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# Acute responses of freshwater and marine species to ethinyl estradiol and fluoxetine

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ACUTE RESPONSES OF FRESHWATER AND MARINE SPECIES TO ETHINYL  
ESTRADIOL AND FLUOXETINE

A Thesis  
Submitted to the Graduate Faculty of the  
Louisiana State University and  
Agricultural and Mechanical College  
in partial fulfillment of the  
requirements for the degree of  
Master of Science

in

The School of Plant, Environmental, and Soil Sciences

by  
Julia Kaye Daigle  
B.S., Louisiana State University, 2006  
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## ABBREVIATIONS

**5-HT** – 5-hydroxytryptamine; serotonin  
**API** – Active Pharmaceutical Ingredient  
**Ch.V.** – Chronic Value  
**CNS** – Central Nervous System  
**COC** – Contaminants of Concern  
**DDT** – dichlorodiphenyltrichloroethane  
**DI** – Deionized [Water]  
**dph** – days post hatch  
**dpf** – days post fertilization  
**EC50** – Median Effective Concentration  
**ECOSAR** – Ecological Structure Activity Relationships  
**ED50** – Median Effective Dose  
**EE2** – Ethinyl estradiol  
**EPA** – Environmental Protection Agency  
**F1** – The first filial (offspring) generation, produced by crossing two parental lines.  
**F0** – The initial parent generation in a multi-generation reproduction study.  
**FDA** – Food and Drug Administration  
**FLX** – Fluoxetine  
**hph** – hours post hatch  
**hpf** – hours post fertilization  
**HSDB** – Hazardous Substance Data Bank  
**IPCH** – International Programme on Chemical Safety  
**LC50s** – Median Lethal Concentration  
**LD50** – Median Lethal Dose  
**LOEC** – Lowest Observable Effect Concentration  
**LOEL** – lowest observable effect level  
**Log K<sub>ow</sub>** – Octanol-water coefficient  
**MIC** – Minimum Inhibitory Concentration  
**NCHS** – National Center for Health Statistics  
**NOEC** – no observable effect concentration  
**NOEL** – no observable effect level  
**PCBs** – polychlorinated biphenyls  
**ppb** – parts per billion (micrograms/Liter)  
**PPCPs** – Pharmaceuticals and Personal Care Products  
**ppm** – parts per million (milligrams/Liter)  
**POTWs** – Publically Owned Treatment Works  
**(Q)SAR** – (Quantitative) Structure Activity Relationship  
**RCRA** – Resource Conservation and Recovery Act  
**SAR** – Structure Activity Relationships  
**SBSS** – Swim Bladder Stress Syndrome  
**SSRI** – Selective Serotonin Reuptake Inhibitor  
**STP** – Sewage Treatment Plant  
**STWs** – Sewage Treatment Works  
**WWT** – Wastewater Treatment  
**WWTP** – Wastewater Treatment Plant  
**USGS** – United States Geological Survey

**USPDI** –United States Pharmacopeia Drug Information



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## ABSTRACT

Damaging and often irreversible effects occurring in aquatic ecosystems have recently been linked to the presence of pharmaceuticals in water bodies. Because this crisis has only recently been identified, existing reports on the consequences of this contamination are scarce. In EPA standard acute effluent toxicity tests, *Ceriodaphnia dubia*, *Daphnia pulex*, *Pimephales promelas*, *Mysidopsis bahia*, and *Menidia beryllina* were subjected to two of the most widely distributed pharmaceuticals in the U.S., ethinyl estradiol (EE2), a synthetic form of estrogen, and fluoxetine HCl (FLX), the active ingredient in Prozac®, for 96 hours to assess and evaluate toxic responses.

After test termination, mortality curves were statistically analyzed to quantify 96 hour median effective concentrations (EC50s), no observable effect concentrations (NOECs), lowest observable effect concentrations (LOECs), and chronic values (Ch.V.s).

Derived EC50s for both drugs identified *C. dubia* as the most sensitive organism. In terms of the Ch.V., *C. dubia* was the most sensitive organism administered EE2 and *M. bahia* given FLX. The most resilient species was *P. promelas* regarding the EC50 after EE2 dosing, *D. pulex* in respect to the Ch.V. after EE2 dosing, and *M. beryllina* pertaining to the EC50 and Ch.V after FLX dosing.

Existing risk assessments and traditional toxicity tests do not incorporate sublethal effects. Because EE2 and FLX have the potential to alter serotonin, dopamine, and estrogenic hormones, biological activities encompassing these chemicals could be affected such as changes in behavior, growth and development, and fecundity. Observations on exposed organisms indicate that there were sublethal effects. The observed increases in abnormal behaviors of exposed organisms included unresponsiveness, irregular swim patterns, erratic activity, and convulsions with observed decreases in feeding habits and aggression. Ethinyl estradiol

appeared to accelerate maturation; elevated concentrations appeared to slightly stimulate maturity while appearing to inhibit molting. The organisms exposed to higher concentrations of EE2 and FLX seemed to lack pigment.

Continuous environmental pollution of these unregulated chemicals can cause stress on aquatic ecosystems and result in disturbances of the normal development and life cycles of aquatic organisms. If these sublethal disruptions in biological activity continually disregarded, catastrophic destructions of entire ecosystems could transpire.

## INTRODUCTION

Pharmaceutical substances were designed with the intention of maintaining health and well being and are used as aids to diagnose, treat, or prevent disease. In order to perform their intended function, pharmaceuticals are designed with degradation resistance, accumulation potential, and toxicity potential in terms of effectiveness on biological key functions. However, through human activity, aquatic ecosystems have been exposed to these chemicals since their introduction into the market. Although drugs are not considered pollutants, they do fit the definition. Pollutants may be classified by the following criteria: origin (natural or synthetic), effects (on organs, species, or entire ecosystems), properties (mobility, persistence, and toxicity), and ease or difficulty of cleanup and/or removal.

Comparatively little attention has been given to pharmaceuticals as potential environmental pollutants because they represent a fraction of man-made pollutants (pesticides and household and personal care products) present in the environment. Additionally, because their existence has only been recently proven, it is still unclear how these chemicals affect ecosystems. This does not belittle their potential to cause harm to the environment because pharmaceuticals are designed to be very efficient in producing a maximum response at a minimum dose. The environmentally detected amounts of drugs have not been linked to significant declines in survivorship. As a result, their potential effects on the environment have gone overlooked. The United States Geological Survey (USGS) has established a baseline for contaminants of concern (COCs) in which pollutants have the potential to affect the health and welfare of aquatic ecosystems and humans. Pharmaceuticals with modes of action depending upon signal transduction, cell division, and key metabolizing enzymes can be hazardous to nontarget organisms that possess or lack the necessary elements to carry out the drugs function. The problem is that although pharmaceuticals were not designed to affect organisms, they have

been recently linked to damaging sublethal effects of nontarget organisms living downstream of wastewater treatment plants (WWTPs). Definitive effects need to be identified in order to develop a solution before effects become out of control and irreversible.

### **Pharmaceuticals as Pollutants**

Every year people use thousands of tons of drugs to treat illnesses, to prevent unwanted pregnancy, or to face the stresses of modern life. The production and consumption of two of the most popular pharmaceuticals in the United States, ethinyl estradiol and fluoxetine, are increasing at an exponential rate. However, environmental concern is not exclusively based on high production rates; the chemicals' properties and behavior in the environment should also be considered. Pharmaceuticals fit the definition of pollutants in which they are introduced in appreciable amounts, exhibit environmental persistence, and cause damaging effects on ecosystems (Ritter 2006).

However, they are often discounted as pollutants and they are not regulated by environmental agencies. Regulations control priority pollutants in wastewater treatment plants' (WWTPs) effluent but because pharmaceuticals are not classified as priority pollutants they are not regulated.

Although pharmaceuticals fit the definition of emerging pollutants, they are disregarded as COCs for a number of reasons. First of all, the production and testing of drugs is under the Food and Drug Administration (FDA). Drugs were seldom considered potential environmental pollutants because the international regulation of pharmaceuticals is regulated by human health agencies which do not focus on environmental concerns. Thus once the chemical is excreted from the user, the fate of active pharmaceutical ingredients (APIs) has been disregarded (Daughton and Ternes 1999). Personal use and excretion of drugs are a less visible source of these potential contaminants because they are unintentionally emitted into the environment.



Pharmaceuticals entering the environment via excretion are under not regulated or monitored by environmental laws. Resource Conservation and Recovery Act (RCRA) does not regulate any household waste under the control of consumers, which includes medicational waste (USEPA 2010). Regulations control priority pollutants in WWT effluent but because pharmaceuticals are not classified as priority pollutants they are not regulated.

Portions of active pharmaceutical ingredients (APIs) that are not fully absorbed by humans nor completely degraded by metabolic processes are secreted in urine or feces and then emitted into raw sewage (Halling-Sorensen et al. 1998; Daughton et al. 1999). Primarily through untreated and treated sewage effluent these compounds have been continuously flowing into the aquatic environment for as long as people have been taking them. Hence they are present anywhere that humans visit or live. Because drugs are polar and nonvolatile, they are unable to escape from the aquatic zone. Aquatic organisms are exposed to pharmaceuticals through the aforementioned pathway for their entire life-cycle making them susceptible to the chemicals' effects.

Because they occur in small amounts, their existence has only recently been proven through advancement in analytical methods. These highly water soluble compounds could not be analyzed using the normal sample clean-up/preconcentration methods, with gas chromatographic separation typically used for aquatic pollutants (Daughton et al. 1999). It was not until the 1990s that analytical methods were substantially advanced with high separatory efficiencies to detect the low levels of pharmaceuticals in the environment (Daughton and Ternes 1999). With the amounts of pharmaceuticals produced and consumed exponentially increasing it is difficult to analyze every API. Most pharmaceuticals and their metabolites are not presented in environmentally oriented mass spectral libraries. Also, any available analytical reference standards are expensive and difficult to obtain (Daughton and Ternes 1999). In order to

minimize environmental contamination, pollution prevention should be favored over remediation or restoration.

### **Risk Assessment**

Because environmental toxicology focuses on acute effects of exposure, traditional toxicity tests simply survey mortality; in this way, these tests are oversimplified because they do not account for body burdens. It should be noted that besides known targets additional or other target tissues and organs may be affected alternatively. This would result in unexpected effects not targeted by the typical regulations. Because environmental concentrations are below those necessary to cause mortality, lethality alone is not a sufficient enough endpoint to determine potential effects on environment.

Risk assessment is flawed because it does not incorporate body burdens; these chemicals cause changes not anticipated by human tests due to physiological differences of nontarget organisms. Traditional toxicity tests usually designate mortality as a significant endpoint and in a few cases size or reproduction.

More specific toxicity analysis should be developed implementing the available knowledge generated during the pharmaceutical development process (e.g. pharmacokinetic behavior and metabolism, probable and possible modes of action, target organs and tissues, and side effects in mammals). It is important to take into account how the drug affects humans by means of which processes are disrupted, which organs and tissues are targeted to anticipate similar effects on nontarget organisms.

Taking into account the vague understanding of specific and nonspecific modes of action, species differentiation, and nontarget organs and tissues affected, specific toxicity analysis is difficult to perform and complicated to anticipate. Given what is known about the drugs through experiment on humans, aquatic experimental outcomes should be targeted towards known target

modes of action based on the assumption of similar modes of action in different species and the various side effects in humans. In order to evaluate which drug produced which result the current study, like many other studies, was not performed in mixtures.

### **Importance of Study**

Because these chemicals are environmentally present in small amounts it is only recently that their existence has been proven. Analytical techniques and instrumentation able to detect polar compounds at minute quantities have been particularly helpful to the advancement in knowledge of environmental occurrence of pharmaceuticals (Fent et al. 2006). Minute amounts of chemicals, typically considered safe because they occur at trace amounts, can elicit unanticipated effects on ecosystems as evident by xenobiotics such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) which have hormone mimicking effects on the reproductive systems of organisms at nanograms per liter (Colbum and Clement 1992).

It is important to identify and investigate potential environmental pollutants before corrective measures are impossible. Since it is unlikely that people will be convinced to quit taking pharmaceuticals what do needs to be executed? The primary studies on this subject must start at the beginning. Hence the purpose of this study is to prove that there is a problem. Environmental issues are typically excused until rehabilitation is almost irremediable brought to light by some catastrophic event, usually mortality. Published literature is minute when you take into consideration the large amount of APIs, metabolites, and other transformation products developed and pumped into the environment, sublethal/lethal effects that may have not occurred, the number of potential non-target organisms (Daughton et al. 1999).

The results from this study can help form databases in which other drugs with similar modes of action or similar species to show effects (lethal and sublethal) and aid in future studies

to show what to look for. Often the type or magnitude of effects at lower concentrations is predicted from the acute tests which incorporate larger doses.

### **Application of Results**

Although they are exposed to low doses throughout their entire life, acute toxicity studies which incorporate the large amounts needed to stimulate mortality are important to identify the effects in worst case scenarios such as spills, wastewater treatment outflow, and if pharmaceutical concentration increases due to bioaccumulation and the increase in potency. Taking into account subtle or seemingly unimportant changes that could affect environment or humans in the future could bring about changes in regulations, toxicity test practices, and public awareness.

Simple observations during acute tests can aid in the analysis of environmental risk and eventually aid in the development of new test regulations. It is probable that sublethal effects could transpire from low concentrations. Although some effects may not be statistically significant this does not lessen their importance.

Experimental ecotoxicological data for pharmaceuticals is scarce. Sanderson et al. (2004) published ecotoxicological data for 2848 active pharmaceutical ingredients using (Quantitative) Structure Activity Relationship ((Q)SAR). Environmental exposure is at least five orders of magnitude less than LC50.

Ecological Structure Activity Relationships (ECOSAR) is a computerized program that predicts the acute (short-term) toxicity and chronic (long-term or delayed) toxicity of chemicals to aquatic organisms such as fish, aquatic invertebrates, and aquatic plants. ECOSAR uses Structure Activity Relationships (SARs) to predict the aquatic toxicity of untested chemicals (i.e. pharmaceuticals) regarding structural similarities of chemicals for which aquatic toxicity data are available. The SARs in ECOSAR express correlations between a compound's physicochemical

properties and its aquatic toxicity within specific chemical classes. While many studies utilize the program to predict the ecotoxicity of pharmaceuticals, it is important to note that the model was created using industrial chemicals and the applicability of the models to predict effects of pharmaceuticals should be carefully considered.

Maden et al. (2009) assessed the confidence in ECOSAR's predictions. Several key factors were considered during the ranking process: total number of classes into which ECOSAR assigned the compound, presence of additional/unusual functionalities that could drive or moderate the toxicity within a selected class, similarity to compounds in the ECOSAR training set, overall size, and log  $K_{ow}$ . The probability of the API falling within the relevant zone of the class was classified by a numerical scale of 1-3. A confidence interval of 1 was given to compounds likely to fall within the domain of the model; there was confidence that the SAR prediction for the pharmaceutical was correct. A confidence interval of 3 was given to compounds unlikely to fall within the domain of the model causing less confidence in the prediction for the compound. Also given the number 3 were compounds ECOSAR allocated into four, five, or six different classes lessening confidence in the correct assignment of class.

Because ethinyl estradiol fell into two ECOSAR classes (phenols and propargyl alc-hindered) and the molecule was different than those in the training set the assigned rank was 3 for phenols in all groups and propargyl alc-hindered class was ranked 3 for fish 96 hour LC50 and no predictions for daphnia 48 hour LC50 and algae 96 hour LC50. ECOSAR predicted a higher potency in phenol class: fish 96 hour LC50, daphnia 48 hour LC50 and algae 96 hour LC50 (Madden et al. 2009).

Fluoxetine was classified as aliphatic amine and given a rank of 3 for fish 96 hour LC50, daphnia 48 hour LC50 and algae 96 hour LC50 signifying that it was unlikely that the API fell

within the domain of the model and there was little confidence in the prediction of the compound (Madden et al. 2009).

## **Objectives**

The current single species study was designed to expose standard EPA organisms to six concentrations of ethinyl estradiol and fluoxetine for 96 hours. Based on EPA's 5<sup>th</sup> edition (2002) acute static renewal test (EPA-821-R-02-012) test organisms (3 species of freshwater organisms and 2 species of marine organisms) were exposed to six concentrations and a control with four replications for 96 hours. A fifth replicate of concentrations void of organisms was used for daily monitoring of water parameters. Median effective concentrations based on lethality (EC50s) were quantified and the toxic responses were compared and contrasted. Mortality was documented daily to assess time framed dose-responses for each test organism. The highest test concentrations were chosen into order to induce at least 50% lethality (EC50). Quantified 96 hour EC50s were established for five standard EPA test organisms determined after test termination.

Because the environmental amounts are below those to stimulate mortality these results of this study extend beyond lethality through the observation of sublethal effects which are not required for acute test methods. In addition to high toxicity potential these two pharmaceuticals are extremely potent in controlling hormonal, serotonergic, and dopaminergic systems in mammals/humans which could interfere with key biological functions including but not limited to fecundity, growth and development, and behavior in nontarget organisms. Organisms are exposed for entire life cycles in environmental settings. In the current study juvenile organisms were exposed during an important life stage of growth and development. Any changes could be important to note because they were occurring within 96 hours.

## Hypothesis

1. Higher concentrations are expected to initiate lethality in at least half of the test organisms. In the current study single species acute toxicity tests were conducted to document and quantify mortality and to assess behavior after exposure to ethinyl estradiol, a synthetic steroid present in most forms of birth control and fluoxetine, a selective serotonin reuptake inhibitor used to treat depression. The higher concentrations are expected to initiate lethality in EPA standard test organisms, *Ceriodaphnia dubia* (water flea), *Daphnia pulex* (water flea), and *Pimephales promelas* (fathead minnow), which are freshwater species and *Mysidopsis bahia* (mysid shrimp) and *Menidia beryllina* (inland silverside) which are marine organisms.

2. Similarly classified organisms are expected to generate comparable mortality data. Mortality data is projected to be comparable to similarly classified organisms. For example, water fleas and mysid should generate toxic responses similar to other crustaceans.

3. The lethal concentrations are expected to be smaller in simpler organisms and larger in more complex organisms. The median lethal concentrations (EC50s) are expected to be smaller in these simpler organisms with shorter life cycles, making them more susceptible and vulnerable to contaminants. Because fish are more complex organisms with more intricate bodily functions and longer life cycles they are expected to yield higher EC50s making them more resilient to these drugs. The concentrations needed to induce mortality in these fish species should be comparable to other fish species and higher than the amounts in crustaceans.

4. The lesser concentrations are likely to generate less lethality while diminishing activity. It is probable that sublethal effects could transpire from lower concentrations. Therefore, in addition to quantifying mortality, observations of behavioral changes were recorded. It was expected that the lesser concentrations used in the present study were likely to generate less lethality while diminishing normal activity.

## **Perspective of Planned Research**

By proving the question that pharmaceuticals can affect organisms over short periods of time, it can be argued that the problem will only increase because aquatic organisms are environmentally susceptible to these chemicals throughout entire life cycles with multiple doses.

Bioconcentration and the addition of new pharmaceutical products on the market may lead to higher concentrations of the compounds studied in the environment. The pharmaceutical companies are producing more effective products for humans in order to perform their intended action pharmaceutical companies by increasing potency, bioavailability, and degradation resistance. This means more negative impacts for environment. Also because now our culture is saying a pill can cure anything, the amounts used are increasing along with the human population and availability of these drugs. Also mixture effects are most often anticipated by the idea of concentration addition in which the combined toxicity is derived from the addition of the individual concentrations of each compound. With this said, in the current study, species exhibited similar sublethal endpoints in both drugs. With this in mind, hypothetically concentrations that do not illicit lethality can cause sublethal effects and based on additions can occur at environmentally detected levels.

The environmental concentrations of EE2 and FLX could potentially increase due to the exponential increase of the amounts being taken. Also these drugs are prescribed for long time use with a large amount of the API excreted and entering the environment. The long half lives, high resistance to degradation, high accumulation potential, and high water solubility allow these chemicals to persist in the environment. Moreover, the pharmaceutical industry is producing more effective active APIs by increasing potency, bioavailability, and degradation resistance. These oppositions increase the potential for these compounds to remain in animal tissue and bioaccumulate enforcing biomagnifications and bioconcentrations.



This study covers a spectrum of aquatic organisms (simple and complex) and their food source to help demonstrate what can happen if an organism is affected and thus the food chain which may in turn affect humans. There are cases in which physiological differentiation can play a role (i.e. aquatic organisms will react differently than mammals). More complex organisms that are distressed at higher concentrations can be affected at lower levels of contamination in the sense that their food source is simpler and will be stressed at lower concentrations. The current study included quantified effects of simple and complex organisms to aid in investigating aquatic ecosystems as a whole. Changes at any level of the food chains will upset environmental balance. Previously documented tests do not incorporate effects of food sources nor do they take into account how these changes affect the test organism. The existence of these studies demonstrates that a problem can occur but by the time the public realizes it may be too late.

In addition to quantified EC50s, concentration boundaries were noted for sublethal effects observed during the test. The organisms were exposed to high concentrations during sensitive life stages which could aid in understanding how they affect nontarget organisms, anticipating effects for future studies, and/or risk assessment at environmentally detected concentrations.

It is the unpredicted effects that are causing harm to ecosystems. Abnormalities have a number of possible explanations or in some cases, cannot be explained. A major concern is the effects which are barely noticeable but accumulate over time to yield extreme changes masked by time and perhaps not distinguishable from natural events. Even subtle alterations in normal life progressions control the promise to directly or indirectly affect entire species populations. Any degree of disturbance of the environmental balance can bring about catastrophes leading to deteriorations of entire ecosystems.

## LITERATURE REVIEW

### Pharmaceutical Industry

Pharmaceutical chemicals are defined as prescribed and nonprescribed “intended to affect the structure or any function of the body of man or other animals.” These substances are responsible for physiological or pharmacological action used to prevent, diagnose, cure, treat, or mitigate diseases (The Food, Drug and Cosmetic (FD&C) Act, sec. 201(g) (1)). Pharmaceuticals can serve as preventatives to disease and disability (e.g. lipid-lowering drugs that lessen the risk of coronary artery disease) and aid in the management of the body’s self-regulating systems (e.g. high blood pressure and thyroid drugs) in which they can replace or supplement needed chemicals (e.g. insulin and vitamins). They can cure some diseases (e.g. antibiotics), serve as new treatments where previously there were none (e.g. drugs for HIV), control symptoms (e.g. analgesics or pain relievers and asthma drugs), or can serve as complements to medical procedures (e.g. anticoagulants during heart valve replacement surgery) (Berndt 2001; Chockley 2001).

There are numerous groups of pharmaceuticals including but not limited to human and veterinary medicinal compounds; nutraceuticals, which are bioactive food supplements; and the classic small molecule drugs which are manufactured primarily through organic synthesis and biotech drugs. At the end of 2009, the Food and Drug Administration had approved more than 1,350 small molecule drugs, 123 biotech drugs (protein/peptide), 71 nutraceuticals and more than 3,243 experimental drugs (Hazardous Substances Databank 2009). Every year thousands of tons of drugs are utilized to treat illnesses, to prevent unwanted pregnancy, or to face the stresses of modern life (National Center for Health Statistics 2004).

The per capita consumption of pharmaceutical drugs is steadily increasing because of expiration of patents resulting in availability of less expensive generics and the discovery of new

uses for existing drugs. Between 1988 and 1994, 39% of Americans were using at least one prescription drug and 12% of Americans were using at least three prescription drugs. In 2005, the percentage of Americans using one prescription increased to 44%, and 17% were using at least three prescription drugs (National Center for Health Statistics 2004).

North America represents about half of global pharmaceutical sales. Pharmaceutical sales in the United States increased from \$152.8 billion in 2000 to \$248 billion in 2004. This dominant market is expected to reach \$330 billion by the year 2010 with an estimated annual growth rate of 8.5%. The exponential increase in pharmaceutical consumption is correlated with increasing population, advancing medical technology and research, increasing life span expectancy, and escalating health care spending (Khetan and Collins 2007).

Pharmaceutical sales in North America have been rising nearly seven times faster than the expanding population growth rate (Khetan and Collins 2007) which has risen an average annual growth rate of 1.2% (National Center for Health Statistics 2006). In 2006, the U.S. population was 300 million compared to 151 million in 1950 (National Center for Health Statistics 2004).

