5181, df = 3). Fertility rates were also not significantly different amongst treatments as determined by an ANOVA (F = 0.5252, p = 0.67, df = 3).

Seven sampling events occurred throughout the 21 day exposure to verify EE2 exposure (Figure 12). The control and NH3 tanks did not have any significant trace concentrations of EE2 and because the 0.25ng/L EE2 concentration is lower than the chemists' detection limits (+/- 0.2ng/L error) only the superstock concentrations were reported. The superstock (7.5ng/L EE2) was 30x the testing concentration in the tank (0.25ng/L EE2). The 0.25ng/L EE2 concentration and mixture concentration had an average superstock EE2 concentration of 7.27ng/L and 8.04ng/L, respectively.



Sampling event #4 for the 0.25ng/L EE2 concentration was not included in the analysis as it was significantly lower than expected and may be due to an analysis error. The coefficient of variation values were below the required 20% test acceptability limit (Ankley et al. 2001) as the EE2 and mixture superstock concentration CV values were 7.71% and 8.13%, respectively.

Ten sampling events for ammonia occurred throughout the 21 day study. The control and EE2 tanks did not have significant trace concentrations of NH3 (Figure 13). The 2.5mg/L total ammonia treatment tanks ranged from 1.96 to 2.47mg/L total ammonia with an average of 2.26mg/L total ammonia (0.03mg/L un-ionized ammonia). The mixture tanks ranged from 1.58 to 2.51mg/L total ammonia with an average of 2.18mg/L total ammonia (0.03mg/L un-ionized ammonia). The total ammonia (0.03mg/L un-ionized ammonia). Mean measured concentrations throughout the study in the treatment tanks appeared sufficiently similar to expected

concentrations and the coefficient of variance (CV) was within acceptable parameters (<20%) for both treatments containing ammonia.



Chemical parameters including dissolved oxygen (ANOVA, F = 0.949, p = 0.425, df = 3), pH (ANOVA, F = 0.417, p = 0.741, df = 3), temperature (ANOVA, F = 0.281, p = 0.839, df = 3), and conductivity (ANOVA, F = 0.210, p = 0.889, df = 3) were determined for the old test solutions and showed no significant differences between any treatments.

Vitellogenin concentrations were analyzed on the male and female livers (Figures 14 and 15). Male vitellogenin concentration was log transformed in order to obtain normality (Shapiro-Wilks, W=0.9759, p = 0.4939). Variances amongst treatments were similar as verified by a non-significant Levene's test for homogeneity of variance (F=0.691, p = 0.5631, df = 3). Male liver vitellogenin concentration was not significantly different between treatments (F = 1.844, p = 0.155, df = 3). Female liver vitellogenin concentrations were not affected by exposure to either contaminant or their mixture (K = 2.09, p = 0.55, df = 3). This data was not normally distributed; therefore a Kruskal Wallis

test was used in the analysis. Again, no transformations were able to normalize the female liver vitellogenin concentrations data set.



Figure 14: Average male vitellogenin liver concentrations. Sample Size (N) = 56. Conducted 4/8/10. Unionized Ammonia (NH3) and 17 α -ethinylestradiol (EE2) Interaction Experiment. Treatments included control, 0.25 ng/L EE2, 0.03mg/L NH3 and a mixture of both toxicants. Error bars are ± 1 standard error. Study duration was 21 days.



Plasma vitellogenin was negligible amongst all treatments in male FHM (Figure 16). The data were normally distributed, therefore a Kruskal-Wallis test was used to determine the

non-significance (K = 5.72, p = 0.12, df = 3). One control male outlier displayed a high amount of plasma vitellogenin and was removed from the analysis (Figure 17).



Figure 16: Average male plasma vitellogenin concentrations. Sample Size (N) = 56. Conducted 4/8/2010, data provided by U.S. EPA in Duluth, MN. Unionized Ammonia (NH3) and 17 α -ethinylestradiol (EE2) Interaction Experiment. Treatments included control, 0.25 ng/L EE2, 0.03mg/L NH3 and a mixture of both toxicants. Error bars are \pm 1 standard error. Study duration was 21 days.



days. This non-response may be due to the concentrations of EE2 being below minimum detection limits by the ELISA assay. Female plasma vitellogenin (Figure 18) was also not effected by exposure to any of the treatments (K = 3.23, p = 0.36, df = 3). The data was also non-normally distributed and analyzed using a Kruskal-Wallis test.



(NH3) and 17 α -ethinylestradiol (EE2) Interaction Experiment. Treatments included control, 0.25 ng/L EE2, 0.03mg/L NH3 and a mixture of both toxicants. Error bars are ± 1 standard error. Study duration was 21 days.

Discussion:

Neither EE2 nor NH3 caused a fecundity effect on FHM reproduction individually. This was expected as both toxicants were being tested at an estimated NOEC. Also, the mixture of these two contaminants did not affect fecundity during the 21 day study. This finding rejects the author's hypothesis that the mixture of these contaminants at their respective NOEC would produce an effect on FHM reproduction. Although a fecundity effect was not apparent, a significant mortality effect was observed in the mixture concentration. This finding is surprising because the concentrations of both EE2 and NH3 were far below any previous reported lethality concentration. Thurston et al. (1986) reported a LOEC of 0.91mg/L NH3 on FHM survival. Lange et al. (2001) determined the LOEC of EE2 on adult FHM survival was greater than 1ng/L. The tested concentrations were chosen to mimic the average WWTP effluent NH3 and EE2 concentrations, so this mortality effect is quite alarming for small short-lived species such as the fathead minnow. In natural systems, Lazorchak & Smith (2004) determined that the average NH3 concentration in U.S. WWTP effluent was around 0.1mg/L. Parrott & Blunt (2005) also stated that EE2 concentrations greater than 1ng/L are found in WWTP effluent around the world. Therefore, such a high mortality rate in a mixture of only two contaminants at their environmentally relevant concentrations is a concern. In light of these findings, the current U.S. EPA water quality criteria should be re-evaluated, and other stressors should be considered because fish are exposed to much greater numbers of contaminants as well as other biological stressors including temperature changes, dissolved oxygen fluctuations, starvation and predation.

The multiple stressor effect on mortality has been documented in other species exposed to different stressors. Schisler & Bergersen (2000) examined the combined effects of disease and temperature on rainbow trout, *Oncorhynchus mykiss*. Elevated water temperatures on their own had minimal effect on mortality of trout; however, mortality was significantly increased in trout infected with whirling disease, *Myxobolus cerabralis*.

The mortality that was experienced during this mixture study could possibly be attributed to a high reproductive output. It is important to point out that the control reproductive success was much higher during this study than any previous flow-through diluter experiment conducted by the U.S. EPA in Cincinnati, OH. This increased reproduction may have been a cause for the unexpected mortality in the mixture concentration because the fish could have spent more energy on reproduction than other similar aged and sized FHM used in previous flow-through diluter experiments; the added stress of EE2 and NH3 in combination may have been enough to cause mortality. Females could have used more energy producing and laying eggs while the males may have spent more energy courting females and guarding the eggs. This experiment should be repeated to make sure the mortality effect is consistent. Gunderson (1997) determined that there was a tradeoff between reproductive effort and adult survival in fish species. The NH3 baseline study also had a high reproductive effort experienced by the control fish when compared to previous flow-through diluter studies; however mortality was not an issue during this study (Chapter 2). If further testing proves the mortality experienced in the mixture study to be a justifiable effect, continued output of WWTP effluent containing concentrations similar to those tested in this study could be causing detrimental effects on wild FHM populations, as well as other fish species.

The mixture study tested a 0.25ng/L 17 α -ethinylestradiol (EE2) and a 0.03mg/L NH3 concentration. The EE2 sample analysis revealed a consistent dosage of superstock throughout the study except for one sample collection, sample #4 (Figure 12). This was deemed as an outlier and was probably due to an error in sample collection or analysis. The EE2 analysis for the 0.25ng/L testing chambers was below the U.S. EPA chemistry group's method detection limits (± 0.2ng/L EE2) and therefore was not considered reliable data. Because the EE2 superstock was within the 60 to 80% nominal test acceptability (Grist et al. 2003) and the 0.03mg/L NH3 concentration in the tanks was also near expected concentrations, a reasonable assumption can be made that EE2 within the tanks was also near expected concentrations.

Another disadvantage of using a flow-through diluter system is that EE2 chemistry data of testing solution is extremely hard to distinguish from background levels of natural estrogens that are produced by the fish. The U.S. EPA has established methods to determine the nominal testing concentrations of EE2 (U.S. EPA 2007). Sixty to eighty percent nominal is acceptable in most studies (Grist et al. 2003). The mixture study produced better than most studies in EE2 analysis as a 96.8% nominal concentration in the EE2 superstock was determined. The 0.25ng/L EE2 concentration in the tanks could not be determined as it is below the method detection limit for the U.S. EPA chemistry group in Cincinnati, OH.

This pilot study suggested an underlying mechanism regulating the impact of ammonia exposure on the induction of vitellogenin in male FHM. Ammonia may be stimulating the production of cortisol, which has been known to decrease the production of vitellogenin in fish (Giesy et al. 2003) and potentially act antagonistically from EE2 effects. Another hypothesis may be that the males are shutting down unnecessary mechanisms, such as the production of vitellogenin, in response to the environmental stressor. Further investigation into the MOA of ammonia on vitellogenin production should also be conducted in order to fully understand why the antagonism exists. By understanding these mechanisms at which vitellogenin production is slowed due to contaminant exposure, computational models could be created in order to predict the effects of other contaminants on vitellogenin production, and eliminate the need to test every multiple stressor combination in a costly experimental design.

The protocol used in this experiment was successful in determining multiple stressor responses to anthropogenic and natural stressors. For example, a mortality effect was not significant in either individual FHM exposure to NH3 or EE2, but a significant effect was found in the exposure to their mixture. EE2 appeared to increase vitellogenin concentration in the livers of male FHM but when EE2 was mixed with NH3, the production of vitellogenin was blocked. These findings indicate the significance of testing with multiple stressors rather than single contaminant exposures and how protocols such as the 21 day flow-through diluter study can be used to test the impacts of multiple stressors. Importantly, the procedures outlined here were designed to test both the individual effect of both toxicants and a mixture of both contaminants, which is necessary to determine synergistic and antagonistic effects.

The findings from this study suggest that multiple contaminant exposures should be tested and used for ecological risk assessments rather than data derived from single contaminant dose response studies. The possibility of chemical interactions within a fish between EDCs, other stressors and mating systems could have a population-level effect through reduced fertility, fecundity, or mortality. Multiple stressors that manipulate reproductive activity and male secondary sexual characteristics may strongly affect sexual selection and mate choice in fish (Nash et al. 2004). This study tested only two out of potentially hundreds of interacting chemicals found in WWTP effluent. WWTP effluent contains a cocktail of chemicals that could have numerous reproductive effects on fish populations. Therefore, understanding how the chemicals within WWTP effluent interact with one another in terms of reproductive effects on fish and other aquatic species should be a high priority for future toxicological studies.