An inverted age structure in the general population and a rise of new target age groups are major contributors to this market increase (Daughton 2003; Khetan and Collins 2007). Because of chronic disorders, older people are more likely to take more pharmaceuticals than younger individuals and have typically been targeted by marketing companies (Beers 2003). However, marketing strategies now encompass all age groups by emphasizing the idea that a pill can cure anything and increase life expectancy. However, patents are expiring allowing generics to become available at a lower cost making them more available to the public (Khetan and Collins 2007).

Two of the most popular pharmaceuticals in the United States have been available for decades. The Federal Drug Administration approved the use of the synthetic estrogen ethinyl estradiol in June 1943 for birth control or hormone treatment, and the Selective Serotonin Reuptake Inhibitor, fluoxetine in December 1987 as an antidepressant (Food and Drug Administration). Ethinyl estradiol ranked 8<sup>th</sup> and fluoxetine ranked 13<sup>th</sup> based on the number of prescriptions dispensed in 2004 in the United States (The Internet Drug Index 2010).

### **Ethinyl Estradiol Mechanics**

The most frequent uses of ethinyl estradiol, a synthetic form of estrogen, are as combination oral contraceptive or hormone replacement therapy in postmenopausal women (Hardman 1996).

It is applicable in the treatment of:

- menopausal and post menopausal symptoms
- moderate to severe vasomotor symptoms (International Agency for Research on Cancer 1999; United States Pharmacopeia Drug Information 1999)
- malignant neoplasm of the breast and prostate (International Agency for Research on Cancer 1999)
- metastatic breast carcinoma in selected men and postmenopausal or oophorectomized women
- female hypogonadism, primary ovarian failure, or ovariectomy
- advanced prostatic carcinoma (United States Pharmacopeia Drug Information 1999)
- Turner's syndrome
- acne in women
- athletic functioning as an enrichment supplement and veterinary supplements (Daughton and Ternes 1999)

Ethinyl estradiol is prepared from natural estrogen (United States Pharmacopeia Drug Information (USPDI) 1999). Desirable therapeutic actions on the female reproductive tract involve stimulation of proliferation and differentiation in the fallopian tube, an increase of the tubal muscular activity, an increase of the water content of cervical mucus, and support in contraction of the uterine myometrium. Estrogens increase the synthesis of DNA, RNA, and various proteins in target tissues. Estrogens reduce the release of gonadotropin-releasing hormone from the hypothalamus, leading to a reduction in release of follicle-stimulating

hormone and luteinizing hormone from the pituitary while the pituitary mass is increased (USPDI 1999).

Ethinyl estradiol is rapidly and completely absorbed from the gastrointestinal tract (USPDI 1999). This lipophilic synthetic steroid diffuses readily through cellular membranes to bind to estrogen receptors in the nucleus in order to regulate gene expression by increasing or decreasing the transcription of hormone regulated genes depending on the tissue receptor which is activated. Ethinyl estradiol activates the estrogenic receptor and regulates gene expression in the female reproductive tract, pituitary, hypothalamus, bone, liver, and other tissues (Hazardous Substances Databank). Receptor activation differs upon which targets are affected (USPDI 1999). The reported bioavailability of circulating EE2 is 40% almost fully bound to plasma albumin. Oral ingestion results in peak plasma concentrations 2-3 hours later with a 12 hour peak thought to represent extensive enterohepatic circulation (USPDI 1999). Unbound molecules are widely distributed to most tissues, especially breast, uterine, vaginal, hypothalamic, and pituitary tissues; with a high affinity for adipose tissue (USPDI 1999). Metabolism involves hydroxylation of the aromatic ring in the liver by the cytochrome P450 3A4 isoenzyme after it is absorbed by the small intestine. Metabolism is slow compared to estrogen. The ethinyl substitution (C-triple bond-CH group) in the C17 position inhibits first-pass metabolism (USPDI 1999). Primary route of biotransformation is through 2-hydroxylation and the formation of 2- and 3-methyl ethers (USPDI 1999).

Following a single oral therapeutic dose biological half life is approximately 7.7 hours (USPDI 1999). The elimination phase half-life has been reported to be 13 to 27 hours (Hardman et al. 1996). In contrast to the metabolites of natural estrogen, a significant proportion of the metabolites of ethinyl estradiol in humans are excreted by the fecal route; ethinyl estradiol itself is excreted in urine and feces in a ratio of about 4:6. About 90% of the metabolites from ethinyl

estradiol are recovered in both feces and urine (International Agency for Research on Cancer 1999). Excretion is via feces due to some enterohepatic circulation of sulfate and glucuronide metabolites; excretion is also via the kidneys (Hazardous Substances Databank 2009). Excretion is mainly through urine; however, the enterohepatic circulation of the sulfate and glucuronide metabolites allows it to also be excreted in feces (Hazardous Substances Databank 2009). The metabolites glucuronide and sulfate conjugate as well as the unabsorbed portion are excreted as both urine and feces.

### **Fluoxetine Mechanics**

Fluoxetine is classified as an antidepressant for the treatment of major depressive disorder and to relieve the symptoms of premenstrual dysphoric disorder (PMDD). Acute depressive episodes typically require 6-12 months of antidepressant therapy while chronic depression may require long-term treatment. Fluoxetine is also used in the treatment of:

- obsessions and compulsions in patients with obsessive-compulsive disorder
- binge-eating and vomiting behaviors in patients with moderate to severe bulimia nervosa (United States Pharmacopeia Drug Information (USPDI) 2003; O'Neil 2001)
- short term management of exogenous obesity for a limited number of patients (McEvoy 2003)

The precise mechanism of antidepressant action of fluoxetine is unclear, but the drug has been shown to selectively inhibit the reuptake of serotonin (5-HT) at the presynaptic neuronal membrane (McEvoy 2003). Fluoxetine, like most Selective Serotonin Reuptake Inhibitors, hinders the 5-hydroxytryptamine transporter (5-HTT), a serotonin transporter in the central nervous system (Kreke and Dietrich 2008). Thus this inhibition increases the concentration of serotonin and reinforces serotonergic neuronal transmission. Fluoxetine specifically inhibits central nervous system (CNS) neuronal re-uptake of serotonin, thus increasing the concentration of the serotonin at the synapse and reinforcing of serotonergic neuronal transmission. Fluoxetine has little effect on other neurotransmitters and may interact with other drugs or circumstances

which cause serotonin release (International Programme on Chemical Safety (IPCH) 1999). Fluoxetine-induced inhibition of serotonin reuptake causes increased synaptic concentrations of serotonin in the CNS, resulting in numerous functional changes associated with enhanced serotonergic neurotransmission (McEvoy 2003). The enhancement of the serotonergic effects may produce a life-threatening serotonin syndrome (USPDI 2003).

Most SSRIs only increase the amount of serotonin. Fluoxetine increased extracellular concentrations of serotonin, norepinephrine and dopamine after acute dosing. The concentration of monoamines was sufficient in blocking 5-HT receptors accounting for the increase of catecholamines (Bymaster et al. 2002).

Fluoxetine hydrochloride administered orally is readily absorbed from the gastrointestinal tract with peak plasma concentrations appearing from 6 to 8 hours later. The systemic bioavailability is greater than 85% and does not appear to be affected by food with plasma protein binding at 94% (IPCH 1999).

Fluoxetine is widely distributed throughout the body with highest concentrations occurring in the lungs and liver; however, distribution of fluoxetine and its metabolites into human body tissues and fluids has not been fully characterized (USPDI 2003). It is known that fluoxetine and norfluoxetine cross the blood-brain barrier in humans and animals one hour after administration with concentrations present in the cerebral cortex, corpus striatum, hippocampus, hypothalamus, brain stem, and cerebellum (McEvoy 2003).

When metabolized, fluoxetine undergoes N-demethylation catalyzed by the cytochrome P-450 isoenzyme CYP2D6/22 in the liver which produces the metabolite norfluoxetine which is just as active as the parent compound. Fluoxetine is metabolized to norfluoxetine, a desmethyl active metabolite similar to FLX, by the liver with peak plasma concentrations occurring 76

hours after dosing (USPDI 2003). Under normal conditions, the concentration of the metabolite is greater than the parent compound (Hiemke and Härtter 2000).

The long and highly variable half-life of FLX ranges from 1 to 4 days after a single dose with an average of nearly 70 hours. However, after receiving high doses over long periods of time prolonged elimination half-lives may occur. Norfluoxetine has a half-life of about 7 to 9 days (IPCH 1999). The primary hypothesized primary route of elimination is further hepatic metabolism to inactive metabolites which are conjugated and then excreted in the urine (Hemeryck and Belpaire 2002). Less than 10% unchanged parent compound is primarily excreted in urine (Hiemke and Härtter 2000).

### **Entrance into the Environment**

Active pharmaceutical ingredients (APIs) enter the environment through a variety of pathways. The major pathway is through WWT facility effluent. Unmetabolized portions or active metabolites that are not absorbed are secreted in urine or feces and then emitted into raw sewage (Halling-Sorensen et al. 1998; Daughton et al. 1999). Flushing unused medications is a minor pathway which appears to be of lesser importance than the primary pathway of excretion (Heberer 2002). Unused or expired pharmaceuticals are often flushed thus contributing to the environmental pollution (Halling-Sorensen et al. 1998). Drugs administered close to the end of one's life can leach from cemeteries and into groundwater (Daughton 2003). Pharmaceuticals may enter the environment through drug-containing waste from manufacturing facilities (Daughton and Ternes 1999).

After oral administration pharmaceuticals can either be fully or partially absorbed from the gastrointestinal tract and then degraded by metabolic processes. After the drug is metabolized by the body it is only slightly changed. These by products are often times conjugated to polar molecules such as glucuronides. Metabolism of the drug may occur to a large or small extent.



Excreted materials may include the metabolites, the parent molecule, or a mixture of both and are then released into the environment via wastewater effluent (Jones et al. 2001; Kummerer and Giampaolo 2006). Most medical substances are metabolized to phase I or phase II metabolites before being excreted from the body with the urine and might arrive in the environment as such. Metabolic Phase I reactions usually consist of oxidation, reduction or hydrolysis, and the products are often more reactive, and sometimes more toxic, than the parent compound. Metabolic phase II reactions involve conjugation, which normally results in inactive water soluble compounds. Both phase I and phase II reactions change the physiochemical properties of the substance because biotransformation produces metabolites that are more water soluble than the parent compound. Thus, often it is not only the parent compound which should be the subject of risk assessment but also the primary metabolites.

Parent compound → into more water soluble phase I metabolites

Parent compound → into more water soluble phase II metabolites

Phase I metabolite → into more water soluble phase II metabolite

Phase II metabolites → into less water soluble parent compound or phase I metabolite

(Halling-Sorensen et al. 1998).

Wastewater treatment plants do not degrade all of the fractions of original pharmaceutical products. Bacteria in the wastewater treatment process cleave these conjugate groups and the metabolites are restored to the parent compound (Heberer 2002; Halford 2008). These products enter the environment via treated or untreated raw sewage (Halling-Sorensen et al. 1998; Daughton et al. 1999; Ternes, 1998; Zuccato et al. 2000; Kummerer 2004; Halford 2008). Sewage systems are not equipped for removal of pharmaceuticals and personal care products (PPCPs). Currently, there are no wastewater treatment facilities that are engineered to remove pharmaceuticals or other unregulated contaminants such as personal care products. Effective

removal of these unregulated chemicals can differ depending on the properties of the chemical and on the individual sewage treatment facilities (USEPA 2010). The high polarity and low volatility of most pharmaceutical means that they are likely to be transported to the water compartment (Breton and Boxall 2003).

Wastewater is often reused for drinking water which can increase the risk of pharmaceuticals reentering the human body as either the parent molecule or a transformed molecule (Khetan and Collins 2007). Most drinking water treatment plants first add coagulant salts and polymers to destabilize colloidal particles. Then the coagulated water is agitated to promote the aggregation of suspended materials. Finally, water is stilled into order to settle suspended solids and floccules for removal. This process, known as coagulation-flocculation-sedimentation (C-F-S), is followed up by chlorination. However, due to the polarity of most pharmaceuticals, they are not typically removed by the C-F-S process (Khetan and Collins 2007).

Leaching into groundwater can occur thus contaminating a potential drinking water supply. Pharmaceuticals are not significantly absorbed in the subsoil due to their polar structure (Khetan and Collins 2007). Sewage treatment plants with longer solids retention times will remove more pharmaceuticals and other chemicals. The sorption coefficients ( $\log K_{oc}$ ) to dissolved organic matter was 4.72 and for a model sediment of Elliott Silt Loam soil was 4.87 (Yammomoto et al. 2005).  $\log K_{oc}$  for FLX is 0.64, 0.97, and 3.70 at the pH 2, 7, and 11 respectively (Brooks et al 2003a).

## **Historical Background**

One of the first concerns of pharmaceuticals adversely affecting the environment was presented by a study investigating the biotransformation of estrogenic hormones by activated sludge which could impact the ecosystem (Stumm-Zollinger and Fair 1965; Tabak and Bunch

1970). In 1976 drugs were detected in sewage at the Big Blue River sewage treatment plant in Kansas City (Hignite and Azarnoff 1977). The first reported occurrence of pharmaceuticals in USA treated wastewater was  $8 \times 10^{-1} - 2$  ppb of clofibrac acid (Garrison et al. 1976). The most severe example of pharmaceutical contamination has been the feminizing of aquatic species to estrogenic compounds. In the mid-1980s fish living in sewage treatment lagoons exhibited feminization (Daughton et al. 1999). In the 1990s, scientists observed that male fish living downstream of WWTP began conceiving early-stage eggs in their testes and producing proteins associated with egg production typically occurring in female fish. Feminized male fish have been reported in rivers and streams in the U.S. and Europe (Halford 2008).

### **Behavior in the Aquatic Environment**

Pharmaceuticals and personal care products are continuously released into the environment. Ecosystems at maximum risks do not have upstream dilution (Brooks et al. 2003b). Active pharmaceutical ingredients (APIs) that may undergo environmental transformation by means of degradation or dilution can be offset by replacement discharges (Daughton et al. 1999).

The behavior and fate of APIs and associated metabolites in the aquatic environment are not completely understood. Low volatility denotes dispersal through aqueous transport with the possibility of food chain distribution (Fent et al. 2006). The maximum solubilities in water have been established for EE2 ( $1 \times 10^{-2}$  ppb) (Yalkowsky and Dannenfelser 1992) and for FLX ( $1 \times 10^{-5}$  ppb (mg/mL)) (O'Neil 2001).

The operation of pharmaceuticals in surface waters is dependent on temperature and light intensity. Low temperatures and shorter daylight hours typical of winter months may lead to decreased biodegradation and decreased photodegradation of chemicals. Surface waters that are prone to snow and ice are less likely to break down pharmaceuticals. In summer months, characterized by higher temperature and high levels of light, chemicals may become less potent

(Khetan and Collins 2007). Metabolites can be transformed to their parent molecule through waste water treatment (WWT) processes or environmental conversions.

The higher the log  $K_{ow}$  (octanol-water coefficient) the more hydrophobic the compound is and the greater probability for bioaccumulation. Lipophilic xenobiotics ( $\log K_{ow} > 3$ ) are likely to enter fish bloodstreams primarily across the gills (Kreke and Dietrich 2008). The reported log  $K_{ow}$  (octanol-water coefficient) for EE2 is 3.67 (Hansch et al. 1995) with a predicted log  $K_{ow}$  of 4.12 by ECOSAR (Sanderson et al. 2004). The reported log  $K_{ow}$  for FLX is 1.0, 1.8, and 2.6 at pH 5, 7, 9 respectively (Eli Lilly and Company 2005) and 1.25, 1.57, and 4.30 at pH 2, 7, and 11 respectively (Brooks et al 2003a). The predicted log  $K_{ow}$  by ECOSAR was 4.65 (Sanderson et al. 2004).

**Bioaccumulation.** Upon entering the environment, the potential for bioaccumulation in food chains occurs (Kummerer and Giampaolo 2006). The pharmaceutical industry is producing more effective active pharmaceutical ingredients (APIs) by increasing potency, bioavailability, and degradation resistance (Khetan and Collins 2007). In order for pharmaceuticals to perform their intended action, they are resistant to biodegradation. However this opposition to biodegradation increases the chances that these drugs bioconcentrate and biomagnify. These drugs may remain in animal tissue and bioaccumulate enforcing biomagnifications (Carbonell et al. 2000).

Increase in accumulation of EE2 based on whole body residue of F1 generation *Pimephales promelas* (fathead minnow) embryos less than 24 hours post fertilization (hpf) exposed to  $5 \times 10^{-2}$  ppb EE2 had a bioconcentration factor (BCF) of 660. Those exposed to  $2 \times 10^{-4}$  ppb had a BCF less than 2400. The concentration  $8 \times 10^{-4}$  ppb yielded a BCF less than 500. Organisms exposed to  $1 \times 10^{-2}$  ppb had a BCF of 610 (Lange et al. 2001). An increase in elimination of EE2 was found in adult *Oncorhynchus mykiss* (rainbow trout) exposed for 0-50

days to  $1 \times 10^{-3}$  – 1 ppb based on residue in plasma of (Schultz et al. 2001). Juvenile *Acipenser fulvescens* (lake sturgeon) showed an increase in accumulation of residue in plasma at  $6 \times 10^{-2}$  ppb with no effect at  $1 \times 10^{-2}$  ppb (Palace et al. 2001).

*Lepomis macrochirus* (bluegill), *Ictalurus punctatus* (channel catfish), and *Pomoxis nigromaculatus* (black crappie) sampled from 100 meters downstream from the effluent discharge in Pecan Creek and *L. macrochirus* and *Cyprinus carpio* (carp) in Clear Creek had levels of fluoxetine in the brain. These fish species studied from two streams in Denton County, Texas, USA represent a bioaccumulation effect. However, the effects of 2 ppb FLX on the nervous system of fish have not yet been investigated (Brooks et al. 2005).

*Oryzias latipes* (Japanese medaka) were exposed to fluoxetine at a nominal concentration of  $6 \times 10^{-1}$  ppb for 7 days and subsequently allowed to depurate in clean water over a 21 day period. Fluoxetine uptake by medaka was observed within the first 5 hours of exposure and the biologically active metabolite, norfluoxetine, was also detected in medaka tissues during this timeframe. Maximum FLX concentration was measured in medaka by the third day of the uptake phase, yielding an uptake rate constant ( $k_1$ ) of  $5.9 \pm 0.5$  per day. During the depuration phase of the experiment, a half life of  $9.4 \pm 1.1$  days was determined for fluoxetine. Using these data, bioconcentration factor (BCF) values of 74 and 80 were estimated for fluoxetine and a pseudo-BCF (the ratio of the concentration of norfluoxetine in medaka and the aqueous fluoxetine concentration) of 117 was calculated for norfluoxetine. These results indicate longer persistence and greater potential for the bioaccumulation of fluoxetine and norfluoxetine in fish tissues than would be predicted from prior half life estimates derived using mammalian species (Paterson and Metcalfe 2008).

## Environmental Concentrations

Persons prescribed these drugs take them for many months, usually years. These medications are recommended for long term treatment which results in continuous pollution into environment. Although pharmaceuticals have been present as long as humans have been taking them, it is through recent advancements in analytical instrumentation and techniques that their presence in the environment has been proven because they typically exist in nanograms per liter (Halford 2008). Analytical techniques and instrumentation able to detect minute quantities of polar compounds have been particularly helpful to the advancement in knowledge of environmental occurrence of pharmaceuticals (Fent et al. 2006).

Minute environmental concentrations of ethinyl estradiol have been detected in different media including but not limited to: surface water (less than  $2 \times 10^{-4}$  parts per billion ( $\mu\text{g/L}$ )), effluent from sedimentation tank ( $3\text{-}5 \times 10^{-4}$  ppb), river water ( $2 - 15 \times 10^{-3}$  ppb), reservoir ( $1 - 3 \times 10^{-3}$  ppb) and drinking water (less than  $5 \times 10^{-3}$  ppb) (Halling-Sørensen et al. 1998). The influent ( $3 \times 10^{-3}$  ppb) and effluent ( $4 \times 10^{-4}$  ppb) of a wastewater treatment facility reached 85% maximum removal by activated sludge (Baronti et al. 2000).

Fluoxetine has been detected in the following media: municipal wastewaters ( $5 \times 10^{-2}$  ppb) (Gange et al. 2006), reclaimed water facility effluent ( $5 \times 10^{-3}$  ppb) (Kinney et al. 2006), waste water treatment plant effluent in Calgary, CA ( $5 \times 10^{-2}$  ppb) (Chen et al. 2006), and stream waters ( $1 \times 10^{-1}$  ppb) (Metcalf et al. 2003b). Weston et al. (2001) detected  $5 \times 10^{-2}$  ppb of FLX in WWT effluent with 79-82% recovery; however, with 67-77% recoveries for norfluoxetine, the metabolite was not detected. Using isotope dilution liquid chromatography/tandem mass spectrometry the amounts of fluoxetine and norfluoxetine were analyzed from a number sewage treatment plant (STP) influents, effluents, and drinking water reservoirs. The detected amounts of FLX were  $2 \times 10^{-2}$  ppb in the influent,  $3 \times 10^{-2}$  ppb in the effluent,  $3 \times 10^{-3}$  ppb in the drinking

water. Norfluoxetine was detected in the influent at  $1 \times 10^{-2}$  ppb, in the effluent at  $4 \times 10^{-3}$  ppb, and in the drinking water at  $1 \times 10^{-3}$  ppb (Vanderford and Snyder 2006).

In 2002 the United States Geological Survey (USGS) performed a national survey in which pharmaceuticals and personal care products (PPCPs) were detected in 139 rivers in 30 states. A plethora of biologically active compounds were detected in 80% of these populated and remote locations with an average recovery of about 60%. Antibiotics and hormones were found with the greatest frequency in North American waterways. Other detected pharmaceuticals included contraceptives, painkillers, anticancer drugs, blood-pressure medications, antidepressants, and drugs used to treat epilepsy. Ethinyl estradiol occurred with a frequency of 16% with an estimated maximum of  $8 \times 10^{-1}$  ppb and an estimated median detectable concentration of  $7 \times 10^{-2}$  ppb. Fluoxetine occurred with a frequency of 1% with an estimated maximum and median detectable level of  $1 \times 10^{-2}$  ppb. When compared to other man-made pollutants, pharmaceuticals represent a minute portion of environmental pollutants (Halford 2008).

Although the concentrations of some of these drugs may be considered small, these drugs were designed to have a profound effect at low concentrations (Koplin et al. 2002). It is estimated that pharmaceuticals are environmentally present at concentrations below those needed to cause considerable harm. It would rash to assume that these compounds were not affecting the ecosystems until there is irrefutable evidence to suggest otherwise (Jones et al. 2004). Studies have shown that concentrations of EE2 and FLX, many above the environmental detected levels, can stimulate health effects and lethality in animals and some plants (Jones et al. 2004).

## Lethal Effects of Ethinyl Estradiol

Ethinyl estradiol produced LC50s for numerous organisms (Table 1). *Gammarus pulex* (scud), when exposed to EE2, had a 72 hour LC50 between  $2 \times 10^3$  ppb -  $4 \times 10^3$  ppb. The mortality increased from day 5-9 but the LC50 remained  $0 - 2 \times 10^3$  ppb. The 10 day LC50 was  $8 \times 10^2$  ppb (Watts et al. 2001). The *Tisbe battagliai* (harpacticoid copepod) had a 10 day and 21 day LC50 greater than or equal to  $1 \times 10^2$  ppb based on mortality (Hutchinson et al. 1999). Pounds et al. (2002) found the same LC50s based on survivorship.

Table 1. Exposure times and concentrations of ethinyl estradiol reported to provoke 50% lethality (LC50S) in aquatic organisms.