Because the field of testing with multiple stressors is relatively new, very little data exists regarding how different estrogenic contaminants interact with one another and therefore it is hard to predict the outcomes of multiple stressor studies. Therefore, more studies testing a variety of other EDC's should be conducted in multiple stressor situations in order to fully understand how estrogenic contaminants interact and affect fish reproduction, especially at environmentally relevant concentrations. Estrogen mimics can occur without chemical exposures due to sub-optimal temperatures, restricted food supply, low pH, environmental pollutants, and/or parasites (Jobling & Tyler 2003). Most multiple stressor studies have analyzed the effects of two natural stressors such as predation and nutrition while very few studies have researched combinations of natural and anthropogenic stressor relationships (Folt et al. 1999). These experiments have shown that the combination of these two stressor types can cause detrimental effects at a level much lower than individual chemical exposures. Testing estrogenic contaminants with other stressor situations such as low dissolved oxygen concentrations and high or low temperatures should also be considered as fish may be exposed to these environmental situations as well.

Hanazato and Dodson (1995) tested the effects of low oxygen concentration when coupled with the effects of a predatory response and a pesticide exposure. Synergistic relationships were determined when a cladoceran, *Daphnia pulex*, was exposed to a combination of 2 or more of these stressors. Growth rates, neonatal development and maturation size were all reduced when a combination of these stressors were present. These findings determined that the synergistic relationships found between environmental factors and chemical exposures are often underestimated in laboratory tests looking at the effects of single contaminant exposures. Most laboratory tests are held at high dissolved oxygen concentrations and their study determined that effects of chemical exposures can be amplified when environmental stressors such as low dissolved oxygen concentrations are present.

Short-lived species such as FHM have a greater risk of population collapse when exposed to EDCs, but chronic exposures of longer-lived species may result in extinction as well (Kidd et al. 2007). FHM and other short-lived species are vital to many aquatic food webs as forage fish (Jenkins & Burkhead 1993). If FHM populations, as well as other small, short-lived species, are threatened and/or reduced, larger and longer-lived species could be at risk due to a decreased food supply. Long-term and lifetime exposures to multiple estrogenic contaminants could also put larger species at risk due to potential endocrine disruption and reduced reproduction success. Long-lived species, such as sturgeon, become sexually mature at an old age compared to short-lived species. The longer lived fish also have shorter spawning seasons and in some cases can have multiyear intervals between spawning events (Gross et al. 2002). These species may be more susceptible to population collapse if their reproductive cycle is adversely altered due to a lower reproductive output. Reproductive effects, in particular the intergenerational effects on fertility, are difficult to assess in long-lived species due to their low reproductive output and long generation times compared to short-lived species. Understanding how multiple stressor exposures affect the reproductive success of fish populations is important because continued estrogenic contaminant inputs into the environment from municipal wastewaters could threaten their sustainability (Kidd et al. 2007).

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CHAPTER FOUR: CONCLUSIONS AND FUTURE DIRECTION

Expectations for this study were to define the multiple stressor effects of EE2 and NH3 on FHM reproduction during a 21 day experiment using a flow-through diluter system. Because the testing of multiple stressors is a relatively new area of study and very few data exist on how chemicals interact during exposure studies, this study served as an exploratory one to provide preliminary data for future multiple stressor experiments as well as to update un-ionized ammonia toxicity relative to FHM reproduction.

The NH3 baseline experiment suggested that U.S. EPA water quality criteria for NH3 may currently be set above the NOEC on fish reproduction. U.S. EPA water quality criteria for waters containing adult, non-salmonid fish is currently set at 0.094mg/L (at a pH of 7.3 and 25°C temperature similar to the parameters of the dilution water tested in this study (Chapter 2)). This concentration is over 56% higher than the LOEC determined by this study (0.06mg/L) in which I found a 29% reduction in cumulative fecundity. A reduction this severe, in addition to other threats to minnow populations such as other contaminant exposures and predation, has the potential to threaten population survival. If fish in the wild are being exposed to concentrations of ammonia high enough to cause severe reproductive effects, theoretically it is possible that entire populations within a water body are at risk.

Density dependent factors play an important role in population regulation and should be considered when determining population risk due to a contaminant. If fish within a healthy water body are producing more eggs than the environment can support, density dependent factors such as competition will limit population growth. It is because of density dependence that a reduction in fecundity as a result of contaminant exposure may not necessarily lead to a direct population collapse. For example, if a population of fish produces 1500 eggs in a given year and the population can only support an increase of 1000 individuals, density dependent factors will result in 500 of those eggs from reaching maturity in order to maintain the population at its carrying capacity. If that same population is exposed to a contaminant that reduces the fecundity from 1500 to 1000 eggs, there still will be 1000 new individuals reaching maturity within that population; hence the contaminant will not have an effect on the number of individuals within that population. It is even possible that the loss in fecundity will produce healthier fish because competition for food availability, sexual mate selection and territorial defense may be reduced. However, it is my view that exposure to a known contaminant which causes reproductive effects in fish should not be considered as potentially beneficial because other detrimental factors resulting from the exposure are likely to occur.

Extrapolating the results of this study which used laboratory fish to individuals found in the wild can be a difficult task at best. The FHM at the U.S. EPA AWBERC facility in Cincinnati, OH have been raised for hundreds of generations in conditions specific to increasing survival and reproduction. The genetic diversity in these fish is minimized because no new genetic sources of FHM have been introduced to this laboratory in years. These fish are not exposed to a variety of stressors seen in wild populations including predation, foraging, low water quality and high and low temperature situations. Wild fish face different evolutionary pressures and also have a much higher genetic diversity than laboratory raised fish because new genes are constantly being added to the gene pool because fish either migrate or are introduced from different water bodies. Extrapolating this data to other species of fish with longer lifespans and lower reproduction can be even more difficult. Long-lived fish tend to have shorter spawning seasons and in some cases can have multiyear intervals between spawning events (Gross et al. 2002). These species may be more susceptible to contaminant exposures as their reproductive output is relatively low compared to short-lived species such as FHM. Reproductive effects of contaminant exposures using long-lived fish are difficult to implement due to the long maturation rate and short and intermittent spawning seasons. Therefore, mechanistic models using laboratory data collected from short-lived fish can be used to predict the exposure response of long-lived species.

The mixture study verified that the estimated NOEC values as determined from the respective baseline experiments were justified. Neither the 0.03mg/L NH3 nor 0.25ng/L EE2 concentrations caused a FHM reproductive effect. The mixture of these two contaminants also did not cause a reproductive effect. This non-response nullifies the hypothesis that the mixture of these two contaminants at their respective environmentally relevant concentration would result in a reproductive effect. The other hypothesis from this study was that vitellogenin production in male FHM would not be induced during exposure to EE2. This hypothesis was verified as the EE2 exposed males showed a slight increase in vitellogenin while the mixture exposed males showed virtually no vitellogenin in either the plasma or liver. This inhibition of vitellogenin production in the males is most likely influenced by the increased stress and production of cortisol which has been shown to reduce vitellogenin production. Another interesting finding that should be explored further is why there wasn't a reduction of vitellogenin production in the females exposed to NH3.

My experiment found significant and alarming evidence that the mixture of EE2 and NH3 significantly increased both male and female mortality. The control treatment only experienced one male and/or female mortality over the course of the 21 day study. In contrast, the mortality increased by 600% for both males and females in the mixture concentration where 6 males and 6 females died (mortality was experienced in 5 of the 7 replicates) during the 21 day study. Twenty-eight percent (28%) of the total fish exposed to the mixture concentration experienced mortality. This is a significant increase in mortality over a relatively short period. The average WWTP effluent contains a 0.1mg/L NH3 and greater than 1.0ng/L EE2 concentration. Previous lethality concentrations for NH3 and EE2 have been reported as 0.91mg/L and greater than 1ng/L, respectively. These previous estimates are far above the concentrations tested in the mixture (0.03mg/L NH3 and 0.25ng/L EE2). It is recommended that this study be repeated to see if a similar mortality effect occurs. If further studies determine that a mortality effect at these environmentally relevant concentrations of NH3 and EE2 exists, it is recommended that the current U.S. EPA water quality criteria be re-visited and possibly amended to incorporate this significant finding.

The mixture study tested environmentally relevant concentrations of EE2 and NH3. Surveys of WWTP effluent have found concentrations of NH3 around 0.1mg/L and EE2 greater than 1ng/L. This study tested 0.03mg/L NH3 and 0.25ng/L EE2. Therefore it is assumed that fish in the wild are being exposed to a mixture of these contaminants at or above the concentrations tested in this study in areas that are heavily impacted by WWTP effluent. The only way to avoid exposure of these chemicals is through more advanced

treatment of WWTP effluent and educating the public on proper disposal of unused or unwanted medication.

The removal of ammonia from wastewater treatment effluent has been thoroughly researched and many types of treatments are available. The traditional method for ammonia removal is through biological treatments such as nitrifying bacteria (Jorgensen & Weatherley 2003). Many biological methods require warm temperatures to sufficiently remove ammonia. Gersberg et al. (1986) found that planting vegetation such as bulrushes and reeds in the wastewater treatment ponds can play a significant role in nitrogen removal. While vegetation may be successful in removing ammonia, during the winter months this method may not be as efficient. Ion exchange is a method that has proven to be successful in removing ammonia at a wide range of temperatures (Jorgensen & Weatherley 2003). Ion exchangers such as zeolite were found to be extremely successful in removal of ammonia in wastewater effluent even during high spikes in ammonia concentration, another variable that biological removal methods cannot handle (Jorgensen & Weatherley 2003).

Because EE2 is the main ingredient in many of today's oral contraceptives and will most-likely always be a part of human birth control and hormone replacement therapy, the control of EE2's release into the environment must take place at the WWTP. Vader et al. (2000) determined that nitrifying activated sludge in the presence of ammonia was able to oxidize EE2 into hydrophilic compounds. The authors reported that this sludge degraded a $50\mu g/L$ concentration of EE2 within 6 days. The enzyme, ammonium monooxygenase, initiated the pathway of ammonium oxidation. While oxidizing ammonia, the bacteria were also reported to cometabolize EE2 (Vader et al.

2000, Shi et al. 2004). It is important to note that in the presence of an ammonia oxidation inhibitor, such as the compound Allylthiourea, oxidation was unable to occur thus limiting the nitrifying bacteria sludge's potential to degrade EE2 (Shi et al. 2004). EE2 hydroxylated metabolites are known to have a substantially lesser estrogenic effect on fish than the EE2 parent compound (Vader et al. 2000).