Exposure Time	Organism	Dose (ppb)	Trend/Response	Reference
24 hours	<i>Gammarus pulex</i> (Scud)	$8 \times 10^3$	Increase in Mortality	Watts et al. 2001
48 hours	<i>Acartia tonsa</i> (Calanoid Copepod)	$1 \times 10^3$	Increase in Mortality	Andersen et al. 2001
48 hours	<i>Gammarus pulex</i> (Scud)	$4 \times 10^3$	Increase in Mortality	Watts et al. 2001
72 hours	<i>Gammarus pulex</i> (Scud)	$> 2 \times 10^3$ $< 4 \times 10^3$	Increase in Mortality	Watts et al. 2001
96 hours	<i>Gammarus pulex</i> (Scud)	$2 \times 10^3$	Increase in Mortality	Watts et al. 2001
96 hours	<i>Neomysis integer</i> (Opossum Shrimp)	$1 \times 10^3$	Increase in Mortality	Verslycke et al. 2004
96 hours	<i>Nitocra spinipes</i> (Harpacticoid Copepod)	$5 \times 10^2$	Increase in Mortality	Breitholtz and Bengtsson 2001
96 hours	<i>Danio rerio</i> (Zebrafish)	$2 \times 10^3$	Mortality	Versonnen et al. 2003
5-9 days	<i>Gammarus pulex</i> (Scud)	$> 0$ $< 2 \times 10^3$	Increase in Mortality	Watts et al. 2001
10 days	<i>Gammarus pulex</i> (Scud)	$8 \times 10^2$	Increase in Mortality	Watts et al. 2001
10 days 21 days	<i>Tisbe battagliai</i> (Harpacticoid Copepod)	$\geq 1 \times 10^2$	Increase in Mortality	Hutchinson et al. 1999
10 days 21 days	<i>Tisbe battagliai</i> (Harpacticoid Copepod)	$\geq 1 \times 10^2$	Decrease in Survival	Pounds et al. 2002

Generally exposure to ethinyl estradiol caused a decrease in hatchability (Table 2). Hatchability for *Danio rerio* (zebrafish) embryos 4-6 hours old with hatch times documented on days 1, 26, and 30 resulted in a 74 hour ET50 of  $1 \times 10^{-3}$  ppb and  $3 \times 10^{-3}$  ppb, and a 75 hour ET50 of  $8 \times 10^{-3}$  ppb (Bogers et al. 2006). The decrease in hatchability of *Pimephales promelas*



(fathead minnow) exposed for 1 day post hatch caused an NOEC greater than or equal to  $5 \times 10^{-2}$  ppb and an LOEC greater than  $5 \times 10^{-2}$  ppb (Lange et al. 2001).

Table 2. Exposure times and concentrations of ethinyl estradiol reported to provoke the endpoints associated with hatchability of aquatic organisms.

Organism	Response	Exposure Time	End Point	Dose (ppb)	Trend	Reference
<i>Danio rerio</i> (Zebrafish)	Hatchability	74 hours	ET50	$1 \times 10^{-3}$ $3 \times 10^{-3}$		Bogers et al. 2006
		75 hours	ET50	$8 \times 10^{-3}$		
<i>Danio rerio</i> (Zebrafish)	Hatchability	58 days	NOEC	$1 \times 10^{-3}$		Hill and Janz 2003
			LOEC	$1 \times 10^{-2}$		
<i>Pimephales promelas</i> (Fathead Minnow)	Hatchability	1 dph	NOEC	$\geq 5 \times 10^{-2}$	Decrease	Lange et al. 2001
			LOEC	$> 5 \times 10^{-2}$		

Generally exposure to ethinyl estradiol caused a decrease in survivorship (Table 3). A study supplying F0 generation *Cyprinodon variegates* (sheepshead minnow) with  $2 \times 10^{-4}$ ,  $2 \times 10^{-3}$ , and  $2 \times 10^{-2}$  ppb resulted in no toxicopathic stress; endurance was similar to controls. Survival was significantly reduced in subadults after 73 days in  $4 \times 10^{-1}$ ,  $8 \times 10^{-1}$ , 2, and 3 ppb producing: an NOEC of  $1 \times 10^{-1}$  ppb and an LOEC of  $3 \times 10^{-1}$  ppb, and an MATC of  $2 \times 10^{-1}$  ppb. F1 generation embryos retained a decrease in survivorship after 73 days, ensuing an NOEC of  $9 \times 10^{-2} - 1 \times 10^{-1}$  ppb, an LOEC of  $3 \times 10^{-1} - 4 \times 10^{-1}$  ppb, and an MATC of  $3 \times 10^{-1}$  ppb (Zillioux et al. 2001). *Tisbe battagliai* (harpacticoid copepod) less than 24 hours old exhibited decreased survivorship launching 10 and 21 day NOECs greater than or equal to  $1 \times 10^2$  ppb and LOECs greater than  $1 \times 10^2$  ppb (Pounds et al. 2002). Hutchinson et al. (1999) found the same endpoints with a decrease in survivorship for the NOEC but found an increase in survivorship for the LOEC. Based on decreased survivorship in *Danio rerio* (zebrafish) embryos after hatching the 1 day NOEC was  $8 \times 10^{-3}$  ppb. An increase in survivorship was noted on days 26 and 30 yielding an NOEC of  $8 \times 10^{-3}$  ppb (Bogers et al. 2006). *Pimephales promelas* (fathead minnow) embryos less than 24 hpf exposed for 28 days post hatch manifested diminished survivorship

yielding an NOEC of  $1 \times 10^{-2}$  ppb and an LOEC of  $5 \times 10^{-2}$  ppb. Dosing for 56 days post hatch provoked an NOEC of  $1 \times 10^{-2}$  ppb with 22.99 – 23% mortality and an LOEC of  $5 \times 10^{-2}$  ppb with 25-31% mortality. At 176-301 days post hatch an NOEC for survivorship was greater than or equal to  $8 \times 10^{-4}$  ppb with an LOEC greater than  $8 \times 10^{-4}$  ppb (Lange et al. 2001).

Table 3. Exposure times and concentrations of ethinyl estradiol reported to provoke the endpoints associated with survivorship of aquatic organisms.

Organism	Response	End Point	Dose (ppb)	Trend	Exposure Time	Reference
<i>Hyalella azteca</i> (Scud)	Survivorship	LOEL	$1 \times 10^2$			Vandenbergh 2003
<i>Tisbe battagliai</i> (Harpacticoid Copepod)	Survivorship	NOEC	$\geq 1 \times 10^2$	Decrease	10 and 21 days	Hutchinson et al. 1999
		LOEC	$> 1 \times 10^2$	Increase		
<i>Tisbe battagliai</i> (Harpacticoid Copepod)	Survivorship	NOEC	$\geq 1 \times 10^2$	Decrease	10 and 21 days	Pounds et al. 2002
		LOEC	$> 1 \times 10^2$			
<i>Cyprinodon variegates</i> (Sheepshead Minnow)	Survivorship	NOEC	$1 \times 10^{-1}$	Decrease	73 days	Zillioux et al. 2001
		LOEC	$3 \times 10^{-1}$			
		MATC	$2 \times 10^{-1}$			
<i>Cyprinodon variegates</i> (Sheepshead Minnow)	Survivorship	NOEC	$9 \times 10^{-2} - 1 \times 10^{-1}$	Decrease	73 days	Zillioux et al. 2001
		LOEC	$3 \times 10^{-1} - 4 \times 10^{-1}$			
		MATC	$3 \times 10^{-1}$			
<i>Danio rerio</i> (Zebrafish)	Survivorship	NOEC	$8 \times 10^{-3}$	Decrease	1 day	Bogers et al. 2006
				Increase	26 and 30 days	
<i>Oncorhynchus tshawytscha</i> (Chinook Salmon)	Survivorship		$4 \times 10^2$	Decrease	194 dph	Piferrer and Donaldson 1992
<i>Pimephales promelas</i> (Fathead Minnow)	Survivorship	NOEC	$1 \times 10^{-2}$	Decrease	28 dph	Lange et al. 2001
		LOEC	$5 \times 10^{-2}$			
<i>Pimephales promelas</i> (Fathead Minnow)	Survivorship	NOEC	$1 \times 10^{-2}$	Decrease	56 dph	Lange et al. 2001
		LOEC	$5 \times 10^{-2}$			
<i>Pimephales promelas</i> (Fathead Minnow)	Survivorship	NOEC	$\geq 8 \times 10^{-4}$	Decrease	176-301 dph	Lange et al. 2001
		LOEC	$> 8 \times 10^{-4}$			
<i>Tilapia aurea</i> (Tilapia)	Survivorship	NOEL	$2 \times 10^5$		40 days	Melard 1995

Generally, exposure to EE2 increased mortality in aquatic organisms (Table 4). Clutch

*Danio rerio* (zebrafish) exhibited a decrease in hatchability resulting in a 58 day NOEC of  $1 \times$

$10^{-1}$  ppb and an LOEC of  $1 \times 10^{-2}$  ppb (Hill and Janz 2003). *Danio rerio* (zebrafish) encountered mortality at 25 (46%), 30 (53%), 40 (53%), and 60 (53-54% mortality) days post hatch. For all days post hatch, the reported NOEL was  $2 \times 10^{-2}$  ppb. The LOEL for 20 days post hatch was  $2 \times 10^{-2}$  ppb (Andersen et al. 2003). *Danio rerio* (zebrafish) fry 2 dph had mortality after exposure for 58 days yielding an NOEC of  $100 \text{ E-}4$  ppb and an LOEC of  $1 \times 10^{-1}$  ppb (Hill and Janz 2003). *Pimephales promelas* (fathead minnow) displayed an increase in mortality on days 0-13.3 in  $5 \times 10^{-2}$  ppb; however, a decrease in mortality was observed on day 21 in  $1 \times 10^{-2}$  ppb (Lange et al. 2003).

Table 4. Exposure times and concentrations of ethinyl estradiol reported to provoke the endpoints associated with mortality of aquatic organisms.

Organism	Response	End Point	Dose (ppb)	Trend	Exposure Time	Reference
<i>Danio rerio</i> (Zebrafish)	Mortality	NOEL	$2 \times 10^{-2}$		10 dph	Anderson et al. 2003
					25 dph	
					30 dph	
					40 dph	
		60 dph				
LOEL	$2 \times 10^{-2}$	20 dph				
<i>Danio rerio</i> (Zebrafish)	Mortality		$5 \times 10^{-3}$ – $5 \times 10^{-2}$	Increase	0-21 days	Van den Belt et al. 2001
<i>Danio rerio</i> (Zebrafish)	Mortality		$3 \times 10^{-4}$ – $9 \times 10^{-2}$	Increase	21 days	Islinger et al. 2003
<i>Danio rerio</i> (Zebrafish)	Mortality	NOEL	1		33 days	Versonnen and Janssen 2004
<i>Danio rerio</i> (Zebrafish)	Mortality	NOEL	$1 \times 10^{-2}$		58 days	MacLachy et al. 2003
<i>Danio rerio</i> (Zebrafish)	Mortality	LOEL	5	(>50% died)	35 dph	Ortiz-Zarragoitia et al. 2006
<i>Danio rerio</i> (Zebrafish)	Mortality	NOEC	$5 \times 10^{-3}$		~ 16 days	Nash et al. 2004
		LOEC	$5 \times 10^{-2}$			
<i>Danio rerio</i> (Zebrafish)	Mortality	NOEC	$3 \times 10^{-2}$	Decrease	40 days	Andersen et al. 2004
<i>Danio rerio</i> (Zebrafish)	Mortality	NOEC	$1 \times 10^{-2}$		58 days	Hill and Janz 2003
		LOEC	$1 \times 10^{-1}$			
<i>Dicentrarchus labrax</i> (Sea Bass)	Mortality		$1 \times 10^4$		40 days 200 days	Blazquez et al. 1998

(Table 4 continued)

Organism	Response	End Point	Dose (ppb)	Trend	Exposure Time	Reference
<i>Etheostoma caeruleum</i> (Rainbow Darter)	Mortality		1	Increase	6-21 days	Elias et al. 2007
<i>Etheostoma caeruleum</i> (Rainbow Darter)	Mortality	No Effect	$2 \times 10^{-1}$		21 days	Elias et al. 2007
<i>Gambusia affinis</i> (Western Mosquitofish)	Mortality	NOEL	$1 \times 10^4$		150 days	Angus et al. 2005
<i>Oryzias latipes</i> (Medaka)	Mortality	NOEL	$1 \times 10^{-4}$ µg/egg		1 day	Hano et al. 2005
		LOEL	$5 \times 10^{-4}$ µg/egg			
<i>Oryzias latipes</i> (Medaka)	Mortality		$1 \times 10^{-4}$ – 1	Increase	85-110 days	Metcalf et al. 2001
<i>Pimephales promelas</i> (Fathead Minnow)	Mortality		$5 \times 10^{-2}$	Increase	0-13.3 days	Lange et al. 2003
<i>Pimephales promelas</i> (Fathead Minnow)	Mortality		$1 \times 10^{-2}$	Decrease	21 days	Lange et al. 2003
<i>Platichthys flesus</i> (Starry, European Flounder)	Mortality		$1 \times 10^{-3}$ – $1 \times 10^{-2}$	Increase	21 days	Allen et al. 1999
<i>Pomatoschistus minutus</i> (Sand goby)	Mortality		$6 \times 10^{-3}$	Increase	0 – 213.08 days	Robinson et al. 2003

There are a few documented cases in which populations did not decline. *Danio rerio* (zebrafish) that were 20 days old post hatch exposed for 40 days evoked a slight decline in mortality forming an NOEC of  $3 \times 10^{-2}$  ppb (Andersen et al. 2004). *Tilapia aurea* (tilapia) yielded 94% survivorship forming a 40 day NOEL of 200000 ppb through dietary exposure (Melard 1995). The following NOELS based on mortalities were recorded in *Danio rerio* (zebrafish): a 33 day exposure to  $1 \times 10^{-1}$  ppb (Versonnen and Janssen 2004), and a 58 day

exposure to  $1 \times 10^{-2}$  ppb (MacLatchy et al. 2003). Sexually immature *Gambusia affinis* (western mosquitofish) fed EE2 through 150 days illustrated an NOEL of  $1 \times 10^4$  ppb (Angus et al. 2005). While an increase in fatality was observed in juvenile *Etheostoma caeruleum* (rainbow darter) in 1 ppb exposed for 6-21 days, exposure after 21 days in  $2 \times 10^{-1}$  ppb caused no effect on mortality (Elias et al. 2007). *Pimephales promelas* (fathead minnow) displayed an increase in mortality on days 0-13.3 in  $5 \times 10^{-2}$  ppb; however, a decrease in mortality was observed on day 21 in  $1 \times 10^{-2}$  ppb (Lange et al. 2003). An increase in survivorship was noted on days 26 and 30 yielding an NOEC of  $8 \times 10^{-3}$  ppb *Danio rerio* (zebrafish) (Bogers et al. 2006).

### Lethal Effects of Fluoxetine

Lethal concentrations (LC50s) were documented for different aquatic organisms (Table 5). A 24 hour LC50 was reported for *Thamnocephalus platyurus* (fairy shrimp)  $8 \times 10^2 \pm 1 \times 10^2$  (Nalcz-Jawecki 2007). Juvenile *Cyprinodon variegatus* (sheepshead minnow) 43 – 63 days old allocated were exposed to test concentrations  $2 \times 10^1 - 2 \times 10^3$ . Those organisms in the highest concentration of  $2 \times 10^3$  ppb sustained 47% mortality, causing the 96 hour LC50 to be greater than  $2 \times 10^3$  ppb (Henry et al. 2004). *Oryzias latipes* (Japanese medaka) larvae  $6 \times 10^3$ ,  $1 \times 10^3$ , and  $2 \times 10^2$  ppb at the respective pHs 7, 8, and 9 (Nakamura et al. 2008). The average 48 hour LC50s for *Pimephales promelas* (fathead minnow) were about 200 ppb for the R-fluoxetine, S-fluoxetine, and the racemic-fluoxetine mixtures.

Table 5. Exposure times and concentrations of fluoxetine reported to provoke 50% lethality (LC50s) in aquatic organisms.

Exposure time	Organism	Amount (ppb)	Trend/Response	Reference
24 hours	<i>Thamnocephalus platyurus</i> (Fairy Shrimp)	$8 \times 10^2$	Lethality	Nalcz-Jawecki 2007
48 hours	<i>Chironomus tentans</i> (Midge)	$2 \times 10^4$	Mortality	Brooks et al. 2003b
48 hours	<i>Hyalella azteca</i> (Scud)	$> 4 \times 10^4$	Mortality	Brooks et al. 2003b

(Table 5 continued)

Exposure time	Organism	Amount (ppb)	Trend/Response	Reference
48 hours	<i>Ceriodaphnia dubia</i> (Water Flea)	$5 \times 10^2$	Increase in Mortality	Henry et al. 2004
48 hours	<i>Ceriodaphnia dubia</i> (Water Flea)	$2 \times 10^2$	Mortality	Brooks et al.2003b
48 hours	<i>Daphnia magna</i> (Water Flea)	$8 \times 10^2$	Mortality	Brooks et al. 2003b
48 hours	<i>Pimephales promelas</i> (Fathead Minnow)	$7 \times 10^2$	Mortality	Brooks et al. 2003b
48 hours	<i>Pimephales promelas</i> (Fathead Minnow)	$\sim 2 \times 10^2$	Mortality	Stanley et al. 2007
48 hours	<i>Oryzias latipes</i> (Japanese Medaka)	$9 \times 10^3$	Mortality	Brooks et al. 2003b
48 hours	<i>Oncorhynchus mykiss</i> (Rainbow Trout)	$2 \times 10^3$	Mortality	FDA-CDER 1996; Eli Lilly and Company 2005
96 hours	<i>Ceriodaphnia dubia</i> (Water Flea)	$3 \times 10^2$	Mortality	Brooks et al. 2003b
96 hours	<i>Daphnia magna</i> (Water Flea)	$9 \times 10^2$	Mortality	Brooks et al. 2003b
96 hours	<i>Cyprinodon variegatus</i> (Sheepshead Minnow)	$> 2 \times 10^3$	Mortality (47%)	Winder et al. 2009
96 hours	<i>Pimephales promelas</i> (Fathead Minnow)	$8 \times 10^2$	Mortality	Brooks et al. 2003b
96 hours	<i>Oryzias latipes</i> (Japanese Medaka)	$2 \times 10^3$		Eli Lilly and Company 2005
96 hours	<i>Oryzias latipes</i> (Japanese Medaka)	$6 \times 10^3$ pH 7		Nakamura et al. 2008
		$1 \times 10^3$ pH 8		
		$2 \times 10^2$ pH 9		

Fluoxetine induced endpoints associated with mortality and/or survival (Table 6). The 24 hour LC50 of  $600 \pm 200$  ppb for *Spirostomum ambiguum* (protozoan) was based on spherical deformation and autolysis. The 24 hour EC50 of  $400 \pm 100$  was based on different deformations of morphological changes such as shortening, bending of the cell, etc (Nalcz-Jawecki 2007). Juvenile *Cyprinodon variegatus* (sheepshead minnow) 43 – 63 days old allocated 2000 ppb sustained 47% mortality, resulting in the 96 hour LC50 greater than 2000 ppb, LOEC 2000 ppb, and NOEC 1000 ppb. In concentrations 0, 50, 300, 500, 1000 ppb mortality remained greater than or equal to 13% (Winder et al. 2009). *Ceriodaphnia dubia* (water flea) suffered 100%

mortality in 2000 ppb after 7-8 days. Introduction to 90 ppb secured 0% mortality. The median concentration of 400 ppb showed a decrease in survivorship (test concentration 20 – 3000 ppb) (Henry et al. 2004).

Table 6. Exposure times and concentrations of fluoxetine reported to provoke the endpoints associated with mortality or survivorship of aquatic organisms.

Organism	Response	Endpoint	Dose (ppb)	Trend	Exposure time	Reference
<i>Spirostomum ambiguum</i> (Protozoan)	Deformation and Autolysis	LC50	600		24 hours	Nalcz-Jawecki 2007
<i>Spirostomum ambiguum</i> (Protozoan)	Deformation	LC50	400		24 hours	Nalcz-Jawecki 2007
<i>Ceriodaphnia dubia</i> (Water Flea)	Mortality	No effect	90	No effect	7-8 days	Henry et al. 2004
	Mortality		2000	Decrease (100%)		
	Survivorship		400	Decrease		
<i>Cyprinodon variegatus</i> (Sheepshead Minnow)	Mortality	NOEC	1000		96 hours	Winder et al. 2009
		LOEC	2000			
<i>Pimephales promelas</i> (Fathead Minnow)	Survivorship R-FLX	NOEC	100	Decrease	7 days	Stanley et al. 2007
		LOEC	200			
<i>Pimephales promelas</i> (Fathead Minnow)	Survivorship Rac-FLX	NOEC	100	Decrease	7 days	Stanley et al. 2007
		LOEC	200			
<i>Pimephales promelas</i> (Fathead Minnow)	Survivorship S-FLX	NOEC	50	Decrease	7 days	Stanley et al. 2007
		LOEC	100			

### Sublethal Effects

Biological diversity of pharmacodynamics, pharmacokinetics, and physiology may produce unanticipated effects on organisms. Modification of the reproductive, immune, and central nervous systems can alter hormones, development, and behavior that have not been anticipated for non-target species (Fent et al. 2006).

**Ethinyl estradiol affects fecundity.** Exposure to EE2 caused aquatic organisms to experience changes in fecundity (Table 7). *Danio rerio* (zebrafish) fry 2 dph acquired had a reduced mean egg production (Lin and Janz 2006). Adult *Danio rerio* (zebrafish) 223 dpf showed a decrease in total number of eggs (Nash et al. 2004). Decreased egg production per gram of females was observed in *Tautoglabrus adspersus* (cunner) (Gutjahr-Gobell et al. 2006). The total number of eggs of *Pimephales promelas* (fathead minnow) adults decreased after EE2 exposure (Brian et al. 2007). An F1 generation study of *Pimephales promelas* (fathead minnow) less than 24 hpf embryos exposed for 301 dph showed a decrease number of eggs per female breeding day (Lange et al. 2001).

The number of eggs per pair of *Pimephales promelas* (fathead minnow) 6-11 months old, increased at  $1 \times 10^{-4}$  and  $1 \times 10^{-3}$  ppb; no eggs were produced in  $1 \times 10^{-1}$  ppb. Effects on fertilization caused an NOEC and LOEC at  $3 \times 10^{-3}$  ppb and  $8 \times 10^{-4}$  ppb respectively. The lowest concentration effecting progeny was  $1 \times 10^{-4}$  ppb (Pawlowski et al. 2004).

In one experiment, 15 dpf *Danio rerio* (zebrafish) larvae were exposed to  $1 \times 10^{-2}$  ppb during different life stages. Based on fertilization success those exposed at 15 and 72 dpf were not affected. The only significant time period was 43 dpf in which fertilization decreased; additionally the mean number of eggs reported was significantly decreased. In another study, 15 dpf organisms were exposed for 28 days at different concentrations resulting in concentration dependent reduction of fertilization success with an NOEL of  $2 \times 10^{-3}$  ppb and an LOEL of  $3 \times 10^{-3}$  ppb; the mean number of eggs per female per day was not significantly affected (Maack and Segner 2004). Based on progeny counts of *Nitocra spinipes* (harpacticoid copepod), a decrease in reproduction was observed (Breitholtz and Bengtsson 2001). An F1 generation study of *Cyprinodon variegates* (sheepshead minnow) embryos showed a decrease in reproduction based on progeny/ number of eggs per female reproductive day (Zillioux et al. 2001).



Increase in eggs per spawning *Pimephales promelas* (fathead minnow) based on gamete production in eggs (Brian et al. 2007). Adult *Pimephales promelas* (fathead minnow) relinquished spawning completely after 28 days in less than  $1 \times 10^{-2}$  ppb (Lange et al. 2001).

Decrease in reproduction frequency and fertility of *Daphnia magna* (water flea) neonates exposed for 25 days (Goto and Hiromi 2003). *Oryzias latipes* (Japanese medaka) had a decrease in fecundity, decreased number of spawning females, and decreased fertilization rate (Ma et al. 2005). Based on the average number of eggs per day per pair a decrease in reproduction/fecundity of *Oryzias latipes* (Japanese medaka) was observed (Seki et al. 2002). Reduction in the percent of *Oryzias latipes* (Japanese medaka) participating in copulatory activity was reported (Balch et al. 2004). *Oryzias latipes* (Japanese medaka) embryos less than or equal to 8 hpf showed increased germ cells counts; gamete production in gonads after injections produced a 10 dph NOEC of  $1 \times 10^{-4}$   $\mu\text{g}/\text{egg}$  and LOEC of  $5 \times 10^{-4}$   $\mu\text{g}/\text{egg}$  (Hano et al. 2005). In a generational effluent study of immature *Pomatoschistus minutus* (sand goby),  $6 \times 10^{-3}$  ppb decreased the percentage of males nesting, percentage of pairs breeding, and overall number of pairs (Robinson et al. 2003).

Table 7. Exposure times and concentrations of ethinyl estradiol reported to provoke the endpoints associated with fecundity or reproduction of aquatic organisms.