Another control of the environmental release of EE2 (and possibly many other pharmaceuticals) can be through educating the public on the proper disposal of expired or unwanted pharmaceutical drugs. Thirty-five percent (35%) of American's dispose of these pharmaceuticals in the sink or toilet (Bound and Voulvoulis 2005). This disposal process increases the likelihood of these chemicals being released into the environment at concentrations above effect levels. By educating the public of the environmental fate of drugs, the improper disposal of these pharmaceuticals could significantly be reduced. Other forms of disposal that could have a lesser effect on the environment include discarding them in household waste where they can be sent out to a landfill (which would allow these drugs to have a lesser chance of reaching the water system) or returning them to an institution that offers a return and disposal service (Bound and Voulvoulis 2005). As part of my graduate program, I created a brochure which outlined the effects of improper prescription disposal and provided alternatives to properly dispose of unwanted or expired medications. This brochure can be obtained electronically through Michigan State University's Extension (MSUE) website at:

http://web2.msue.msu.edu/bulletins/Bulletin/PDF/E3110.pdf
Future Directions:

Toxicity testing needs to incorporate multiple stressor effects to better assess how a mixture of chemicals in WWTP effluent interact with one another in situ and potentially affect the reproduction of aquatic organisms. Future studies should focus on testing a variety of EDC's in multiple stressor situations in order to fully understand how estrogenic contaminant interactions. especially at environmentally relevant concentrations, impact fish reproduction. Estrogen mimics occur without chemical exposures due to biological stressors in the environment. Testing estrogenic contaminants with biological stressors should also be considered if a full understanding of multiple stressor response is to be acquired. Most multiple stressor studies have focused on testing the effects of two biological stressors while very few studies have researched combinations of biological and chemical relationships.

Further investigation into the mode of action of ammonia on vitellogenin production should also be conducted in order to fully understand why the antagonism between ammonia exposure and vitellogenin production exists. Models have been established relating vitellogenin to egg production. If NH3's mode of action on vitellogenin production was understood, data from studies such as this could be used to predict population trajectories. These models could also be used in place of expensive and time consuming flow-through diluter system experiments if the effects of similar contaminants with similar modes of action were being analyzed. The mixture study determined that there is a mortality effect to exposed fish in a mixture of NH3 and EE2 at their respective environmentally relevant concentration. Further investigation in to why this mortality is occurring and why the synergistic response between these two contaminants exists should also be thoroughly researched. These future studies would also be useful in determining if the high mortality experienced in this study could be attributed to the high reproductive output across all concentrations when compared to previous flow-through diluter experiments. APPENDICES

APPENDIX A: MODEL ORGANISM AND DESIGN OF THE FLOW-THROUGH DILUTER SYSTEM

Model Organism:

The FHM used in this study were cultured in-house at the U.S. EPA Cincinnati-AWBERC location using established methods (U.S. EPA 2006). FHM 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 (Jensen et al. 2001, Ankley and Villeneuve 2006), have a broad distribution in both lentic and lotic environments across North America (Ankley and Villeneuve 2006), 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 FHM in a laboratory setting is relatively easy (U.S. EPA 2006). FHM are a good model organism because of their life-history: they are fractional spawners; produce clutches of 50-100 eggs every 3-5 days and have a rapid life cycle reaching maturity within 4-5 months (Ankley et al. 2001). Controlling their reproductive life cycle is readily achieved through alterations in temperature and photoperiod (Jensen et al. 2001). Previous studies also suggest that FHM response to chemicals is comparable to responses observed in a variety of threatened and endangered fish (Ankley et al. 2001). Extrapolating data from the laboratory to the field has been a challenging task for ecotoxicologists. Laboratory FHM have played a key role in making this linkage as they have been an important species in predicting the bioavailability of inorganics and other contaminants through the use of modeling and caged deployments (Ankley & Villeneuve 2006).

Flow-Through Diluter System:

An established U.S. EPA protocol for conducting flow-through diluter experiments (Ankley et al. 2001) was implemented to explore how both EE2 and NH3 act together to disrupt reproduction of the FHM. A summary of the experimental design including test conditions and endpoints of interest is outlined in Appendix C. Laboratory line 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 500 gallon 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 toxicant concentrations including a control to the glass aquarium testing chambers.

The setup of the flow-through diluter system allowed for a continuous renewal of toxicant to thirty chambers (Figure 19). Each day, three amounts of highly concentrated stock solution were added to three separate 18L dilution water pots to produce the diluted stock concentrations, from here on referred to as a superstock. This superstock was mixed up fresh daily in order to provide a continuous diluted stock for the flow-through diluter system. For each treatment including the control, a 22qt stainless steel stockpot containing a 30x toxicant (or control) superstock was placed on an electric stir plate. The superstock from each stockpot was drawn at a rate of 10ml/min using a 4 cartridge Watson-Marlow 205u series peristaltic pump. The superstocks were then diluted with laboratory line water, which was pulled from a single 24qt stainless steel stockpot at a rate of 290ml/min using a Watson-Marlow 500 series peristaltic pump, to create the

testing solutions. Two 32-cartridge Watson-Marlow peristaltic pumps then distributed the testing solutions into randomly selected 12L testing chambers each at a rate of 20ml/min, which resulted in fresh testing solution being supplied to the tanks at rate of 40ml/min. This flow-rate was sufficient enough for 6 water changes per day. The setup allowed for a fail-safe system in the event that one pump fails or if a tube breaks during the exposure period. In this event, the other 32 cartridge pump would still distribute the testing solution into each testing chamber at a rate of 20ml/min, thus allowing the dosing to continue.

The diluter system was designed to test twenty-eight testing chambers and two blank tanks. The blank tanks were needed to measure background water chemistry in the event of high control mortality. The testing chambers were randomly selected for the treatments using the online randomizer found at http://www.randomizer.org. Each chamber was equipped with a drainage system to allow for a continuous renewal of 9.5L of testing solution. The temperature within the chambers was monitored weekly and heated using a water bath to $25 \pm 1^{\circ}$ C. The water bath was heated to 27° C using a submersible heating cord. Seven replicates, each consisting of 2 male and 4 female adult FHM were used in each of three testing concentrations and control. Three spawning tiles, made of PVC pipe sections, were also placed into each replicate chamber. Having three tiles in each chamber allowed both males to each court a female for spawning and allowed an additional chamber to serve as a hiding place for the other females.