Organism	Measured Response	End point	Dose (ppb)	Trend	Exposure time	Reference
<i>Daphnia magna</i> (Water Flea)	Reproduction Frequency	NOEC	$5 \times 10^2$	Decrease	25 days	Goto and Hiromi 2003
<i>Nitocra spinipes</i> (Harpacticoid Copepod)	Reproduction/ Progeny counts	NOEC	$5 \times 10^1$	Decrease	15-18 days	Breitholtz and Bengtsson 2001
<i>Cyprinodon variegates</i> (Sheepshead Minnow)	Reproduction/ Progeny counts	NOEC	$2 \times 10^{-2}$	Decrease in eggs per female F1 generation study	73 days	Zillioux et al. 2001
		LOEC	$9 \times 10^{-2}$ – 1			
		MATC	$6 \times 10^{-2}$			

(Table 7 continued)

Organism	Measured Response	End point	Dose (ppb)	Trend	Exposure time	Reference
<i>Danio rerio</i> (Zebrafish)	Fertilization Success	No effect	$1 \times 10^{-2}$		15 dpf	Maack and Segner 2004
					72 dpf	
<i>Danio rerio</i> (Zebrafish)	Fertilization Success	NOEL	$2 \times 10^{-3}$	Decrease *Mean number of eggs per female per day not significant	28 days	Maack and Segner 2004
		LOEL	$3 \times 10^{-3}$			
<i>Danio rerio</i> (Zebrafish)	Gamete Production	LOEL	$1 \times 10^{-3}$	Decrease in mean egg production	58 days	Lin and Janz 2006
<i>Danio rerio</i> (Zebrafish)	Gamete Production	NOEL	$5 \times 10^{-3}$	Decrease in total number of eggs	10 days	Nash et al. 2004
		LOEL	$5 \times 10^{-2}$			
<i>Oryzias latipes</i> (Medaka)	Fecundity	NOEC	$3 \times 10^{-1}$	Decrease based on average number of eggs per day per pair	0-21 days	Seki et al. 2002
		LOEC	$5 \times 10^{-1}$			
<i>Oryzias latipes</i> (Medaka)	Fecundity	LOEL	$1 \times 10^{-1}$	Decreased *Decreased number of spawning females  *Decreased fertilization rate	28 days	Ma et al. 2005
<i>Oryzias latipes</i> (Medaka)	Gamete Production	NOEC	$1 \times 10^{-4}$ µg/egg	Increase in germ cells counts	10 dph	Hano et al. 2005
		LOEC	$5 \times 10^{-4}$ µg/egg			
<i>Oryzias latipes</i> (Medaka)	Mounting/ Copulation/ Intercourse	NOEL	$2 \times 10^{-3}$	Decrease	121.76 – 182.64 days	Balch et al. 2004
		LOEL	$1 \times 10^{-2}$			
<i>Pimephales promelas</i> (Fathead Minnow)	Gamete Production	NOEC	$1 \times 10^{-3}$ – $2 \times 10^{-3}$	Decrease in total number of eggs	21 days	Brian et al. 2007
<i>Pimephales promelas</i> (Fathead Minnow)	Gamete Production	NOEC	$5 \times 10^{-4}$	Increase in eggs per spawning	21 days	Brian et al. 2007
<i>Pimephales promelas</i> (Fathead Minnow)	Number Spawning	NOEL	$6 \times 10^{-4}$ and $2 \times 10^{-3}$		21 day	Brian et al. 2007

(Table 7 continued)

Organism	Measured Response	End point	Dose (ppb)	Trend	Exposure time	Reference
<i>Pimephales promelas</i> (Fathead Minnow)	Fertilization	NOEC	$3 \times 10^{-3}$	Increase	21 days	Pawlowski et al. 2004
		LOEC	$8 \times 10^{-4}$			
	Number of eggs per pair		$1 \times 10^{-4}$ $1 \times 10^{-3}$			
	No eggs produced		$1 \times 10^{-1}$			
	Progeny	LOEC	$1 \times 10^{-4}$			
<i>Pimephales promelas</i> (Fathead Minnow)	Spawning	NOEC	$< 1 \times 10^{-2}$	Total Lack of spawning	28 days	Lange et al. 2001
<i>Pimephales promelas</i> (Fathead Minnow)	Progeny (number of eggs per female breeding day)	NOEC	$\geq 8 \times 10^{-4}$	Decrease	301 dph	Lange et al. 2001
		LOEC	$> 8 \times 10^{-4}$			
<i>Pomatoschistus minutus</i> (Sand goby)	% Males nesting		$6 \times 10^{-3}$	Decrease	generational effluent study	Robinson et al. 2003
	% Pairs breeding			Decrease		
	Overall number of pairs			Decrease		
<i>Tautoglabrus adpersus</i> (Cunner)	Egg Production	NOEL	$5 \times 10^2$	Decrease per gram female *Increase of egg viability at 0.05 mg/kg	14 days	Gutjahr-Gobell et al. 2006
		LOEL	$3 \times 10^3$			
<i>Dreissena polymorpha</i> (Zebra Mussels) Males	Spawning		$5 \times 10^{-7}$ – $5 \times 10^{-4}$ M of FLX	spawned within the first hour		Fong 1998
<i>Dreissena polymorpha</i> (Zebra Mussels) Females	Spawning	LOEC 200 E-5 P	$5 \times 10^{-6}$ and $10^{-5}$ M of FLX			Fong 1998
<i>Dreissena polymorpha</i> (Zebra Mussels) Males	Sperm Motility		$5 \times 10^{-3}$ – $5 \times 10^{-5}$ M of FLX	not motile *But, sperm recovered motility when placed in fresh lake water		Fong 1998
<i>Dreissena polymorpha</i> (Zebra Mussels) Males	Growth and Development		$1 \times 10^{-3}$ M of FLX	looked unhealthy		Fong 1998

\*Additionally observed trends

**Fluoxetine affects fecundity.** Exposure to FLX caused changes in the fecundity of aquatic organisms (Table 8). Reproduction effects modulated by serotonin vary between different phyla (Fent et al. 2006). In vivo exposure of 0.5 mg/mL of FLX for 1 hour induced egg laying in *Caenorhabditis elegans* (nematoda roundworm) (Dempsey et al. 2005). *Chironomus tentans* (diptera: chironomidae) egg clutches exposed to 6 ppb of Fluoxetine contained significantly more eggs ( $515 \pm 194$ ) than the control ( $316 \pm 214$ ) (Williams and Herrup 1988). *Ceriodaphnia dubia* (water flea) exposed for 7 days had LOEC 400 and NOEC 90 ppb for both the reduction in number of broods produced and the reduction of neonates (Henry et al. 2004). A similar NOEC of 60 ppb based on reduced number of neonates in was observed by Brooks et al. (2003).

Fluoxetine administered at 1 ppb amplified 5-HT and induced stimulation of parturition of *Sphaerium straitium* (fingernail clam) (Fong et al. 1998). *Procambarus clarkii* (red swamp crayfish) injected with 15  $\mu\text{g/g}$  body weight fluoxetine in vivo at 1, 5, 10, and 15 days, increased ovarian index and oocyte size, and amplified 5-HT-induced effects (Kulkarni et al. 1992).

Serotonin promotes oocyte maturation in *Oryzias latipes* (Japanese medaka) (Iwamatsu et al. 1993) and in some bivalves and crustaceans as well as induced spawning (Fong 2001) at 50 nM in males and 5  $\mu\text{M}$  in female *Dreissena polymorpha* (Zebra mussel) (Fong 1998) and at 1 ppb in vivo for *Macoma balthica* (Baltic clam) and created an extension of spawning season (Honkoop et al. 1999).

*Chironomus riparius* (midge) showed no effect in reproduction measured concentrations up to 60 ppb (Péry et al. 2008). *Oryzias latipes* (Japanese medaka) exposed for 4 weeks to fluoxetine at concentrations of 100–5000 ppb, had no effect on vitellogenin plasma content, plasma steroids, fecundity, egg fertilization or hatching rate (Foran et al. 2004). This indicates that FLX concentrations up to 5 ppb do not impair reproduction in this fish. A significant

decrease of reproduction was observed in *Potamopyrgus antipodarum* (snail) at 10 ppb with a reproduction success LOEC of  $5 \times 10^{-2}$  ppb (Péry et al. 2008). Fecundity of *Ceriodaphnia dubia* (water flea) was decreased by fluoxetine treatments of 200 ppb (Brooks et al. 2003 a; b). However, *C. dubia* fecundity was significantly increased after 7 day exposure of 60 ppb fluoxetine (Brooks et al. 2003b) producing an NOEC of 60 ppb and an LOEC of 100 ppb (Brooks et al. 2003). Doses of FLX (1400, 2800, 5600, 11200, 22400 ppb) appeared to stimulate reproduction in *Hyalella azteca* (scud) (Brooks et al. 2003 a, b). It is assumed that enhanced fecundity may be brought about by increased serotonin levels (Brooks et al. 2003b). Studies indentified that minimal quantities may trigger advanced fecundity; however, increased egg and neonate production is often associated with reduced egg and neonate body size (Bodar et al. 1988; Ebert 1993).

Table 8. Exposure times and concentrations of fluoxetine reported to provoke the endpoints associated with fecundity or reproduction of aquatic organisms.

Organism	Measured Response	End point	Dose (ppb)	Trend	Exposure time	Reference
<i>Caenorhabditis elegans</i> (Nematoda Roundworm)	Egg laying		0.5 mg/mL of FLX	Induce	1 hour	Dempsey et al. 2005
<i>Chironomus tentans</i> (Diptera: Chironomidae)	Egg clutches		6 of FLX	Significantly more eggs than the control		Williams and Herrup 1988
<i>Ceriodaphnia dubia</i> (Water Flea)	Progeny counts/ Number	NOEC	$9 \times 10^1$	Decrease  *Reduced number of neonates  *Reduced number of broods	7 days	Henry et al. 2004
		LOEC	$4 \times 10^2$			
<i>Ceriodaphnia dubia</i> (Water Flea)	Number of neonates	NOEC	$6 \times 10^1$	Decrease		Brooks et al. 2003
<i>Ceriodaphnia dubia</i> (Water Flea)	Fecundity		$2 \times 10^2$	Decrease		Brooks et al. 2003 a; b

(Table 8 continued)

Organism	Measured Response	End point	Dose (ppb)	Trend	Exposure time	Reference
<i>Ceriodaphnia dubia</i> (Water Flea)	Fecundity	NOEC	$6 \times 10^1$	Increase	7 days	Brooks et al. 2003 a; b
		LOEC	$1 \times 10^2$			
<i>Chironomus riparius</i> (Midge)	Reproduction	No effect	$6 \times 10^1$			Péry et al. 2008
<i>Hyalella azteca</i> (Scud)	Reproduction		$1 \times 10^3$	Stimulates		Brooks et al. 2003 a,b
			$3 \times 10^3$			
			$6 \times 10^3$			
			$1 \times 10^4$			
			$2 \times 10^4$			
<i>Dreissena polymorpha</i> (Zebra Mussel)	Spawning		50 Nm male 5 $\mu$ M in female	Induced Spawning *Promotes oocyte maturation		Fong 1998
<i>Macoma balthica</i> (Baltic Clam)	Spawning		1	Induced *Extension of spawning season		Honkoop et al. 1999
<i>Potamopyrgus antipodarum</i> (Snail)	Reproduction		$1 \times 10^{-1}$	Decrease		Péry et al. 2008
<i>Potamopyrgus antipodarum</i> (Snail)	Reproduction Success	LOEC	$5 \times 10^{-1}$			Péry et al. 2008
<i>Procambarus clarkia</i> (Red Swamp Crayfish)	Ovarian index and oocyte size		15 $\mu$ g/g body weight	Increase *Amplified 5-HT-induced effects	1, 5, 10, and 15 days,	Kulkarni et al. 1992
<i>Sphaerium straitium</i> (Fingernail Clam)	Parturition		1	Induced *Amplified 5-HT		Fong et al. 1998
<i>Oryzias latipes</i> (Japanese Medaka)	Oocyte maturation		Serotonin			Iwamatsu et al. 1993
<i>Oryzias latipes</i> (Japanese Medaka)	Vitellogenin plasma content	No effect	$1 \times 10^2$ – $5 \times 10^2$		4 Weeks	Foran et al. 2004
	Plasma steroids					
	Fecundity					
	Egg fertilization					
	Hatching rate					

\*Additionally observed trends

**Ethinyl estradiol affects growth and development.** Aquatic organisms exposed to EE2 exhibited changes in normal growth and development (Table 9). The following organisms showed a decrease in whole body growth based on length, and weight: *Oryzias latipes* (medaka) (5 µg/organism) (Papoulias et al. 2001), *Pomatoschistus minutus* (sand goby) ( $6 \times 10^{-3}$  ppb) (Robinson et al. 2003), and *Danio rerio* (zebrafish) (58 day LOEC  $1 \times 10^{-2}$  ppb) (Lin and Janz 2006).

A decrease in length in *Danio rerio* (zebrafish) (Andersen et al. 2004) and in *Dicentrarchus labrax* (sea bass) (Blazquez et al. 1998).

Increased length was recorded in *Acartia tonsa* (calanoid copepod) (Andersen et al. 2004), *Oncorhynchus tshawytscha* (chinook salmon) (Piferrer and Donaldson 1992), and in *Salvelinus namaycush* (lake trout) (Werner et al. 2003).

Decrease in weight in *Anguilla Anguilla* (common eel) (Versonnen et al 2004) and in *Carassius auratus* (goldfish) (Martyniuk et al. 2006). Bogers et al. (2006) observed an increase in whole body weight in *Danio rerio* (zebrafish).

A decrease in length and weight was observed in fathead minnows exposed for 37 days. Additional exposure produced effects on length. Those exposed until 301 days post hatch showed no effect on length (Lange et al. 2001).

*Danio rerio* (zebrafish) were reported to have a decrease in weight but an increase in length (Lin and Janz 2006). Decrease in progeny developmental stage in *Danio rerio* (zebrafish) based on swim-up breeding trial begun 240 days post hatch after 6 months recovery with no exposure, a decrease in stage development of the progeny (offspring) was found (Lin and Janz 2006).

*Acartia tonsa* (calanoid copepod) exposed for 5 days showed a decrease in larval development (Andersen et al. 2001). *Hyalella azteca* (scud) exposed to 10 ppb exhibited a

decrease in sexual development of Gnathopod (modified jaw) but showed an increase in length of antenna (Vandenbergh et al. 2001). *Oncorhynchus mykiss* (rainbow trout) (Verslycke et al. 2002) and *Acipenser flvescens* (lake sturgeon) showed an increase in the weight of the gonad in relationship to body. Also observed was an increase in liver weight in relationship to body in *Acipenser flvescens* (lake sturgeon) (Palace et al. 2001), *Oncorhynchus mykiss* (rainbow trout) (Verslycke et al. 2002), and *Ictalurus punctuates* (channel catfish) (Nimrod and Benson 1996).

Table 9. Exposure times and concentrations of ethinyl estradiol reported to provoke the endpoints associated with growth and development of aquatic organisms.

Organism	Measured Response	Exposure time	End point	Dose (ppb)	Trend	Reference
<i>Acartia tonsa</i> (Calanoid Copepod)	Larval Development	5 days	EC50	$9 \times 10^1$		Anderson et al. 2001
<i>Acartia tonsa</i> (Calanoid Copepod)	Larval Development	5 days		$4 \times 10^1$ – $2 \times 10^2$	Decrease	Anderson et al. 2001
<i>Acartia tonsa</i> (Calanoid Copepod)	Length	18 days	NOEC	$3 \times 10^{-2}$	Increase	Anderson et al. 2004
<i>Hyalella azteca</i> (Scud)	Sexual Development of Gnathopod (modified jaw)	28 days		$1 \times 10^1$	Decrease	Vandenbergh et al. 2003
<i>Hyalella azteca</i> (Scud)	Length of Antenna	28 day	NOEC	$1 \times 10^1$	Increase	Vandenbergh et al. 2003
<i>Anguilla Anguilla</i> (Common Eel)	Weight	9 day	NOEL	$1 \times 10^1$	Decrease	Versonnen et al. 2004
<i>Anguilla Anguilla</i> (Common Eel)	Weight	15 day	NOEL	1 nM	Decrease	Versonnen et al. 2004
<i>Acipenser flvescens</i> (Lake Sturgeon)	Weight of Gonads		25 days	$6 \times 10^{-2}$ – 1	Increase	Palace et al. 2001
<i>Acipenser flvescens</i> (Lake Sturgeon)	Weight of Liver		25 days	$1 \times 10^{-2}$ – $6 \times 10^{-2}$	Increase	Palace et al. 2001
<i>Carassius auratus</i> (Goldfish)	Weight	15 days	NOEL	1 nM	Decrease	Martyniuk et al. 2006
<i>Danio rerio</i> (Zebrafish)	Length and Weight	58 days	LOEC	$1 \times 10^{-2}$	Decrease	Lin and Janz 2006
<i>Danio rerio</i> (Zebrafish)	Weight	40 days	NOEC	$3 \times 10^{-2}$	Decrease	Andersen et al. 2004
	Length		LOEC	$3 \times 10^{-2}$	Decrease	



(Table 9 continued)

Organism	Measured Response	Exposure time	End point	Dose (ppb)	Trend	Reference
<i>Danio rerio</i> (Zebrafish)	Weight	30 days		$1 \times 10^{-3}$ – $8 \times 10^{-3}$	Increase	Bogers et al. 2006
<i>Danio rerio</i> (Zebrafish)	Length	58 days	NOEC	$1 \times 10^{-2}$	Increase	Lin and Janz 2006
	Weight		NOEC	$1 \times 10^{-2}$	Decrease	
<i>Danio rerio</i> (Zebrafish)	Developmental stage of Progeny	58 days	LOEC	$1 \times 10^{-2}$	Decrease	Hill and Janz 2003
<i>Dicentrarchus labrax</i> (Sea Bass)	Length	200 days	LOEC	$1 \times 10^4$	Decrease	Blazquez et al. 1998
<i>Ictalurus punctatus</i> (Channel Catfish)	Weight of Liver	7 days		$7 \times 10^2$	Increase	Nimrod and Benson 1996
<i>Oncorhynchus mykiss</i> (Rainbow Trout)	Weight of Gonads	7 days		$1 \times 10^3$ – $5 \times 10^3$	Increase	Verslycke et al. 2002
<i>Oncorhynchus mykiss</i> (Rainbow Trout)	Weight of Liver	7 days		$1 \times 10^4$ and $3 \times 10^4$	Increase	Verslycke et al. 2002
<i>Oncorhynchus tshawytscha</i> (Chinook Salmon)	Length	184 days post hatch	NOEC	$4 \times 10^2$	Increase	Piferrer and Donaldson 1992
<i>Oryzias latipes</i> (Medaka)	Length and Weight	105 days		5 µg/ organism	Decrease	Papoulias et al. 2001
<i>Pimephales promelas</i> (Fathead Minnow)	Length and Weight	37 days		$1 \times 10^{-2}$	Decrease	Lange et al. 2001
				1		
<i>Pimephales promelas</i> (Fathead Minnow)	Length	56 days post hatch	LOEC	$3 \times 10^{-3}$	Decrease	Lange et al. 2001
<i>Pimephales promelas</i> (Fathead Minnow)	Length	301 days post hatch	NOEC and LOEC	$> 8 \times 10^{-4}$	Decrease	Lange et al. 2001
<i>Pomatoschistus minutus</i> (Sand Goby)	Length and Weight	0-213.08 days		$6 \times 10^{-3}$	Decrease	Robinson et al 2003
<i>Salvelinus namaycush</i> (Lake Trout)	Length	21 days	NOEC	$4 \times 10^3$	Increase	Werner et al. 2003
			LOEC	$2 \times 10^{-2}$ – 4		

**Fluoxetine affects growth and development.** Aquatic organisms exposed to FLX exhibited changes in normal growth and development (Table 10). *Chironomus riparius* did not exhibit any significant effects on growth for concentrations up to 6000 ppb in the sediment (Nentwig 2006). The median effective concentration (MICs), the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation, were established for the following: *S. capricornutum* (green algae), *Chaetomium globosum* (fungus), *Aspergillus flavus* (mold), *Pseudomonas acidovorans* (soil bacteria), *Azotobacter chroococcum* (N-fixing bacteria), and *Nostoc sp.* (blue-green algae) (Eli Lilly and Company 2005). The growth of *Hyalella azteca* (Scud) produced an NOEC of 30 ppb and an LOEC of 100 ppb (Péry et al. 2008). Brooks et al. (2003) found an LOEC of 6000 ppb.

Although F1 generation *Daphnia magna* (water flea) showed much more pronounced effects in the sense that they were longer length, they still had the same NOEC and LOEC as the F0 generation. A slight decrease in length was observed at 3 ppb. At this concentration the organisms' length was between that of the NOEC and LOEC; however, this concentration was not significant because the length of the organisms in the NOEC (10 ppb) was close to those in the control (Péry et al. 2008).

Based on growth, *Chironomus tentans* (midge) had an LOEC of 1000 ppb (Brooks et al. 2003). *Pimephales promelas* (fathead minnow) exposed to FLX exhibited decreased growth and feeding rates (Stanley et al. 2007).

Female *Gambusia affinis* (western mosquitofish) were significantly longer and larger (wet weight) than males at the end on the 91 day exposure period. The number of females remained 49 – 54% in each treatment and the control. Few fish had elongated anal fins; however, neither control nor exposed fish developed a mature gonopodium with completely formed hooks. Sexual maturity was delayed in female and males from the juvenile stage (59

days) to the adult stage (159 days) as indicated by the development of a black spot (female) and elongated anal fin (males). In this experiment the number of females in control and all treatments was 54 -57%. At 70 ppb, the number of organisms developing a black spot on the posterior portion of the abdomen was significantly lower as well as the number growing an elongated anal fin (Henry and Black 2008).

Table 10. Exposure times and concentrations of fluoxetine reported to provoke the endpoints associated with growth and development of aquatic organisms.

Organism	Measured Response	End point	Dose (ppb)	Trend	Exposure time	Reference
<i>Aspergillus flavus</i> (Mold)	Growth Rate	MIC	$6 \times 10^4$			Eli Lilly and Company 2005
<i>Azotobacter chroococcum</i> (N-fixing Bacteria)	Growth Rate	MIC	$6 \times 10^4$			Eli Lilly and Company 2005
<i>Chaetomium globosum</i> (Fungus)	Growth Rate	MIC	$6 \times 10^4$			Eli Lilly and Company 2005
<i>Pseudomonas acidovorans</i> (Soil Bacteria)	Growth Rate	MIC	$1 \times 10^6$			Eli Lilly and Company 2005
<i>Nostoc sp.</i> (Blue-Green Algae)	Growth Rate	MIC	$3 \times 10^5$			Eli Lilly and Company 2005
<i>S. capricornutum</i> (Green Algae)	Growth Rate	MIC	$3 \times 10^1$			Eli Lilly and Company 2005
<i>Chironomus riparius</i>	Growth	No effect	$6 \times 10^3$			Nentwig 2006
<i>Chironomus tentans</i> (Midge)	Growth	LOEC	$1 \times 10^3$			Brooks et al. 2003
<i>Hyaella azteca</i> (Scud)	Growth	NOEC	$3 \times 10^1$			Péry et al. 2008
		LOEC	$1 \times 10^2$			
		LOEC	$6 \times 10^3$			Brooks et al. 2003
<i>Daphnia magna</i> (Water Flea) (F0 and F1 generation)	Length	NOEC	$1 \times 10^1$	F1 generation longer length	21 days	Péry et al. 2008
		LOEC	$3 \times 10^1$			
<i>Gambusia affinis</i> (Western Mosquitofish)	Growth, length and wet weight		$5 \times 10^{-2}$ $5 \times 10^{-1}$ 5	Females significantly longer and larger	91 days	Henry and Black 2008

(Table 10 continued)

Organism	Measured Response	End point	Dose (ppb)	Trend	Exposure time	Reference
<i>Gambusia affinis</i> (Western Mosquitofish)	Black spot on the posterior portion of the abdomen (females)		70	Decrease	100 days	Henry and Black 2008
	Number growing an elongated anal fin (males)		70	Decrease	49-59 days	
		No response	0, 7, 70	Not different from control	73-100 days	
<i>Pimephales promelas</i> (Fathead Minnow)	Growth R-FLX	NOEC	$1 \times 10^2$	No effect	7 days	Stanley et al. 2007
		LOEC	$2 \times 10^2$			
<i>Pimephales promelas</i> (Fathead Minnow)	Growth Rac-FLX	NOEC	$1 \times 10^1$	Decrease	7 days	Stanley et al. 2007
		LOEC	$5 \times 10^1$			
<i>Pimephales promelas</i> (Fathead Minnow)	Growth S-FLX	NOEC	$1 \times 10^1$	Decrease	7 days	Stanley et al. 2007
		LOEC	$5 \times 10^1$			
<i>Pimephales promelas</i> (Fathead Minnow)	Feeding Rates R-FLX	NOEC	$1 \times 10^2$	Decrease	7 days	Stanley et al. 2007
		LOEC	$2 \times 10^2$			
<i>Pimephales promelas</i> (Fathead Minnow)	Feeding Rates Rac-FLX	NOEC	$5 \times 10^1$	Decrease	7 days	Stanley et al. 2007
		LOEC	$1 \times 10^2$			
<i>Pimephales promelas</i> (Fathead Minnow)	Feeding Rates S-Flx	NOEC	$1 \times 10^1$	Decrease	7 days	Stanley et al. 2007
		LOEC	$5 \times 10^1$			

### Impacts on Nontarget Organisms

The U.S. Geological Survey has established a baseline for pharmaceuticals and personal care products as “emerging pollutants” in the United States (Kolpin et al. 2002). The baseline states that these contaminants have the potential to affect the health and welfare of aquatic ecosystems and humans (Khetan et al. 2007). Pharmaceuticals with modes of action depending upon signal transduction, cell division, and key metabolizing enzymes can be hazardous to

nontarget organisms that possess these necessary human features to carry out the drugs function (Nelson et al. 1996). If nontarget organisms lack the necessary attributes for the specified mode of action, disparate modes of action will transpire resulting in unanticipated effects (Fent et al. 2006).

Biochemical reactions that have been identified in humans may not justify irregularities observed in animals. Because of the limitations in understanding biochemical signaling pathways, drugs also act by other routes and on nontargeted tissues and organisms. Even if the pharmaceutical were capable of reacting with a single type of receptor, the tissue distribution of the receptor may not be fully understood. With this in mind, potential actions and biochemical effects on nontarget aquatic ecosystems are not entirely realized or identified (Daughton and Ternes 1999). Although these chemicals are present in extremely low concentrations, this does not belittle their potential to cause harm to the environment. Pharmaceuticals are designed to produce a response at low levels (Halford 2008). Minute amounts of chemicals, typically considered safe because they occur at trace amounts, can elicit unanticipated effects on ecosystems as evident by xenobiotics such as DDT and PCBs which have hormone mimicking effects on the reproductive systems of organisms at nanograms/L (Colbum and Clement 1992).

### **Ethinyl Estradiol Performance in Animals**

The binding affinity for the estrogen receptor in fish is correlated with the relative potency of the sex steroid (Papoulias et al. 1999). Research led by John Sumpter, an ecotoxicologist at England's Brunel University, linked the presence of the synthetic birth control compound 17 $\alpha$ -ethinylestradiol to the feminizing phenomenon (Desbrow et al. 1998). In the 1990s reports from the United Kingdom and the United States fish living downstream from wastewater treatment plants exhibited several reproductive abnormalities including but not limited to changes in the levels of sex steroids, hermaphroditism, and increased levels of

vitellogenin (the female egg yolk precursor) in male fish (Beven et al. 1996; Folmer et al. 1996; Harries et al. 1996; Purdom et al. 1994; and Jobbing et al. 1998). These abnormalities are deemed feminization because fish that are genetically male exhibit female sex characteristics (Papoulias et al. 1999).

*Pimephales promelas* (fathead minnow) embryos 48-60 hours post fertilization exposed 106 – 150 days post hatch developed secondary sex characteristics (Parrott and Blunt 2005). After injections of EE2 in *Oryzias latipes* (medaka) embryos less than or equal to 8 hpf exposed for 100 dph began to exhibit abnormal morphology of the gonads after injection. The XY males displayed phenotypic female characteristics; ovaries actively produced maturing oocytes in the postvitellogenic phase and papillary processes on the anal fin were void (Hano et al 2005).

Precocious sexual development of genital papillae was observed in juvenile *Etheostoma caeruleum* (rainbow darter) 120 days post hatch and progressively increasing. Also reported with the same endpoints was an increase in imposex/intersex conditions (Elias et al. 2007).

Mature *Oryzias latipes* (medaka) about 6 months post hatch manifested an increase in morphology of intersex gonads based on testis-ova (Seki et al. 2002). *Dicentrarchus labrax* (sea bass) exposed to 10000 ppb through diet displayed smaller size gonads than control and instances of intersex and sterile gonads (Blazquez et al. 1998). *Margariscus margarita* (pearl dace) had a reduction of the developmental stage of ovaries with reduction of vitellogenic oocytes (Palace et al. 2006).

Lai et al. (2002) found that  $6 \times 10^{-3}$  ppb of EE2 produced 100% female *Danio rerio*. *Tilapia aurea* (tilapia) fed 50,000 ppb EE2 for 19 days yielded 100% sex reversal (Nakamura and Takahashi 1973; Papoulias et al. 1999). The estimated dosage to produce 50% XY female *Oryzias latipes* (medaka) is 2000 ppb EE2, 6000 ppb estradiol (E2), and 20000 mg/g estrone (Yamamoto 1975) in (Papoulias et al. 1999). Primary germ cell number and gonad size of

medaka signify that sex reversal has occurred by about 6 days post hatch (Papoulias et al. 1999). Sex reversal demonstrates the plasticity and vulnerability of gonads (Papoulias et al. 1999).

A decrease in the morphology of testes was observed in *Oncorhynchus mykiss* (rainbow trout) after 62 days (Schultz et al. 2003). Testes size diminished in relationship to the body of *Oncorhynchus mykiss* (rainbow trout) (Jobling et al. 1996) and in *Danio rerio* (zebrafish) greater than 240 days post hatch (Lin and Janz 2006).

Decrease in renal somatic index, GSI (gonadosomatic indices: gonad weight/body weight x 100), and HSI (Hepatosomatic indices liver weight) were indicated in sexually mature *Gobiocypris rarus* (Chinese rare minnow) 7 months old. Males were generally more sensitive than females (Zha et al. 2007).

Fewer motile sperm in adult *Danio rerio* (zebrafish) after 21 days (Santos et al. 2007) with an NOEL based on sperm motility of  $5 \times 10^{-4}$  and an LOEL of  $5 \times 10^{-3}$ . A decreased sperm count was observed giving an LOEL of 2 ppb in juvenile *Poecilia reticulata* (guppy) less than or equal to 7 days old; however, a significant increase of the sperm count was observed at  $1 \times 10^{-2}$  ppb (Nielsen and Baatrup 2006). A reduction in number of normal development of tubercles (which are features of males) after 21 days in mature *Pimephales promelas* (fathead minnow) 6-11 months old instituted an NOEC of  $1 \times 10^{-4}$  ppb and an LOEC of  $7 \times 10^{-4}$  ppb (Pawlowski et al. 2004). Tubercles of adult male *Pimephales promelas* (fathead minnow) developed abnormally after exposure to EE2 for 21 days. A decrease in the number of normal developed tubercles occurred at  $5 \times 10^{-4}$  ppb and  $1 \times 10^{-3} - 2 \times 10^{-3}$  ppb. The lesser concentrations caused a higher instance of prominent tubercles. The higher concentrations caused a decrease in the normal shape/prominence of tubercles. Decrease in normal shape/prominence of tubercles NOEC  $5 \times 10^{-4}$  ppb; increase in normal shape/prominence of tubercles/size NOEL  $5 \times 10^{-4}$  ppb (Brian et al. 2007). An NOEL based on the sexual development of the mean number of tubercles

in *Pimephales promelas* (fathead minnow) adults (Bogers et al. 2007). These studies reaffirm what Kidd et al. (2007) conveyed; even at low concentrations, if estrogen is present at a measurable concentration feminization has the potential to occur.

Table 11. Exposure times and concentrations of ethinyl estradiol reported to provoke the endpoints associated with abnormalities of aquatic organisms.

Organism	Measured Response	End point	Dose (ppb)	Trend	Exposure time	Reference
<i>Danio rerio</i> (Zebrafish)	Sex Reversal		$6 \times 10^{-3}$	100% females		Lai et al. 2002
<i>Danio rerio</i> (Zebrafish)	Testes Size		$1 \times 10^{-2}$	Decrease	>240 dph	Lin and Janz 2006
<i>Danio rerio</i> (Zebrafish)	Sperm motility	NOEL	$5 \times 10^{-4}$	Decrease	21 days	Santos et al. 2007
		LOEL	$5 \times 10^{-3}$			
<i>Dicentrarchus labrax</i> (Sea Bass)	Gonad Size		$1 \times 10^4$	Decrease; sterile gonads	200 days	Blazquez et al. 1998
<i>Etheostoma caeruleum</i> (Rainbow darter)	Sexual Development of Genital Papillae	NOEC	$2 \times 10^{-2}$	No Effect	21 days	Elias et al. 2007
		LOEC	2	Increase		
<i>Gobiocypris rarus</i> (Chinese Rare Minnow)	Size of Kidney	LOEC	$1 \times 10^{-3}$	Decrease	28 days	Zha et al. 2007
<i>Margariscus margarita</i> (Pearl dace)	Developmental Stage of Ovaries	LOEL	$5 \times 10^{-3}$	Decrease	882.76 days	Palace et al. 2006
<i>Oncorhynchus mykiss</i> (Rainbow Trout)	Weight of Testes		$2 \times 10^{-3}$	Decrease	21 days	Jobling et al. 1996
<i>Oryzias latipes</i> (Medaka)	Abnormal Morphology of Gonads	NOEC	$1 \times 10^{-4}$ $\mu\text{g}/\text{egg}$		100 dph	Hano et al. 2005
		LOEC	$5 \times 10^{-4}$ $\mu\text{g}/\text{egg}$			
<i>Oncorhynchus mykiss</i> (Rainbow trout)	Morphology of Testes	LOEC	1	Decrease	62 day	Schultz et al. 2003
<i>Oryzias latipes</i> (Medaka)	Morphology of Intersex Gonads	NOEC	$3 \times 10^{-2}$	Increase	21 days	Seki et al. 2002
		LOEC	$6 \times 10^{-2}$			
<i>Oryzias latipes</i> (Medaka)	Sex Reversal – estimated dose to produce 50% XY females	EE2	$2 \times 10^3$			Yamamoto 1975 in Papoulias et al. 1999
		Estradiol (E2)	$6 \times 10^3$			
		Estrone	20,000 mg/g			



(Table 11 continued)

Organism	Measured Response	End point	Dose (ppb)	Trend	Exposure time	Reference
<i>Oryzias latipes</i> (Medaka)	Sex Reversal – primary germ cell number and gonad size	NOEC	$1 \times 10^{-4}$		6 days post hatch	Papoulias et al. 1999
<i>Pimephales promelas</i> (Fathead Minnow)	Development of Secondary Sex Characteristics	NOEL	$5 \times 10^{-2}$		106 – 150 dph	Parrott and Blunt 2005
<i>Pimephales promelas</i> (Fathead Minnow)	Sexual Development —number of normal developed tubercles	NOEC	$5 \times 10^{-4}$ and $1 \times 10^{-3} - 2 \times 10^{-3}$	Decrease	21 days	Pawlowski et al. 2004
		LOEC	$7 \times 10^{-4}$			
<i>Pimephales promelas</i> (Fathead Minnow)	Number of normal developed tubercles	NOEC	$1 \times 10^{-3} - 2 \times 10^{-3}$	Decrease	21 days	Brian et al. 2007
	Normal shape/prominence of tubercles	NOEC	$5 \times 10^{-4}$	Decrease		
	Normal shape/prominence of tubercles/size	NOEL	$5 \times 10^{-3}$	Increase		
<i>Pimephales promelas</i> (Fathead Minnow)	Sexual Development – Mean number of tubercles	NOEL	$3 \times 10^{-4}$		28 days	Bogers et al. 2007
		LOEL	$1 \times 10^{-3}$			
<i>Poecilia reticulata</i> (Guppy)	Sperm count	LOEL	2	Decrease	108 days	Nielsen and Baatrup 2006
			$1 \times 10^{-2}$	Significant Increase		
<i>Tilapia aurea</i> (Tilapia)	Sex reversal		$5 \times 10^4$	100% sex reversal	19 days	Nakamura and Takahashi 1973; Papoulias et al. 1999

### Fluoxetine Performance in Animals

It is plausible to assume that SSRIs could produce similar pharmacological responses in fish as those seen in humans and other mammals (Kreke and Dietrich 2008). However, due to interactions with other neurotransmitters and hormones along with the complexity of the serotonergic system, “the downstream effects of SSRI action on known pharmacological target

molecules in fish may differ considerably” (Kreke and Dietrich 2008). Selective Serotonin Reuptake Inhibitors may produce effects in fish not seen in humans due to phylogenetic differences in transduction pathways (i.e. those not present or differently regulated) (Kreke and Dietrich 2008). A broad range of effects and the ability for bodily chemicals to neutralize and return to normal after increases of serotonin suggests that a traditional dose-response relationship may not be expected with chronic exposure (Brooks et al. 2003a).

Fluoxetine may adversely influence the function of the nervous system and associated hormonal systems of organisms possessing serotonin. In addition to operating as a neurotransmitter, serotonin functions as a hormone directly impacts the immune system, alters appetite, modulates sexual function, and influences the establishment of social hierarchies through disruption in sexual behavior and aggressive interactions (Barton et al. 2002; Fent et al. 2006).

The pineal hormone melatonin is a derivative of serotonin and is thought to modulate aggressive behavior and may be an explanation for submissive behavior of organisms exposed to SSRIs (Lee et al. 2004). A study on *Oncorhynchus mykiss* (rainbow trout) using melatonin, tryptophan, and the SSRI citalopram, concluded that it is the 5-HT that is the antiaggressive agent in fish. The melatonin group had no significant effect while the other two groups decreased aggression and lowered plasma controls (Metcalf et al. 2001). Derivatives of serotonin can affect sensory perception (i.e. visual perception) thus disrupting the circadian system which regulates organisms’ measurement and interpretation of day duration. Animals that rely on photoperiodic signals can be affected by this. Slight changes in extracellular 5-HT may alter behavior output by modulating other signaling cascades and could result in abnormalities in circadian rhythmic activities including but not limited to sleep, feeding patterns, hunting,

reproductive behavior, core body temperature, brain wave activity, cell regeneration, and hormone production (Kreke and Dietrich 2008).

After four week exposure levels of 100 and 500 ppb FLX, female circulating steroid concentrations (E2) increased significantly in Japanese medaka (Weston et al. 2003). The modified circulating steroids confirms unpredicted responses including but not limited to sexual dimorphisms in P450 activity and potential sexual dimorphisms in serotonin systems (Hernandez-Rauda et al. 1999). In some bivalves and crustaceans induced spawning (Fong 2001). The induction of mussel spawning points to interference in serotonergic action. In invertebrates, serotonin may stimulate ecdysteroids, ecdysone and juvenile hormone, responsible for controlling oogenesis and vitellogenesis (Nation, 2002). Serotonin stimulates sex steroid syntheses and controls the development of oogenesis, including vitellogenesis in fish species (Arcand-Hoy and Benson 2001). Serotonin was indicated to enhance the effects of gonadotropin-releasing hormone on gonadotropin release from the pituitary gland (Khan and Thomas 1994).

## **Problem**

It has been apparent that these pharmaceuticals not only illicit lethality but also abnormal behaviors that have a number of possible explanations or in some cases cannot be explained. Nontarget organisms can experience similar effects shown in mammalian toxicity tests if they possess the necessary target receptors and/or biomolecules present in humans and mammals (Fent et al. 2006). Aside from the major mode of action which in most instances are fully identified; however because pharmaceuticals have multiple secondary modes of actions, nontarget tissues and organs can become disrupted, thus complicating the approach to quantify possible end points.

Despite receiving attention and necessary action by regulatory agencies like FDA and the European Union, there is a lack for substantial procedures regarding impending monitoring of drug concentrations in the environment and the palpable adverse effects (Rahman et al. 2007).

Published literature is minute when you take into consideration the large amount of APIs, metabolites, and other transformation products developed and pumped into the environment, sublethal/lethal effects that may have not occurred, the number of potential non-target organisms (Daughton et al. 1999).

When compared to other man-made pollutants (pesticides, household and personal care products), pharmaceuticals represent a minute portion of environmental pollutants. Aquatic organisms are exposed to pharmaceuticals through the aforementioned pathways for their entire life-cycle thus making them important targets. However, little is known about effects on these organisms. Organisms that are affected on subtoxic levels effects may result in long-term changes that may become manifested in succeeding generations of a given species in an ecosystem (Halford 2008). Acute data alone may not be suitable for specifically addressing the question of environmental effects and subsequently in the hazard and risk assessment (Fent 2003). A major concern is those effects which may be barely noticeable but accumulate over time to yield extreme changes which would be masked by time and perhaps not distinguishable from natural events. Even faint variations in routine life development have the capability to directly or indirectly affect entire ecosystems through chain reactions originating from disturbances in a single population or species. Upsetting the environmental balance can result in unforeseen catastrophes through deteriorations of entire ecosystems.

## MATERIALS AND METHODS

Several single species studies using the EPA Acute Static Renewal Test (EPA-821-R-02-012) (USEPA 2002) were conducted. Test organisms were exposed to six concentrations of Ethinyl estradiol and fluoxetine for 96 hours and a control with four replications each. A total of 35 beakers were used for each single species test. Twenty four beakers housed exposed organisms while four beakers housed controlled organisms. Seven beakers containing the fifth replicate of concentrations were void of organisms and used for daily monitoring of water parameters. Daily monitoring of organisms were documented daily to assess time framed dose-responses for each test organism at each concentration. The highest test concentrations were chosen into order to induce at least 50% lethality (LC50) based on reported studies of similarly classified organisms. After test termination, mortality curves were analyzed to quantify 96 hour LC50s, NOECs, LOECs, and Ch.V.s for each of the five standard EPA test organisms exposed to ethinyl estradiol and fluoxetine. *Ceriodaphnia dubia* (water flea), *Daphnia pulex* (water flea), and *Pimephales promelas* (fathead minnow), which are freshwater species, and *Mysidopsis bahia* (mysid shrimp) and *Menidia beryllina* (inland silverside) which are marine species were chosen because currently there are no acute toxicity tests incorporating EE2 and FLX induced effects on these standard EPA organisms. The test organisms include simple and complex aquatic species both of freshwater and marine habitats, thus encompassing a wide spectrum of test organisms.

In addition to assessing, comparing, and contrasting mortality, this study extended beyond lethality through the observation of sublethal effects which are not required for acute test methods. Abnormalities observed during the test were documented and concentration boundaries were established for sublethal effects.

## Methodology

Ethinyl estradiol (USP grade) and fluoxetine HCl (98% purity), both obtained from AK Scientific, Inc. (Mountain View, California), were used in preparing 6 concentrations with 5 replicates (1 replicate void of organisms for water quality testing). Organisms were kept in a controlled laboratory environment for 96 hours amid an 18/6 hour light/dark period with a light intensity of 50-100 ft/c (REED LM-81LX Light Meter).

The immature/juvenile organisms were obtained from CK Associates Aquatic Toxicology Laboratories (Baton Rouge, Louisiana) on the morning of test initiation and housed in the controlled laboratory space for approximately three hours to acclimate to room temperature which remained constant at  $25 \pm 1^\circ\text{C}$ . *Ceriodaphnia dubia*, *Daphnia pulex*, *Pimephales promelas*, *Mysidopsis bahia*, and *Menidia beryllina* were housed in borosilicate beakers arranged in a randomized block order and covered with sleeves of Plexiglas® to prevent external contamination.

Table 12. Age and number of test organisms deposited into the elected beaker size containing the corresponding amount of dosing solution.

Test Organism	Age	Number of Organisms in Container	Beaker Size (mL)	Amount of Solution in Beaker (mL)
<i>Ceriodaphnia dubia</i>	< 24 hours old	5	30	15
<i>Daphnia pulex</i>	< 24 hours old	5	30	20
<i>Pimephales promelas</i>	1 day old	10	400	250
<i>Mysidopsis bahia</i>	1 day old	10	400	250
<i>Menidia beryllina</i>	13 days old	10	400	250

## Glassware

Borosilicate glassware was used to prevent any chemicals from changing composition or possible leaching into pores of containers which could subsequently affect test results. Prior to test initiation, the glassware was soaked overnight in a bath of NOCHROMIX® and sulfuric

acid. NOCHROMIX® is a patented, white crystalline, inorganic oxidizer packaged in pre-measured, hermetically sealed pouch. When mixed with sulfuric acid, it forms a clear, strong cleaning solution. The glassware was then transferred to a deionized (DI) water bath and rinsed with DI water to dilute and wash away residual material. After rinsing with laboratory grade acetone, the glassware was rinsed again with DI water. They were let to air dry on clean white contact paper.

### **Water Quality**

Forty liters of aerated DI water was used to prepare stock solutions, concentrations, and controls. Because *Mysidopsis bahia* and *Menidia beryllina* are marine organisms, salinity had to be added to the DI water used for these organisms. For each 1 liter of DI water, 15 grams of Instant Ocean® was added.

Daily monitoring of water parameters was performed in the fifth replicate of the control (aerated DI water) and six concentrations, all void of organisms. Alkalinity (as CaCO<sub>3</sub> in ppm) and Hardness (ppm) were approximated colormetrically using Hach AquaCheck Dip-and-Read Water Quality Test Strips. Temperature was taken by means of Fisher Scientific Traceable Digital Thermometer with stainless steel probe in °C with. A certificate from ISO 17025 calibration laboratory was included and accredited by A2LA to confirm accuracy. The pH meter used was ORION Research 230A Digital, Portal pH probe. Dissolved oxygen was measured with OAKTON® DO 300 Waterproof Data Meter.

### **Dosing**

Stock solutions were prepared at test initiation and 48 hour test renewal by mixing the measured amounts of chemicals with aerated deionized water. Chemicals were left in refrigerator and left to sit on table top for about 1 hour to remove any moisture before weighing.

The scale was accurate to 0.000 mg. The concentrations were prepared by diluting the stock solution by 6.5, 12.5, 25, 50, and 100% with room temperature aerated DI water.

Table 13. Test concentrations of ethinyl estradiol and fluoxetine after appropriate dilutions were made to the measured amount used in the stock solution.

Test Organism	Drug	Concentration in µg/L (parts per billion)						Amount measured for stock solution (mg)
		1	2	3	4	5	Stock 6	
<i>Ceriodaphnia dubia</i>	EE2	200	400	900	2000	4000	7000	7.010
	FLX	7	20	30	60	100	200	0.936
<i>Daphnia Pulex</i>	EE2	300	600	1000	3000	5000	10000	10.361
	FLX	60	100	200	500	900	1000	1.404
<i>Pimephales promelas</i>	EE2	100	200	400	800	2000	3000	3.202
	FLX	10	20	40	90	200	400	0.353
<i>Mysidopsis Bahia</i>	EE2	80	200	300	600	1000	2000	2.398
	FLX	20	50	100	200	400	800	0.760
<i>Menidia Beryllina</i>	EE2	100	200	400	800	2000	3000	3.201
	FLX	50	100	200	400	900	2000	1.716

### Transferring organisms

**Test initiation.** Using 10 mL borosilicate large tip opening serological pipettes, organisms were randomly distributed 1 at a time to differing concentrations and replicates.

**Test renewal.** *Ceriodaphnia dubia* and *Daphnia pulex* were transferred into clean 30 mL beakers with the corresponding amount and concentration by using the wide tip pipette. The test solution of *Pimephales promelas*, *Mysidopsis bahia*, and *Menidia beryllina* was removed with serological pipettes until about 5 mL remained. The beakers were then refilled with fresh solutions to bring the volume back to 250 mL.

### Feeding

Test organisms were fed daily with feedings 2 hours before test initiation and 2 hours before renewal. The required foods were also obtained from CK Associates which included a CEROPHYLL®, a mixture of green unicellular algae and yeast, and Trout Chow for water fleas



and *Artemia nauplii* less than 24 hours old for other organisms. Before feeding regimes, *Artemia nauplii* were rinsed with DI water to remove the saline solution. Excess *Artemia* was removed before feeding the test organisms the next day. The excess food was gathered by pipetting the clumps of remaining food while leaving as much solution as possible.

Table 14. Amount of standard food given to test organisms. Organisms were fed at least two hours before test initiation and twice a day on subsequent days until test termination.