- A: Laboratory line water inlet
- B: Either EE2 or NH3 stock solution (18L)
- C: Control/dilution water holding tank (20L) with float valve
- D: Control/dilution water 500 series Watson-Marlow pump
- E: Toxicant and dilution water mixing tank
- F: 32 cartridge 205u series Watson-Marlow pump
- G: Excess sink with carbon filtration
- H: Toxicant delivery 4 cartridge 205u series Watson-Marlow pump

- I: Lighting system
- J: Fish holding chamber (14L) with drain system
- K: Drain with carbon filtration
- L: Laboratory line water flow for heating bath
- M: Heating cord

Figure 19: Flow-through diluter system used to expose fathead minnow breeding pairs to mixtures of 17α -ethinylestradiol and un-ionized ammonia for 21 days

APPENDIX B: SUMMARY OF TEST CONDITIONS FOR *PIMEPHALES PROMELAS*

<u>TEST PARAMETER</u> Test Type	<u>CONDITION</u> Flow-through
Test Duration	14 day acclimation period 21 day exposure period
Temperature	25°C (±1°C)
Photoperiod	16 h light: 8 h dark
Test Chamber Size	12 L
Test Solution Volume	9.5 L
Renewal of Test Solution	Continuous
Age of Test Organisms	4-6 month old Pimephales promelas
Weight of Test Organisms	Male: 3.0 – 4.0g Female: 1.0 – 2.0g
No. Organisms/Test Chamber	2 male: 4 female
No. Replicate Test Chambers	7
No. Organisms/concentration	14 male: 28 female
Feeding Regime	1ml frozen <i>Artemia salina</i> (1x daily) 20ml newly hatched <i>Artemia salina</i> (2x daily)
Control and/or Dilution Water	Laboratory Line Water
Sample/Test Material	EE2, NH3, and EE2 + NH3
Endpoints	Survival, Fecundity, Fertility, Gonadosomatic Index, Fatpad Index, Vitellogenin Analysis
Test Acceptability	90% or greater control survival 85% or greater control fertility <20% CV for analytical chemistries

REFERENCES

REFERENCES

- Adams, S.M. (2005). Assessing cause and effect of multiple stressors on Marine systems. *Marine Pollution Bulletin*, 51, 649-657.
- Alexander, H.C. Latvaitis, B.P., & Hopkins, D.L. (1986). Site-specific toxicity of unionized ammonia in the Tittabawassee River at Midland, Michigan: Overview. *Environmental Toxicology and Chemistry*, 5, 427 – 435
- Ankley, G.T., Jensen, K.M., Kahl, M.D., Korte, J.J., & Makynen, E.A. (2001). Description and evaluation of a short-term reproductive test with the fathead minnow (*Pimephales promelas*). Environmental Toxicology and Chemistry, 20(6): 1276-1290.
- Ankley, G.T. & Villeneuve D.L. (2006). The fathead minnow in aquatic toxicology: past, present, and future. *Aquatic Toxicology*, 78: 91-102.
- Armstrong, B.M. (2010). How to properly dispose of unwanted or expired medication. Michigan State University Extension Bulletin, E-3110.
- Bonga, S.E. (1997). The stress response in fish. *Physiological Reviews*, 77, 591 625.
- Bound, J.P. & Voulvoulis, N. (2005). Household disposal of pharmaceuticals as a pathway for aquatic contamination in the United Kingdom. *Environmental Health Perspectives, 113(12),* 1705-1711.
- Di Giulio, R.T. & Hinton, D.E. (2008). The toxicology of fishes. CRC Press, Taylor and Francis Group.
- Filby, A.L., Thorpe, K.L., Maack, G. & Tyler, C.R. (2007). Gene expression profiles revealing the mechanisms of anti-androgen and estrogen-induced feminization in fish. *Aquatic Toxicology*, *81*, 219-231.
- Folt, C.L., Chen, C.Y., Moore, M.V. & Burnaford, J. (1999). Synergism and antagonism among multiple stressors. *Limnology and Oceanography*, 44 (3:2), 864-877.
- Gersberg, R.M, Elkins, B.V., Lyon, S.R. & Goldman, C.R. (1986). Role of aquatic plants in wastewater treatment by artificial wetlands. *Water Research*, 20(3), 363 368.
- Giesy, J.P., Snyder, E.M., Nichols, K.M., Snyder, S.A., Villalobos, S.A., Jones, P.D., & Fitzgerald, S.D. (2003). Examination of reproductive endpoints in goldfish (*Carassius auratus*) exposed in situ to municipal sewage treatment plant effluent discharges in Michigan, USA. *Environmental Toxicology and Chemistry*, 22(10), 2416-2431.