Test Organism	Food	Amount of Food
<i>Ceriodaphnia dubia</i>	CEROPHYLL® and Trout Chow	0.1 mL each
<i>Daphnia pulex</i>	CEROPHYLL® and Trout Chow	0.1 mL each
<i>Pimephales promelas</i>	<i>Artemia nauplii</i>	0.2 mL
<i>Mysidopsis bahia</i>	<i>Artemia nauplii</i>	0.2 mL (100 Mysid per organism)
<i>Menidia beryllina</i>	<i>Artemia nauplii</i>	0.2 mL

### Endpoints

Observations were made daily beginning with control organisms. The entire beaker was carefully moved to a light table and all observations, lethal and sublethal, were recorded at the time of observation.

**Lethal.** Deceased organisms were usually present at the bottom of the beaker and were removed with large tip opening pipettes. Organisms floating listlessly were gently probed with large tip opening pipettes to confirm survival. The number of surviving organisms were recorded daily in each container and averaged within concentrations at test termination. To ensure that toxicity of the drug not cannibalism was causing mortalities, the number of dead organisms removed daily was also recorded.

**Sublethal.** During observations, any recorded abnormalities were compared to the control and surrounding concentrations to confirm irregularities. After test termination changes in behavior and/or abnormalities were summarized, compared, and contrasted throughout concentrations and organisms.

## Statistical Analysis

Daily observations of survival were plotted on Dose Response Plot and analyzed using ToxCalc5. The mortality percentages presented in the successive chapters employ the untransformed data which are based on averages of the four replicates. The average survivorship was transformed to Arcsine Square Roots to establish which concentrations caused survivorship statistically different from the control. The purpose of transforming the data was to set survivorship of the control equal to 100%. Based on Shapiro Wilk's Test with a variance of 0.01 this transformed data was used to establish normal or non-normal distribution. If non-normal distribution of transformed data was found or no equality of variance could be determined, the hypothesis test used was Steel's Many One Rank Test with a 0.05 variance. This hypothesis test determined the no observable effect concentration (NOEC), lowest observable effect concentration (LOEC), and the chronic value (Ch.V.) based on survivorship. The ChV. is the determined from a ratio of the NOEC and LOEC and is presumed "safe." It should be lower than the EC50. Based on the definition of the chronic value the EC50 is usually slightly higher than Ch.V.

To establish the dose-response curve, the Maximum Likelihood-Probit Method was used to determine the Effective Concentration (EC<sub>x</sub>) with upper and lower 95% fiducial limits when applicable. The fiducial limits are the boundaries within which a parameter is considered to be located. In this case the EC represents the effective concentration of the desired percent mortality (x). In a normal dose-response curve, the y-axis is usually the mortality response and the x-axis is the dose of the drug. As the dose increases the effect should increase. Hormetic responses show an opposite effect in small doses compared to large doses. The lower doses stimulate more lethal effects than the higher doses. The result is a J-shaped or an Inverted U-shaped dose response curve.

## RESULTS

### Lethal Effects

Daily observations of survival were documented to assess the time framed dose-responses for each test organism at each concentration. The dose-response curve was used to quantify the effective concentration at which an average of 50% of test organisms encountered lethal effects (EC50 or LC50). The upper and lower 95% fiducial limits were the applicable boundaries within which the ECx was considered to be located. The y-axis of the dose-response curve was the response, the percent mortality, caused by the dose of the drug which is represented in the x-axis. A normal dose-response curve resembles an S-shape; this implies that as the dose or concentration increases, so should the response. The opposite effect in which lethal effects were induced by lower concentrations resulted in a J-shaped or an Inverted U-shaped dose response curve and categorized as a hormetic response.

After test termination, lethal effects to ethinyl estradiol (Table 15) and fluoxetine (Table 16) were documented as percent mortality which incorporates the average untransformed lethality of test organisms caused after 96 hours of exposure. Derived from the transformed data were the statistically significant concentrations, the NOECs, and the LOECs. The chronic value was statistically elected from the NOEC/LOEC ratio. By definition, this concentration is not considered to be harmful thus it should be lower than the EC50.

### Lethal Effects of Ethinyl Estradiol to *Ceriodaphnia dubia*

The normal dose-response of *Ceriodaphnia dubia* exposed to 200 – 7000 ppb of ethinyl estradiol yielded an EC50 of 400 ppb which had 95% fiducial limits of 400 and 300 ppb (Figure 1). Based on the 20 organisms allotted for each of the concentrations, the average mortality was compared to the control (0% mortality). The only concentration not statistically significant when compared to the control was the NOEC, 200 ppb, which only had 20% mortality. Statistically

significant figures included the LOEC, 400 ppb, which had 60% mortality, and 900, 2000, 4000, and 7000 ppb all bared 100% mortality (Table 15). The chronic value, 300 ppb, was only slightly lower than the EC50.

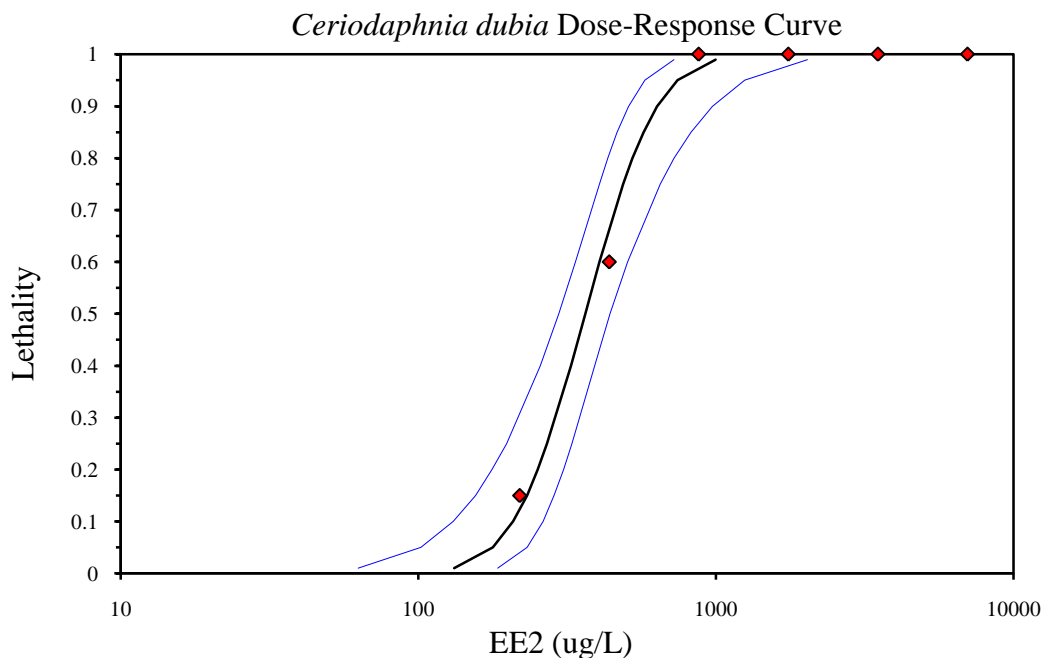


Figure 1. The *Ceriodaphnia dubia* dose-response curve and 95% fiducial limits derived from the average mortality of four replications caused by 96 hours of exposure to 200 – 7000 ppb of Ethinyl estradiol. The EC50 of 400 ppb had 95% fiducial limits of 400 and 300 ppb when compared to 0 because no mortality was observed in the control.

### **Lethal Effects of Ethinyl Estradiol to *Daphnia pulex***

The dose-response of *Daphnia pulex* after 96 hours of exposure to ethinyl estradiol concentrations of 300 -10000 ppb, the EC50, 6000 ppb, with limits of 5000 and 8000 ppb resulted from the control of 0.1 (Figure 2). Due to 10% mortality in the control the statistically insignificant concentrations included the NOEC, 3000 ppb, (20% mortality) as well as the lesser concentrations which exhibited a hormetic curve: 300 (25%), 600 (5%), and 1000 ppb (30%). Significantly different concentrations included the LOEC, 5000 ppb, which caused 40% mortality and 10000 ppb triggered 95% mortality (Table 15). The Ch.V., 4000 ppb, was based on a ratio of the NOEC and the LOEC.

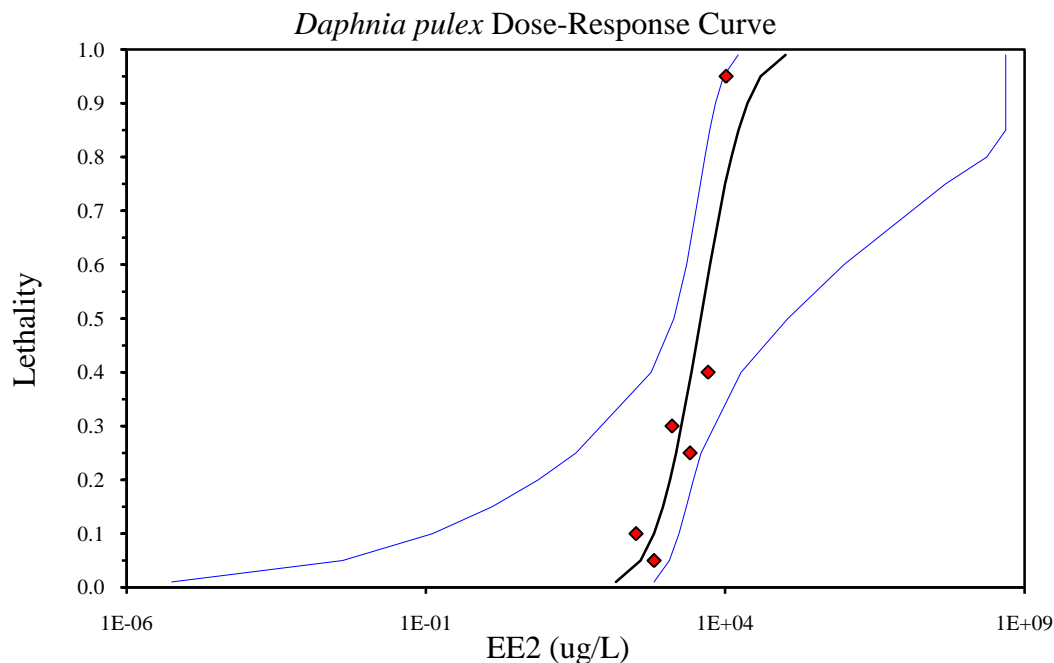


Figure 2. The *Daphnia pulex* dose-response curve and 95% fiducial limits derived from the average mortality of four replications caused by 96 hours of exposure to 300 -10000 ppb of ethinyl estradiol. The EC50 of 6000 ppb had 95% fiducial limits of 5000 and 8000 ppb when compared to 0.1 because 10% mortality was observed in the control.

### **Lethal Effects of Ethinyl Estradiol to *Pimephales promelas***

The normal dose-response of *Pimephales promelas* exposed to 100 –3000 ppb of EE2 resulting in the anticipated EC50 of 11000 ppb. No fiducial limits were determined; however, a significant heterogeneity was detected at  $p = 3.87 \times 10^{-4}$  (Figure 3). The dose response plot exhibited a bell curve with disruption in the plot at the following insignificant values compared to the control (0% mortality): 100 (5%), 200 (3%), 400 (0%), and 800 ppb (3%). The NOEC, 2000 ppb, ended with only 8% mortality. The only significantly different concentration was the LOEC, 3000 ppb, which was highest concentration. Because this level only yielded 40% mortality (Table 15), the resulting Ch.V. was 2000 ppb.

### **Lethal Effects of Ethinyl Estradiol to *Mysidopsis bahia***

The normal dose-response of *Mysidopsis bahia* exposed to 80 – 2000 ppb had an EC50 was 400 ppb with a large range of fiducial limits, 40 - 2000 ppb with control 0.075 (Figure 4).

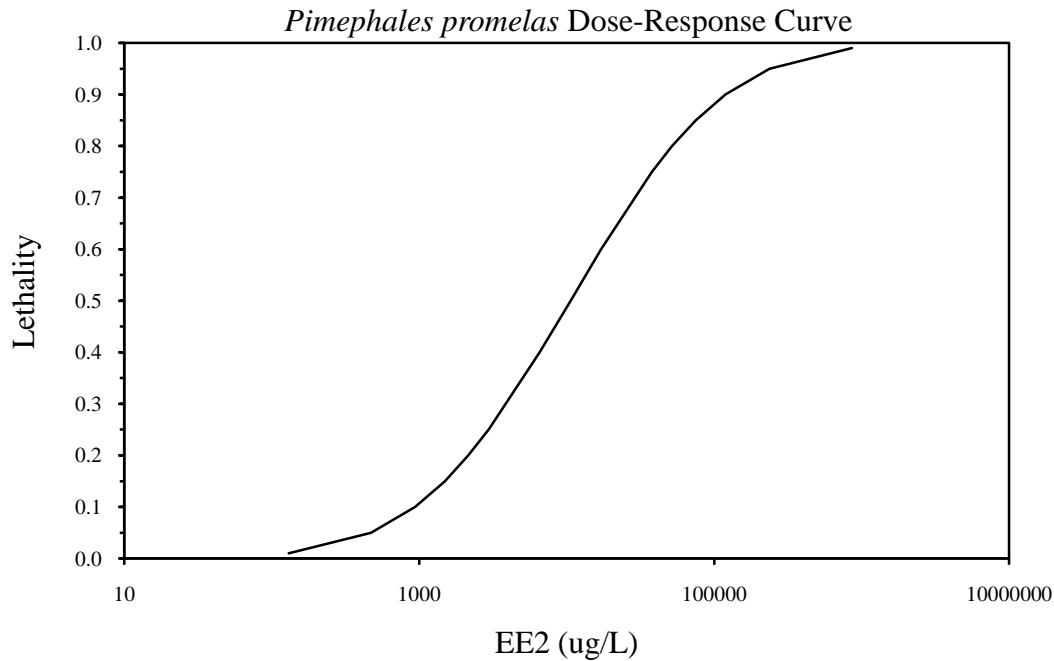


Figure 3. The *Pimephales promelas* dose-response curve derived from the average mortality of four replications caused by 96 hours of exposure to 100 –3000 ppb of ethinyl estradiol. The EC50 of 11000 ppb had no fiducial limits when compared 0 because no mortality was observed in the control. Given that a non-normal response was created a significant heterogeneity was detected at  $p = 3.87 \times 10^{-4}$ .

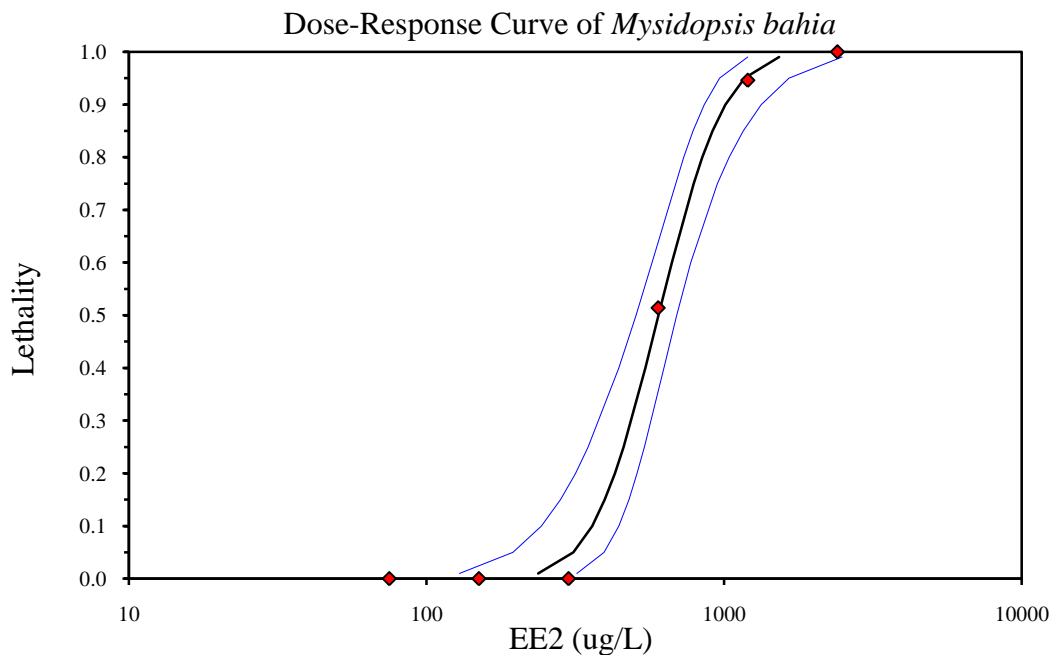


Figure 4. The *Mysidopsis bahia* dose-response curve and 95% fiducial limits derived from the average mortality of four replications caused by 96 hours of exposure to 80 – 2000 ppb of ethinyl estradiol. The EC50 of 400 ppb had 95% fiducial limits of 40 - 2000 ppb when compared to 0.075 because 7.5% mortality was observed in the control.

Mortality remained fairly constant from control – 300 ppb (5-7% mortality); however, organisms exposed to 80 ppb faced less mortality than control. The NOEC, 300 ppb, and 200 ppb experienced identical changes in mortality of about 13%. At the LOEC, 600 ppb, mortality plummeted to about 68% assigning a Ch.V. of 400 ppb. Significantly different concentrations of 1000 and 2000 ppb suffered about 95-100% mortality respectively (Table 15).

### Lethal Effects of Ethinyl Estradiol to *Menidia beryllina*

The normal dose-response of *Menidia beryllina* exposed to 100 – 3000 ppb caused the EC50, 2000 ppb, with fiducial limits of 1000 and 2000 ppb, which was compared to 0.025 due to mortality in the control (Figure 5). A slight hormetic effect was observed in concentrations less than 400 ppb rendering them insignificant. The control and 200 ppb organisms experienced about 2.5% mortality while 100 ppb had 5% and 400 ppb had 0% mortality. Additionally the NOEC, 800 ppb, was also insignificant affecting about 13%.

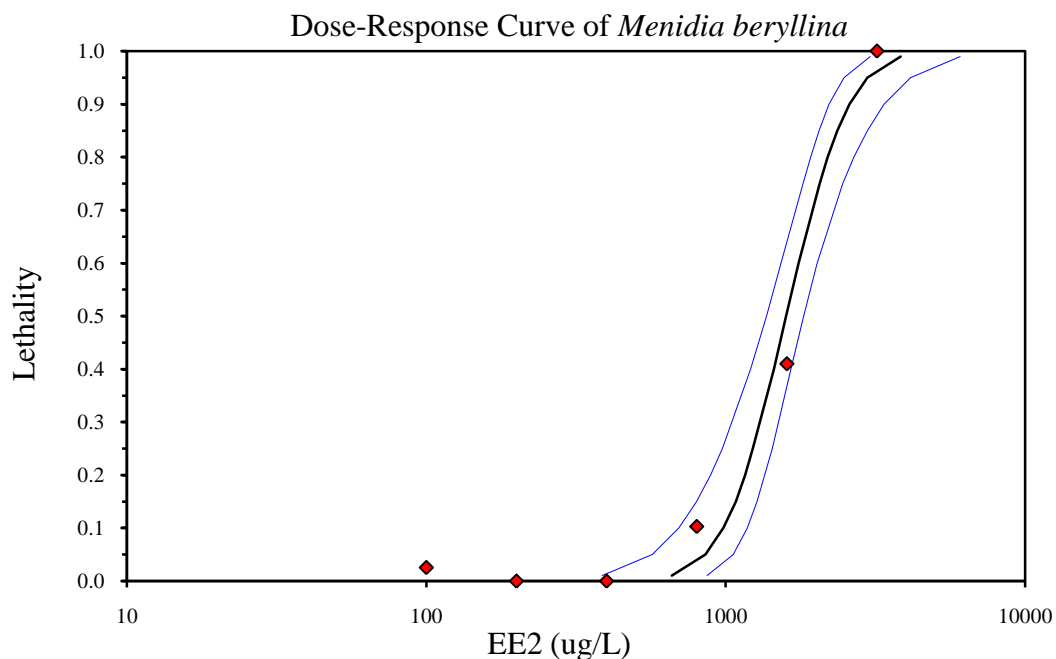


Figure 5. The *Menidia beryllina* dose-response curve and 95% fiducial limits derived from the average mortality of four replications caused by 96 hours of exposure to 100 – 3000 ppb of ethinyl estradiol. The EC50 of 2000 ppb had 95% fiducial limits of 1000 and 2000 ppb, when compared to 0.025 because 2.5% mortality was observed the control.

The higher concentrations including the LOEC, 2000, (about 43%) and 3000 ppb (100%) were significantly different than the control (Table 15). A Ch.V. of 1000 ppb was prompted.

Table 15. Untransformed data presented as the average percent mortality caused after 96 hours of exposure to ethinyl estradiol including the statistically derived concentrations, the NOEC, and the LOEC associated with each single species test.

Ethinyl estradiol Concentration vs. Mortality									
<i>C. dubia</i>		<i>D. pulex</i>		<i>P. promelas</i>		<i>M. bahia</i>		<i>M. beryllina</i>	
Dose (ppb)	Mortality (%)	Dose (ppb)	Mortality (%)	Dose (ppb)	Mortality (%)	Dose (ppb)	Mortality (%)	Dose (ppb)	Mortality (%)
Control 0	0	Control 0	10	Control 0	0	Control 0	6.67	Control 0	2.5
200 NOEC	20	300	25	100	5	80	5.28	100	5
*400 LOEC	60	600	5	200	2.5	2000	5	200	2.5
*900	100	1000	30	400	0	300 NOEC	8.06	400	0
*2000	100	3000 NOEC	20	800	2.5	*600 LOEC	53.17	800 NOEC	12.5
*4000	100	*5000 LOEC	40	2000 NOEC	7.5	*1000	93.75	*2000 LOEC	42.5
*7000	100	*10000	95	*3000 LOEC	40	*2000	100	*3000	100

\*Statistically significant concentrations were derived using TOXCALC 5 by transforming the average survivorship to Arcsine Square Roots. Based on the Shapiro Wilk's Test with a variance of 0.01 this transformed data was used to determine statistically significant concentrations. NOECs and LOECs were established using Steel's Many One Rank Test with a 0.05 variance.

### Lethal Effects of Fluoxetine to *Ceriodaphnia dubia*

The non-normal dose-response of *Ceriodaphnia dubia* exposed to 7 – 200 ppb contained no fiducial limits to the assigned EC50 of 5 ppb, but a significant heterogeneity was detected at  $p = 2.68E-10$ . Control was 0.1 due to mortality in the control (Figure 6). Concentrations not significant from the control (10%) included 7 ppb which had 25% mortality and the NOEC, 100 ppb, (60%). Median concentrations of 20, 30, and 60 ppb triggered 100% mortality at the end of 96 hours and made them significantly different than the control. The highest concentration was deemed the LOEC, 200 ppb, which stimulated 95% mortality. Because of the decrease in



mortality at the NOEC, the LOEC was not the first significant figure. The Ch.V. of 200 ppb was based on this effect (Table 16).

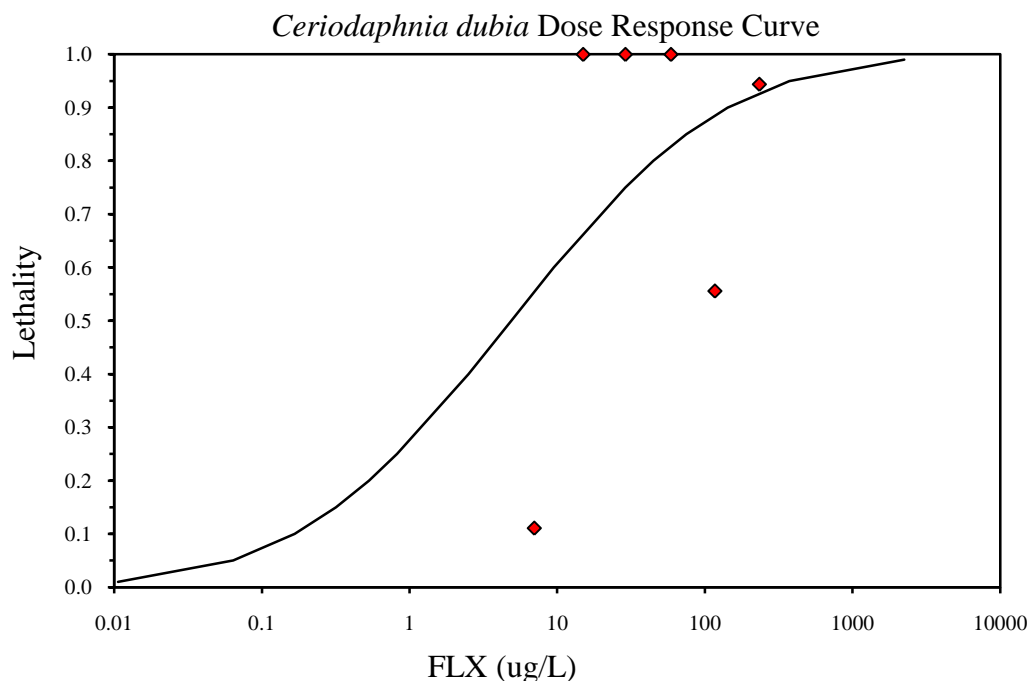


Figure 6. *Ceriodaphnia dubia* dose-response curve derived from the average mortality of four replications caused by 96 hours of exposure to 7 – 200 ppb of fluoxetine. The EC50 of 5 ppb had no 95% fiducial limits when compared to 0.1 because 10% mortality was observed the control. Given that a non-normal response was created a significant heterogeneity was detected at  $p = 2.68 \times 10^{-10}$ .

### Lethal Effects of Fluoxetine to *Daphnia pulex*

The normal dose-response of *Daphnia pulex* exposed to 60 – 1000 ppb The EC50, 200 ppb, with upper and lower 95% fiducial limits of 300 and 100 ppb were compared to a control of 0.1 (Figure 7). The control and 100 ppb retained 10% mortality while the median concentration, 60 ppb, gained 15% mortality. These concentrations along with the NOEC, 200 ppb, (80%) remained insignificant. The LOEC, 500 ppb, which lead to 90% mortality, and 900 and 1000 ppb both with 100% mortality were all significantly different than the control (Table 16). The Ch.V. was determined to be 300 ppb.

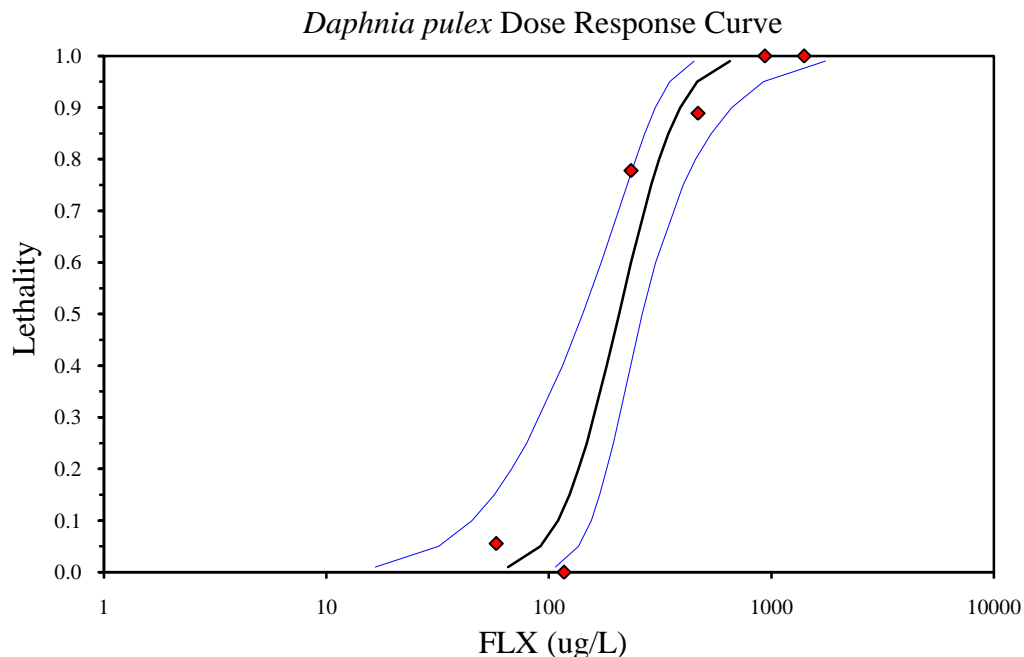


Figure 7. The *Daphnia pulex* dose-response curve and 95% fiducial limits derived from the average mortality of four replications caused by 96 hours of exposure to 60 – 1000 ppb of fluoxetine. The EC50 of 200 ppb had 95% fiducial limits of 300 and 100 ppb when compared to 0.1 because 10% mortality was observed the control.

### Lethal Effects of Fluoxetine to *Pimephales promelas*

The dose-response of *Pimephales promelas* Exposed to 10 – 400 ppb The EC50 was 200 ppb with an upper limit of 200 and a lower limit of 200 ppb (Figure 8). The control together with the lesser concentrations of 10, 20, and 40 ppb, all yielded 0% mortality which scored them as insignificant. The NOEC, 90 ppb, concluded in only 3% mortality. The two concentrations statistically different than the control included the LOEC, 200 ppb, which caused a 40% change and 400 ppb which affected 100% of the organisms (Table 16). The Ch.V. transpired as 100 ppb.

### Lethal Effects of Fluoxetine to *Mysidopsis bahia*

The non-normal dose-response of *Menidia beryllina* exposed to 20 – 800 ppb EC50 was 500 ppb with no limits available established a significant heterogeneity of  $p = 2.96 \times 10^{-3}$  (Figure 9).

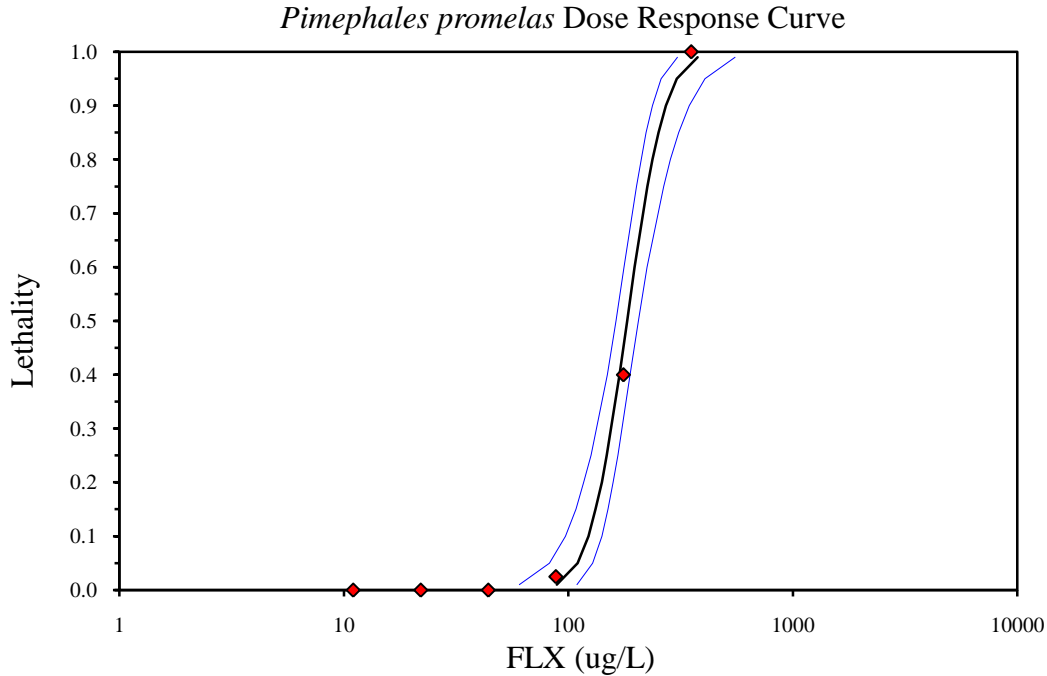


Figure 8. The *Pimephales promelas* dose-response curve and 95% fiducial limits derived from the average mortality of four replications caused by 96 hours of exposure to 10 – 400 ppb of fluoxetine. The EC50 of 200 ppb had 95% fiducial limits of about 200 ppb when compared to 0 because no mortality was observed the control.

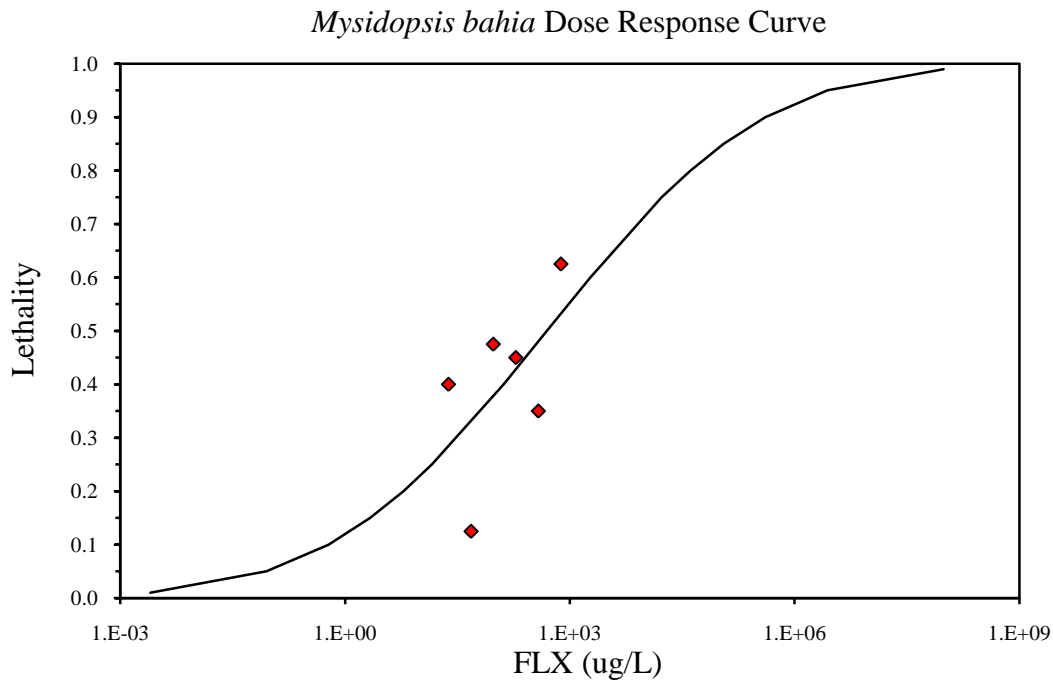


Figure 9. The *Mysidopsis bahia* dose-response curve derived from the average mortality of four replications caused by 96 hours of exposure to 20 – 800 ppb of fluoxetine. The EC50 of 500 ppb had no 95% fiducial limits when compared 0 because no mortality was observed in the control. Given that a non-normal response was created a significant heterogeneity was detected at  $p = 2.96 \times 10^{-3}$ .

There was an immense decrease in mortality at 20 ppb (40%) when compared to the next highest concentration of 50 ppb, the NOEC, (13%). This NOEC was the only concentration not significantly different from control (0% mortality). A hormetic effect occurred between concentrations 100, the LOEC, and 400 ppb. Mortality decreased from 47.5% - 35%. Increased mortality was observed at the next highest concentration, 800 ppb (62.5%). The Ch.V. was 70 ppb because the NOEC exhibited a lower mortality than lesser concentrations (Table 16).

### Lethal Effects of Fluoxetine to *Menidia beryllina*

The dose-response of *Menidia beryllina* exposed to 50 – 2000 ppb EC50, 500 ppb, with upper and lower limits of 500 and 600 ppb. The control for comparison was 0.025 (Figure 10).

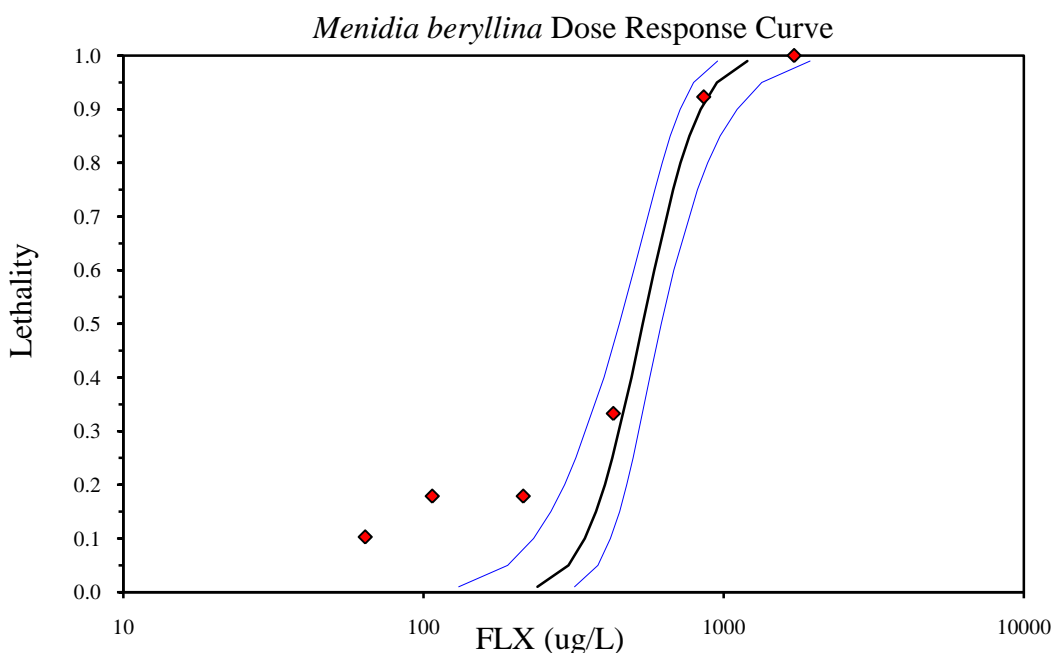


Figure 10. The *Menidia beryllina* dose-response curve and 95% fiducial limits derived from the average mortality of four replications caused by 96 hours of exposure to 50 – 2000 ppb of fluoxetine. The EC50 of 500 ppb had 95% fiducial limits of 500 and 600 ppb when compared to 0.025 because 2.5% mortality was observed the control.

Concentrations not significantly different than the control (about 3%) included 50 ppb (about 13%) and the NOEC, 200 ppb, (20%); however, 100 ppb also had 20% mortality but was considered significant. Remaining concentrations were also regarded as significant. Compared

to the LOEC, 400 ppb, which caused 35% mortality, exposure to 900 ppb caused a severe incline in mortality (about 93%) along the highest concentration of 2000 ppb (100%) (Table 16). The Ch.V. of 300 ppb was less than EC50, 500 ppb.

Table 16. Untransformed data presented as the average percent mortality caused after 96 hours of exposure to fluoxetine including the statistically derived concentrations, the NOEC, and the LOEC associated with each single species test.

Fluoxetine Concentration vs. Mortality									
<i>C. dubia</i>		<i>D. pulex</i>		<i>P. promelas</i>		<i>M. bahia</i>		<i>M. beryllina</i>	
Dose (ppb)	Mortality (%)	Dose (ppb)	Mortality (%)	Dose (ppb)	Mortality (%)	Dose (ppb)	Mortality (%)	Dose (ppb)	Mortality (%)
Control 0	10	Control 0	10	Control 0	0	Control 0	0	Control 0	2.5
7	25	60	15	10	0	*20	40	50	12.5
*20	100	100	10	20	0	50 NOEC	12.5	*100	20
*30	100	200 NOEC	80	40	0	*100 LOEC	47.5	200 NOEC	20
*60	100	*500 LOEC	90	90 NOEC	2.5	*200	45	*400 LOEC	35
100 NOEC	60	*900	100	*200 LOEC	40	*400	35	*900	92.5
*200 LOEC	95	*1000	100	*400	100	*800	62.5	*2000	100

\*Statistically significant concentrations were derived using TOXCALC 5 by transforming the average survivorship to Arcsine Square Roots. Based on the Shapiro Wilk's Test with a variance of 0.01 this transformed data was used to determine statistically significant concentrations. NOECs and LOECs were established using Steel's Many One Rank Test with a 0.05 variance.

### Sublethal Effects

Because the environmental amounts are below those to stimulate mortality these results of this study extend beyond lethality through the observation of sublethal effects which are not required for acute test methods. In addition to high toxicity potential these two pharmaceuticals are extremely potent in controlling hormonal, serotonergic, and dopaminergic systems in mammals/humans which could interfere with key biological functions including but not limited to fecundity, growth and development, and behavior in nontarget organisms. Organisms are exposed for entire life cycles in environmental settings. In the current study juvenile organisms

were exposed during an important life stage of growth and development. Any changes could be important to note because they were occurring within 96 hours.

### **Sublethal Effects of Ethinyl Estradiol**

*Ceriodaphnia dubia* decreased activity as time and concentration increased. At the end of 96 hours the *C. dubia* exposed to 200 ppb, the NOEC, had 1 larval offspring in each container; additionally, deceased organisms observed in microscopic photos had larvae in the brood chamber. The number of offspring ranged from 1-7 for organisms exposed to 400 ppb, the LOEC.

*Daphnia pulex* exposed to 300 ppb became lethargic and lifeless after 72 hours exposure. Those granted 300 and 1000 ppb for 24 hours began swimming around the perimeter of the beaker. At 72 hours in 5000 ppb, organisms crashed into the sides of the beakers and attempted escape. Eggs were first observed on the tails of *D. pulex* at 24 hours in 10000 ppb. At 72 hours in 1000 ppb, *D. pulex* appeared to have eggs in the brood chamber and looked much larger by comparison. Offspring were observed in 600 and 3000 ppb. At test termination, ephippia were present at the base of the beaker in concentrations greater than 3000 ppb. Ephippia are fertilized resting eggs encased in a tough covering to withstand extreme conditions. These “winter eggs” are released by the female under times of stress and rest at the bottom of a water body until favorable conditions return. At 72 hours, organisms in 300 ppb were missing. In concentrations 300 and 1000 ppb at the end of 96 hours, dead daphnia appeared to have been eaten by survivors. Prior to test renewal in 10000 ppb, the water appeared cloudy possibly from algae dying.

*Pimephales promelas* exposed to EE2 decreased general activity as time and concentration increased. Most of the minnows in 400 – 3000 ppb became extremely lethargic as time increased. However, during the first 24 hours, organisms in 800 ppb became frantic. Few swim abnormalities were observed in 400 and 2000 ppb; the organisms began swimming

inconsistently throughout the water column and jumpy and became disoriented for the entire test. At concentrations above 800 ppb the organisms remained quite drab in color. During the first few days of the test, roughly 2-5 minnows yielded aggressive and probing actions at 800 and 2000 ppb. Those administered 800 ppb ceased aggressive activity as time increased; on the other hand organisms in 2000 ppb continued this behavior for duration of the test. In 100 and 200 ppb deceased minnows appeared damaged and mutilated by the remainders. The underbelly of a minnow in 3000 ppb appeared injured. Interestingly, this concentration triggered an enormous engorgement of swim bladders. Upon microscopic investigation it was shown that swim bladder size increased in a dose dependent manner.

*Mysidopsis bahia* displayed aggressive behaviors in 1000 ppb at 24 hours and 80 ppb at 72 hours. Hostility was delayed in lesser concentrations but in higher concentrations this activity decreased with time. In concentrations 300 ppb and below, cannibalism decreased as concentrations increased. Cannibalism ceased after 24 hours in concentrations above 600 ppb. Feeding decreased at concentrations 1000-2000 ppb; however, the artemia, were alive and very active. The food source appeared to much more active in concentration 2000 ppb but were motionless after one day. Mysid subjected to 200 ppb were noticeably larger and darker after 72 hours observation. Similarly, denser coloration was observed in all unexposed organisms and sporadically throughout lesser concentrations; however, those subjected to EE2 were more massive.

*Menidia beryllina* became motionless after 72 hours of exposure which became more appreciated in elevated concentrations. In 400 ppb fish exhibited extremely restless behavior that decreased with time. Swim abnormalities of organisms in 2000 ppb were observed at 48 hours in which they began flipping backwards. Throughout the test the number of organisms that experienced tremors in 2000 and 3000 ppb correlated with lethality. For example, in the highest

test concentration, the organisms began seizing at 24 hours and the next day suffered 100% mortality. Accelerated maturation was observed in those exposed to 100-200 ppb by evidence of pigmentation at the head. One organism in 2000 ppb had a yellow body with a clear tail which supposedly occurs when they reach maturity. Engorged abdomens developed at 48 hours in 200-400 ppb.

### **Sublethal Effects of Fluoxetine**

*Ceriodaphnia dubia* began lifelessly floating on edge of the beaker at 20 – 200 ppb. For the entirety of the test, organisms in 7 ppb were very active and anxious; this concentration caused organisms to increase in size compared to the control or elevated concentrations.

*Daphnia pulex* in 200 ppb at became immobilized after 48 hours. At concentrations above 500 ppb, the majority of organisms remained lethargic throughout the entirety of the test. Abnormal swim behaviors were observed at 48 hours in 60 ppb in which the daphnia exhibited extremely jerky swimming motions. Throughout the test, organisms in this concentration appeared to be combating and/or mating; however, neither eggs nor offspring were detected. In 100 ppb at test termination, organisms appeared to be rotating backwards and swimming in circles around beaker. Daphnia appeared to increase size in 900 and 1000 ppb. In the lesser concentrations, abdomens were distended.

*Pimephales promelas* were floating listlessly on the surface of the water table; nonresponsiveness became more appreciated as concentration and time increased. In 20 - 40 ppb minnows displayed stationary vertical positioning. A small number of minnows in 10 – 90 ppb appeared disoriented; however this activity decreased as time and concentration increased. Restless behavior decreased as concentration and time increased. At test termination fathead minnows in 90 – 200 ppb became aggressive by probing the inactive organisms at the surface. Interestingly enough, organisms in these concentrations had crooked spines and began swimming



in corkscrew motion. The spine curvature was confirmed by microscopic observation; additionally confirmed were the larger and lighter swim bladders of exposed organisms.

*Mysidopsis bahia* exposed to FLX displayed very little activity; however, those in 190 ppb were larger and more active than mysid in 100, 400, and 800 ppb. These later concentrations triggered abnormal behaviors. At 24 hours organisms in 800 ppb executed tumbling behaviors. Those in 100 ppb exhibited this same behavior at 48 hours but were inflicted with crooked tails. Organisms exposed to concentrations above 200 ppb, exhibited decreased feeding and pigmentation. During the first 24 hours, cannibalism was observed in all concentrations of FLX except 50 ppb, the NOEC. As concentrations increased, casualties due to cannibalism decreased in response to the decrease in antagonistic behavior

*Menidia beryllina* exposed to lower concentrations were hovering at the surface showing very little activity. During first 24 hours, organisms in higher concentrations exhibited fits of agitation. After test renewal, immobilization increased with time and concentration; at test termination all exposed organisms were lethargic. In addition, prior to 48 hour test renewal, catatonic behavior and vertical positioning was more prominent in all organisms as time and concentration increased. Exposure to 900 and 2000 ppb convulsing and seizing; CNS toxicity correlated with fatality. Few fish suffered inflamed abdomens.

## DISCUSSION

Because the basis of the study rested on the hypothesis that similarly classified organisms would exhibit similar responses in mortality, the organisms' responses were compared and contrasted against different levels: dose-response plots, the percent mortality detected at a median concentration, the lowest concentration that induced 100% mortality, the median effective concentration based on lethality (EC50 or LC50), and the chronic value derived from a ratio of the no observable concentrations and the lowest observable effect concentrations based on mortality. After test termination, mortality curves were analyzed to quantify 96 hour LC50s, NOECs, LOECs, and Ch.V.s for each of exposed to ethinyl estradiol and fluoxetine.

### **Lethal Effects Based on Dose-Response Plot**

To compare and contrast the dose induced mortality, the average untransformed percent mortality were plotted against the test concentration with the NOECs and LOECs identified in ethinyl estradiol (Figure 11) and fluoxetine (Figure 13). A normal response should correlate with the dose and form a straight line. This was observed in dose-response plots of *C. dubia* exposed to ethinyl estradiol (Figure 11) and in *D. pulex*, *P. promelas*, and *M. beryllina* exposed to fluoxetine (Figure 13).

A hormetic response or a disruption in the linear dose-response correspondence was observed in *D. pulex*, *P. promelas*, *M. bahia*, and *M. beryllina* exposed to EE2 (Figure 11) and in *C. dubia* and *M. bahia* exposed to FLX (Figure 13).

**Dose-response plot effects of ethinyl estradiol.** *C. dubia* exhibited a normal dose response with concentrations above 900 ppb exhibiting 100% mortality. A disruption in the dose response plot for *D. pulex* showed a decrease in mortality in concentrations 600 and 3000 ppb. *P. promelas*, *M. bahia*, *M. beryllina* exhibited hormetic response. *D. pulex* exhibited a similar curve but the disruption in the curve is different. A significant decrease in mortality from the

NOEC to the LOEC was observed in *C. dubia* and *M. bahia* producing similar EC50 and chronic values around 300 - 400 ppb. At 3000 ppb *M. beryllina* had 100% mortality while *P. promelas* had only 40% mortality making it very resilient. Interestingly at the lesser concentrations, 100 - 800 ppb both fish exhibited identical hormetic changes in mortality (Figure 11).