- Grist, E.P., Wells, N.C., Whitehouse, P., Brighty, G., & Crane, M. (2003). Estimating the effects of 17a-ethinylestradiol on populations of the fathead minnow *Pimephales* promelas: Are conventional toxicological endpoints adequate? *Environmental* Science and Technology, 37, 1609-1616.
- Gross, M.R., Repka, J., Robertson, C.T., Secor, D.H & Van Winkle, W. (2002). Sturgeon conservation: Insights from elasticity analysis. *American Fisheries Society* Symposium, 28, 13 - 30
- Gunderson, D. R. (1997). Trade-off between reproductive effort and adult survival in oviparous and viviparous fishes. *Canadian Journal of Fisheries and Aquatic Sciences*, 54, 990 998.
- Hanazato, T. & Dodson, S.I. (1995). Synergistic effects of low oxygen concentration, predator kairomone, and a pesticide on the cladoceran *Daphnia pulex*. *Limnology and Oceanography*, *40*(*4*), 700 709.
- Hawkins, M.B., Thornton, J.W., Crews, D., Skipper, J.K., Dotte, A., & Thomas, P. (2000). Identification of a third distinct estrogen receptor and reclassification of estrogen receptors in teleosts. *Proceedings National Academy of Sciences, USA*, 97, 10751 – 10756.
- Hodges, S.W. (2007). Movement, survival and refuge use of three minnow species (*Campostoma anomalum, Notropis boops, and Semotilus atromaculatus*) during seasonal drying in an intermittent Ozark mountain stream. (Doctoral dissertation, University of Arkansas, 2007). Abstract retrieved November 27, 2010 from ProQuest database.
- Islinger, M. Willimski, D., Völkl, A. & Braunbeck, T. (2003). Effects of 17aethinylestradiol on the expression of three estrogen-responsive genes and cellular ultrastructure of liver and testes in male zebrafish. *Aquatic Toxicology*, 62, 85-103.
- Jenkins, R.E & Burkhead, N.M. (1993). Freshwater Fishes of Virginia. American Fisheries Society, Bethesda, Maryland.
- Jensen, K.M., Korte, J.J., Kahl, M.D., Pasha, M.S., & Ankley, G.T. (2001). Aspects of basic reproductive biology and endocrinology in the fathead minnow (*Pimephales* promelas). Comparative Biochemistry and Physiology Part C, 128: 127-141.
- Jenssen, B.M. (2006). Endocrine-disrupting chemicals and climate change: A worst-case combination for Arctic marine mammals and seabirds? *Environmental Health Perspectives*, *114*(1): 76-80.
- Jobling, S., & Tyler, C.R. (2003). Endocrine disruption, parasites and pollutants in wild freshwater fish. *Parasitology*, *126*, S103 S108

- Jorgensen, T.C. & Weatherley, L.R. (2003). Ammonia removal from wastewater by ion exchange in the presence of organic contaminants. *Water Research*, *37*(8), 1723 1728.
- Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., & Flick, R.W. (2007). Collapse of a fish population after exposure to a synthetic estrogen. *Proceedings of the National Academy of Sciences*, 104(21): 8897-8901
- Kime, D.E. (1998). Endocrine disruption in fish. Kluwer Academic, London.
- King County. (2007). Survey of endocrine disruptors in King County surface waters. Prepared by Richard Jack and Deb Lester, Water and Land Resources Division. Seattle, Washington.
- Korte, J.J., Mylchreest, E. & Ankley, G.T. (2004). Comparative evaluation of ELISAs for detecting vitellogenin in the fathead minnow (*Pimephales promelas*) – a response to Tyler et al. *Comparative Biochemistry and Physiology Part C*, 138: 533-536.
- Lange, R., Hutchinson, T.H., Croudace, C.P., Siegmund, F., Schweinfurth, H., Hampe, P., Panter, G.H., & Sumpter, J.P. (2001). Effects of the synthetic estrogen 17αethinylestradiol on the life-cycle of the fathead minnow (*Pimephales promelas*). *Environmental Toxicology and Chemistry*, 20(6), 1216 – 1227.
- Lazorchak, J. M. & Smith, M.E. (2004). National Screening Survey of EDCs in Municipal Wastewater Treatment Effluents. EPA/600/R-04/171. U.S. Environmental Protection Agency, Cincinnati, OH.
- Ma, T., Wan, X., Huang, Q., Wang, Z. & Liu, J. (2005). Biomarker responses and reproductive toxicity of the effluent from a Chinese large sewage treatment plant in Japanese medaka (*Oryzias latipes*). *Chemosphere*, *59*, 281-288.
- Mayes, M.A., Alexander, H.C. & Hopkins, D.L. (1986). Acute and chronic toxicity of ammonia to freshwater fish: a site-specific study. *Environmental Toxicology & Chemistry*, 5, 437-442
- McNaught, A.D. & Wilkinson, A. (1997). Compendium of Chemical Terminology. Accessed on May 21, 2009 from the *International Union of Pure and Applied Chemistry* website at http://goldbook.iupac.org/index.html
- Mercer, B.W., Ames, L.L., Touhill, C.J., Van Slyke, W.J. & Dean, R.B. (1970). Ammonia removal from secondary effluents by selective ion exchange. *Water Pollution Control Federation*, 42(2), R95-R107
- Miller, D.H., Jensen, K.M., Villeneuve, D.L., Kahl, M.D., Makynen, E.A., Durhan, E.J. & Ankley, G.T. (2007). Linkage of biochemical responses to population-level effects: A case study with vitellogenin in the fathead minnow (*Pimephales* promelas). Environmental Toxicology and Chemistry, 26(3), 521 – 527

- Murphy, C.A., Rose, K.A. & Thomas, P. (2005). Modeling vitellogenesis in female fish exposed to environmental stressors: predicting the effects of endocrine disturbance due to exposure to a PCB mixture and cadmium. *Reproductive Toxicology*, *19*, 395 409
- Nash, J.P., Kime, D.E., Van der Ven, L.T.M., Wester, P.W., Brion, F., Maack, G., Stahlschmidt-Allner, P. & Tyler, C.R. (2004). Long-term exposure to environmental concentrations of the pharmaceutical ethynylestradiol causes reproductive failure in fish. *Environmental Health Perspectives*, 112(17): 1725-1733
- Osborne, N., Sherry, J., Rendell, J.L, & Currie, S. (2007). The role of hsp90 in 17αethynylestradiol-induced endocrine disruption in rainbow trout hepatocytes. *Ecotoxicology & Environmental Safety*, 68, 13 – 19.
- Parks, L.G., Cheek, A.O., Denslow, N.D., Heppell, S.A., McLachlan, J.A., Leblanc, G.A., & Sullivan, C.V. (1999). Fathead minnow (*Pimephales promelas*) vitellogenin: purification, characterization and quantitative immunoassay for the detection of estrogenic compounds. *Comparative Biochemistry and Physiology Part C*, 123, 113-125.
- Parrott, J.L. & Blunt, B.R. (2005). Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. *Wiley InterScience*. 131-141.
- Passell, H.D., Dahm, C.N. & Bedrick, E.J. (2007). Ammonia modeling for assessing potential toxicity to fish species in the Rio Grande, 1989 – 2002. *Ecological Applications*, 17(7), 2087 – 2099.
- Pawlowski, S., van Aerle, R., Tyler, C.R. & Braunbeck, T. (2004). Effects of 17aethinylestradiol in a fathead minnow (*Pimephales promelas*) gonadal recrudescence assay. *Ecotoxicology and Environmental Safety*, 57: 330-345.
- Randall, D.J. & Tsui, T.K.N. (2002). Ammonia toxicity in fish. *Marine Pollution* Bulletin, 45, 17 23.
- Robbins, J.W.D., Howells, D.H. & Kriz, G.J. (1972). Stream pollution from animal production units. *Journal of Water Pollution Control Federation*, 44(8), 1536 1544.
- Schisler, G.J. & Bergersen, E.P. (2000). Effects of multiple stressors on morbidity and mortality of fingerling rainbow trout infected with *Myxobolus cerebralis*. *Transactions of the American Fisheries Society*, 129, 859 – 865.

- Schlosser, I.J. (1995). Dispersal, boundary processes, and trophic level interactions in streams adjacent to beaver ponds. *Ecology*, *76*, 908-925.
- Schultz, I.R., Orner, G., Merdink, J.L. & Skillman, A. (2001). Dose-response relationships and pharmacokinetics of vitellogenin in rainbow trout after intravascular administration of 17a-ethynylestradiol. *Aquatic Toxicology*, 51, 305-318.
- Shi, J., Fujisawa, S., Nakai, S. & Hosomi, M. (2004). Biodegredation of natural and synthetic estrogens by nitrifying activated sludge and ammonia-oxidizing bacterium *Nitrosomonas europaea*. *Water Research*, 38, 2323-2330.
- Skillman, A.D., Nagler, J.J., Hook, S.E., Small, J.A. & Schultz, I.R. (2006). Dynamics of 17a-ethynylestradiol exposure in rainbow trout (*Oncorhynchus mykiss*): absorption, tissue distribution, and hepatic gene expression pattern. *Environmental Toxicology and Chemistry*, 25(11): 2997-3005.
- Starrett, W.C. (1951). Some factors affecting the abundance of minnows in the Des Moines River, Iowa. *Ecology*, 32(1), 13 27.
- Thurston, R.V., Russo, R.C. & Meyn, E.L. (1986). Chronic toxicity of ammonia to fathead minnows. *Transactions of the American Fisheries Society*, 115, 196 207
- U.S. EPA. (1999). 1999 update of ambient water quality criteria for ammonia. EPA/822/R-99/014. Office of Water. Washington DC.
- U.S. EPA. (2002). A short-term method for assessing the reproductive toxicity of endocrine disrupting chemicals using the fathead minnow (*Pimephales promelas*). EPA/600/R-01/067. Washington D.C.
- U.S. EPA. (2005). Standard operating procedure for Tri Reagent[®] RNA isolation protocol. National Exposure Research Laboratory number MIRB 031.2
- U.S. EPA. (2006). Standard operating procedure for fathead minnow culture method (*Pimephales promelas*) Number T03-100. Dynamac/NERL/EP-D-06-096.
- U.S. EPA. (2007). Standard operating procedure for the analysis of estrogen compounds from water samples by GC/MS. National Exposure Research Laboratory Number MIRB 051.0E.
- U.S. EPA (2009). Standard operating procedure for the qualitative "real time" PCR (QPCR) vitellogenin protocol using HS DyNamo[™] SYBR[®] Green Kit. National Exposure Research Laboratory Number MIRB 032.3.
- U.S. EPA. (2009). 2009 update of ambient water quality criteria for ammonia freshwater. EPA/822/D-09/001. Office of Water. Washington DC.

- Vader, J.S., van Ginkel, C.G., Sperling, F.M.G.M., de Jong, J., de Boer, W., de Graaf, J.S., van der Most, M. & Stokman, P.G.W. (2000). Degradation of ethinyl estradiol by nitrifying activated sludge. *Chemosphere*, 41, 1239-1243.
- Weber, L.P., Balch, G.C., Metcalfe, C.D. & Janz, D.M. (2004). Increased kidney, liver, and testicular cell death after chronic exposure to 17a-ethinylestradiol in medaka (*Oryzias latipes*). *Environmental Toxicology and Chemistry*, 23(3), 792-797.
- Wolfand, J. (2007). Active ingredient in oral contraceptives (17a-ethinylestradiol) alters male competitive courtship behaviors and secondary sexual characteristics in fathead minnows (*Pimephales promelas*). Journal of U.S. Stockholm Junior Water Prize. Volume 2. Water Environment Federation.
- Wood, C.M. (2001). Chapter 1: Target responses of the gill. IN Target organ toxicity in marine and freshwater teleosts, Volume 1 Organs. Edited by: Schlenk, D. &Benson, W.H. London, Taylor & Francis: 1-85.