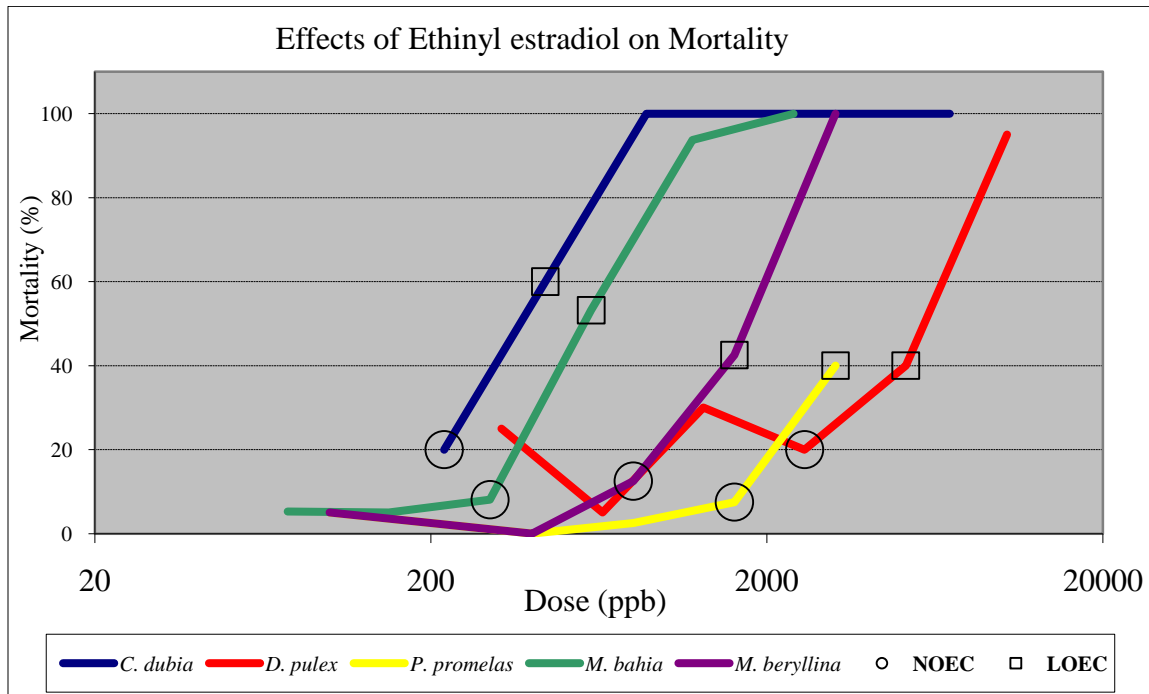


Figure 11. The observed percent mortality and the analyzed NOEC and LOEC determined at test termination for all test organisms exposed to test concentrations of ethinyl estradiol.

**Dose-response plot effects of fluoxetine.** In concentrations 20 – 200 ppb *C. dubia* exhibited a hormetic effect at 48 hours and produced about 100% mortality at test termination. A disruption in the dose-response curve in 100 ppb (about 60%) could explain why the chronic value is higher than EC50. *D. pulex* had a normal dose response curve with the NOEC of 200 ppb manifesting 80% mortality. *P. promelas* had little or no mortality from 0 - 90 ppb and exhibited a normal response not including the drastic increase in mortality (3 – 100%) which occurred in 90 – 400 ppb. *M. bahia* exhibited a slight hormetic change at 100 – 400 ppb (47.5 – 35%) and reduction in mortality at 50 ppb (NOEC, the only non significant concentration) rendering the second significant concentration as the LOEC. *M. beryllina* depicted a normal

dose response curve with very little change between first three concentrations (50, 100, 200 ppb) with a dire variation in mortality after LOEC of 400. Aside from a slight hormetic change, *D. pulex* and *M. beryllina* exhibited normal dose response curves. *P. promelas* demonstrated this normalcy apart from the radical increase in mortality (3 – 100%) from 800 – 3000 ppb (Figure 12).

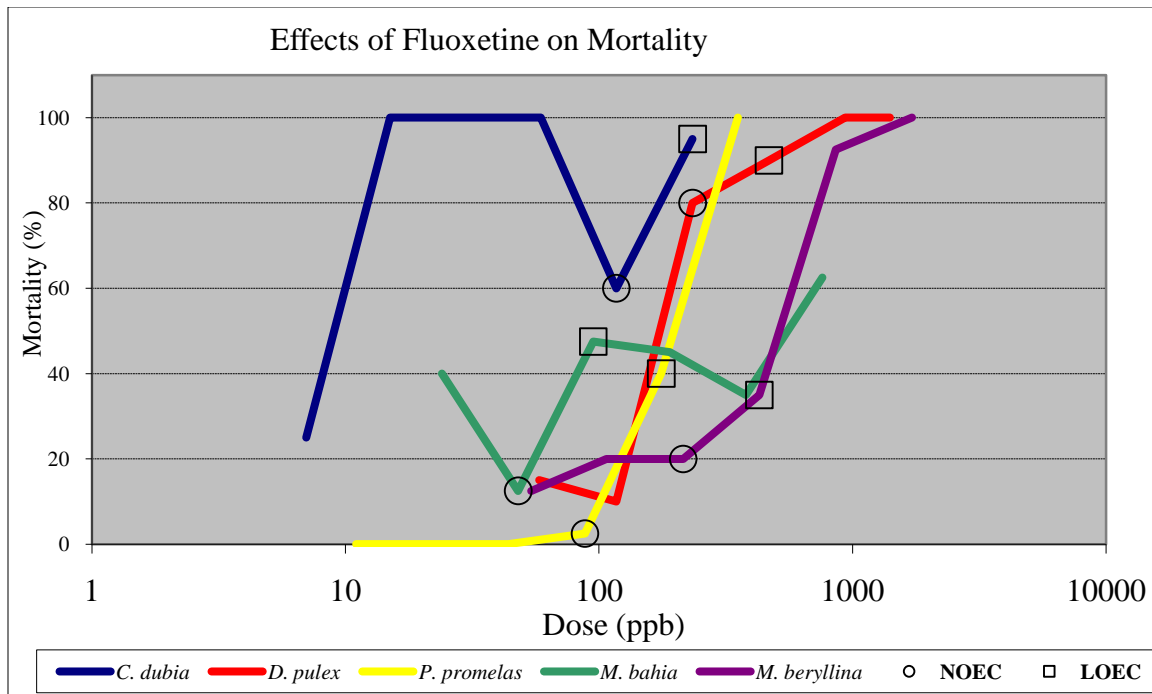


Figure 12. The observed percent mortality and the analyzed NOEC and LOEC determined at test termination for all test organisms exposed to test concentrations of fluoxetine.

### Lethal effects based on Median Concentrations and Concentrations Causing Complete Mortality

The effects on mortality were projected to be comparable to similarly cataloged organisms. The concentrations needed to induce mortality were expected to be smaller in crustaceans *Ceriodaphnia dubia* (water flea), *Daphnia pulex* (water flea), and *Mysidopsis bahia* (mysid shrimp) because they are simpler organisms with shorter life cycles, making them more susceptible and vulnerable to the toxicants. Because fish are more complex organisms with more

intricate bodily functions and longer life cycles the *Pimephales promelas* (fathead minnow) and *Menidia beryllina* (inland silverside) were expected to be more resilient to these drugs.

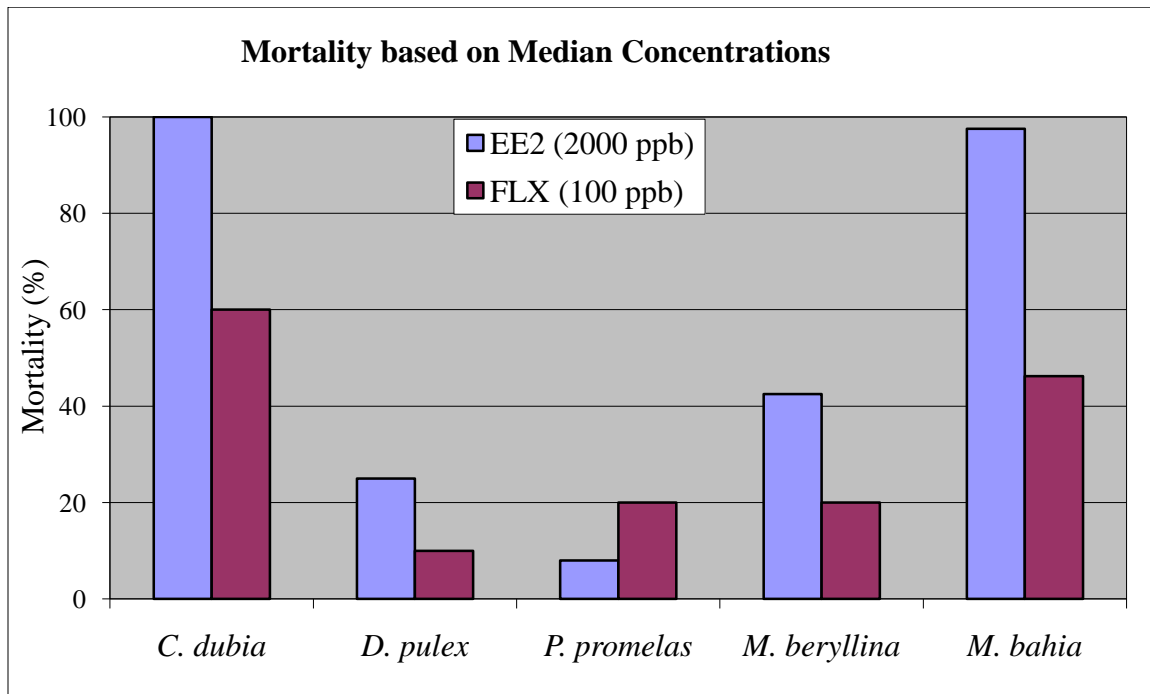


Figure 13. A comparison of the percent mortality in all species of test organisms caused after 96 hours of exposure to the median concentrations 2000 ppb of ethinyl estradiol and 100 ppb of fluoxetine.

**Median concentrations of ethinyl estradiol.** Based on an EE2 concentration of 2000 ppb, *P. promelas* had 8% mortality, *D. pulex* (30-20%), *M. beryllina* (42.5%), *M. bahia* (95-100%) and *C. dubia* (100%).

**Median concentrations of fluoxetine.** At a concentration of 100 ppb FLX mortalities were recorded as *D. pulex* (10%), *P. promelas* (3-40%), *M. beryllina* (20%), *M. bahia* (47.5 – 45%), and *C. dubia* (60%).

**Complete mortality in ethinyl estradiol.** The lowest concentrations of EE2 causing 100% mortality were as follows: *C. dubia* (900 ppb), *M. bahia* (2000 ppb), *M. beryllina* (3000 ppb), *P. promelas* (8000 ppb), and *D. pulex* (10000 ppb).

**Complete mortality in fluoxetine.** The lowest concentration causing 100% were as follows: *C. dubia* (20 ppb), *P. promelas* (400 ppb), *D. pulex* (900 ppb), *M. bahia* (1000 ppb), and *M. beryllina* (2000 ppb).

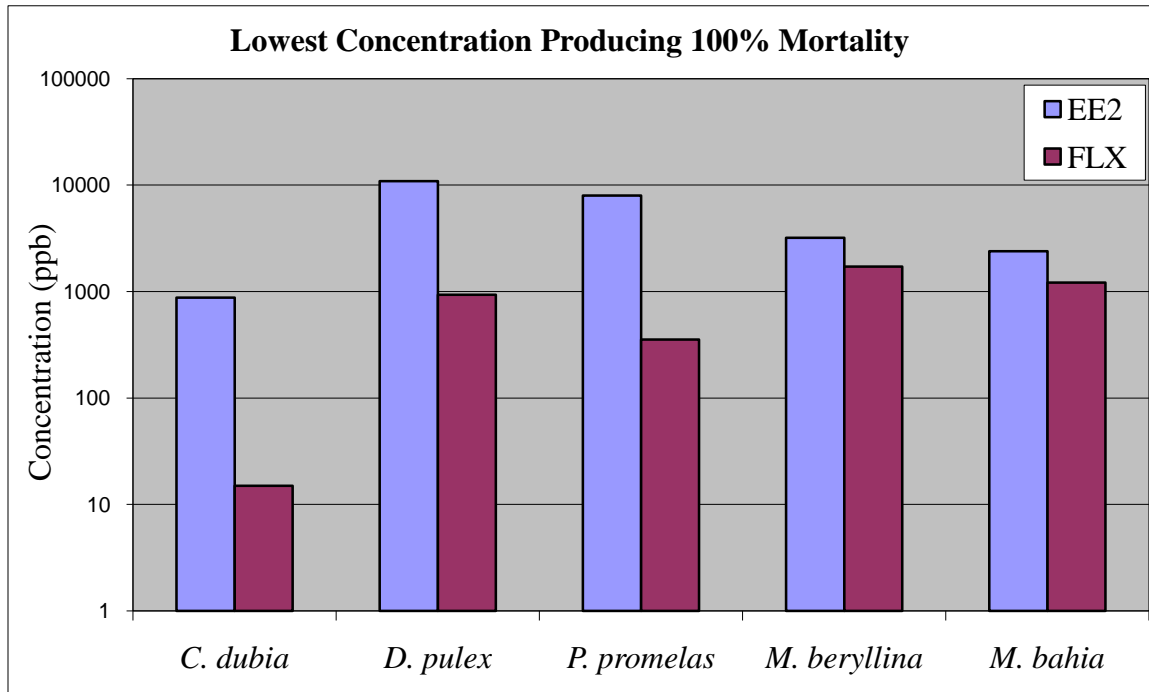


Figure 14. A comparison of the lowest concentrations of ethinyl estradiol and fluoxetine producing 100% mortality in all species of test organisms.

### Median Effective Concentrations Causing Lethality

The lethal concentrations (LC50s) were expected to be smaller in the crustaceans than in the fish. The highest test concentrations were chosen into order to induce at least 50% lethality (LC50) based on literature review of similarly classified organisms (EE2, Table 1; FLX, Table 5). Quantified 96 hour LC50s were established for the five test organisms and were compared against literature review.

**Ethinyl estradiol EC50s** The most sensitive species based on EC50: *C. dubia* (400 ppb) > *M. bahia* (600 ppb) > *M. beryllina* (2000 ppb) > *D. pulex* (6000 ppb) > *P. promelas* (10000 ppb).

**Similarly classified organisms – crustaceans** The three crustaceans produced the following 96 hour LC50s after exposure to EE2: *Ceriodaphnia dubia* (water flea) (400 ppb) > *Mysidopsis bahia* (mysid shrimp) (600 ppb) > *Daphnia pulex* (water flea) (6000 ppb).

Compared to the similarly classified test organisms, the *Nitocra spinipes* (harpacticoid copepod), a crustacean whose habitat ranges from fresh water to hypersaline conditions, displayed an increase in mortality yielding a 96 hour LC50 of 500 ppb (Breitholtz and Bengtsson 2001) which was between the quantified LC50s of the two crustaceans, *C. dubia* (400 ppb) and *M. bahia* (600 ppb), while being significantly lower than the *D. pulex*.

The marine shrimp, *Neomysis integer* (opossum shrimp) showed an increase in mortality at 1000 ppb (Verslycke et al. 2004). The *M. bahia* is also a marine shrimp but yielded a much lower LC50 (600 ppb).

Watts et al. (2001) showed that the *Gammarus pulex* (scud) displayed an increase in mortality at 2000 ppb. This freshwater shrimp exhibited an LC50 between that of the *M. bahia* (600 ppb) and the *D. pulex* (6000 ppb). Interestingly this freshwater shrimp had a 96-hour LC50 identical to the marine fish, the *M. beryllina* (2000 ppb).

**Similarly classified organisms – fish.** The two fish species produced the following 96 hour LC50s after exposure to EE2: *Menidia beryllina* (inland silverside) (2000 ppb) > *Pimephales promelas* (fathead minnow) (10000 ppb).

Versonnen et al. (2003) showed that *Danio rerio* (zebrafish), a tropical freshwater fish belonging to the minnow family, caused an LC50 of 2000 ppb. This 96 hour LC50 is identical to the marine fish *M. beryllina* (2000 ppb) but much lower than the freshwater minnow (10000 ppb).

**Fluoxetine EC50s.** The most sensitive species based on EC50: *C. dubia* (5 ppb) > *P. promelas* (200 ppb) > *D. pulex* (200 ppb) > *M. bahia* (500 ppb) > *M. beryllina* (500 ppb).

The hypothesis was that similarly classified organisms would have close to the same LC50s.

**Similarly classified organisms – crustaceans.** The three crustaceans produced the following 96 hour LC50s after exposure to FLX: *Ceriodaphnia dubia* (water flea) (5 ppb) > *Daphnia pulex* (water flea) (200 ppb) > *Mysidopsis bahia* (mysid shrimp) (500 ppb).

Brooks et al. (2003b) found that *Ceriodaphnia dubia* produced an LC50 of 300 ppb. This concentration is close to the other species of water flea, the *D. pulex* (200 ppb) and the freshwater fish the *P. promelas* (200 ppb). While still close to the other crustacean, *M. bahia* (500 ppb), the exact same species was much lower than the literature review due to the observed hormetic effects.

Brooks et al. (2003b) also documented the LC50 in another species of water flea, *Daphnia magna*, as 900 ppb. This concentration was much higher than any of the determined LC50s of the test species used in the current study.

**Similarly classified organisms – fish.** The two fish species produced the following 96 hour LC50s after exposure to FLX: *Pimephales promelas* (fathead minnow) (200 ppb) > *Menidia beryllina* (inland silverside) (500 ppb).

Brooks et al. (2003b) documented the LC50 of *Pimephales promelas* (fathead minnow) as 800 ppb. In the current study, all test organisms had 96 hour EC50s much lower than this concentration. The concentration 400 ppb caused 100% mortality in *P. promelas*. According to the raw data, *M. beryllina* experienced 35% mortality at 400 ppb which increased to about 93% at 900 ppb. Interestingly, at 800 ppb the crustacean, *M. bahia* suffered around 63% mortality.

Winder et al. (2009) found that *Cyprinodon variegatus* (sheepshead minnow) a marine/brackish water fish only caused 47% mortality in 2000 ppb. In the current study, the highest LC50 was 500 ppb in the *M. beryllina* (inland silverside) with a documented 100% mortality observed at 2000 ppb.



The fish species *Oryzias latipes* (Japanese medaka) had a 96 hour LC50 of 2000 ppb (Eli Lilly and Company 2005). The highest concentration used in the current study of *M. beryllina* was 2000 ppb which caused 100% mortality. The concentration that caused 100% mortality in *P. promelas* was 400 ppb.

Nakamura et al. (2008) documented the LC50s of *Oryzias latipes* (Japanese medaka) as 6000 at a pH of 7, 1000 at a pH of 8, and 200 at a pH of 9. In the current study, all test organisms had 96 hour LC50s much lower than those found at pHs 7 and 8; however, the LC50 at the pH of 9 (200 ppb) was identical to the fish *P. promelas* (200 ppb) and the water flea *D. pulex* (200 ppb) and comparable to the fish *M. beryllina* (500 ppb) and the shrimp *M. bahia* (500 ppb).

### **Chronic Values**

The chronic value was statistically elected from the NOEC - LOEC ratio according to the Steel's Many One Rank Hypothesis Test. By definition, this concentration is not considered to be harmful thus it should be lower than the EC50.

**Ethinyl estradiol chronic values.** The most sensitive species based on Ch.V: *C. dubia* (300 ppb) > *M. bahia* (400 ppb) > *M. beryllina* (1000 ppb) > *P. promelas* (2000 ppb) > *D. pulex* (4000 ppb).

All test organisms exposed to EE2 follow the idea that the EC50 is usually slightly higher than Ch.V. (Figure 15). In *C. dubia* and *M. bahia* the EC50 was only slightly higher than the Ch.V. in consequence of the normal dose response curve as well as the NOECs causing more than 20% mortality LOECs causing about 60-68% mortality and the highest concentrations bringing about 100% mortality. The EC50 of *P. promelas* is more sizable than the Ch.V. attributable to only 40% mortality occurring in the LOEC which was the highest concentration and only 8% mortality in the NOEC. The LOEC *D. pulex* also produced 40% mortality formulating an EC50 more considerable than the Ch.V. Additionally, a hormetic response was

observed from the lowest concentration throughout the NOEC. The EC50 of *M. beryllina* was fairly larger than the Ch.V. because the LOEC had 43% with next highest concentration producing 100%.

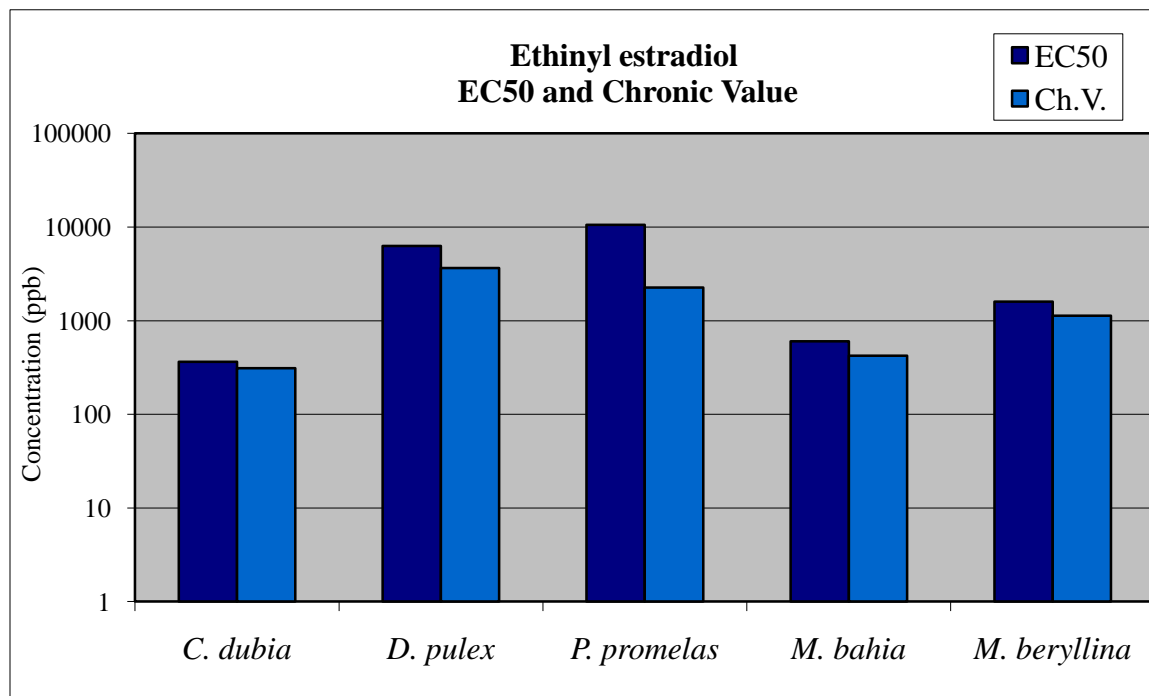


Figure 15. A comparison of the statistically analyzed EC50s and chronic values of organisms after 96 hours of exposure to ethinyl estradiol.

**Fluoxetine chronic values.** All the Ch.V.s were pretty close ranging from 70-300 ppb. The most sensitive Ch.V: *M. bahia* (70 ppb) > *P. promelas* (100 ppb) > *C. dubia* (200 ppb) > *D. pulex* (200 ppb) > *M. beryllina* (300 ppb).

Based on the definition of the chronic value the EC50 is usually slightly higher than Ch.V. (Figure 16). The EC50 is lower than Ch.V. in *C. dubia* because there was 100% mortality in concentrations less than the NOEC (60%) and LOEC (95%). Additionally, in *D. pulex*, there was 80% in the NOEC and 90% in the LOEC and 10-15% in control and lesser concentrations. In *P. promelas* the EC50 was greater than the Ch.V. despite the drastic change in mortality between the three highest concentrations which included the NOEC and the LOEC, which only caused 40% mortality. The much higher EC50s in *M. bahia* and *M. beryllina* for can be

attributed to the disruption in the normal dose-response curve and because the LOEC is not the first significantly different concentration.

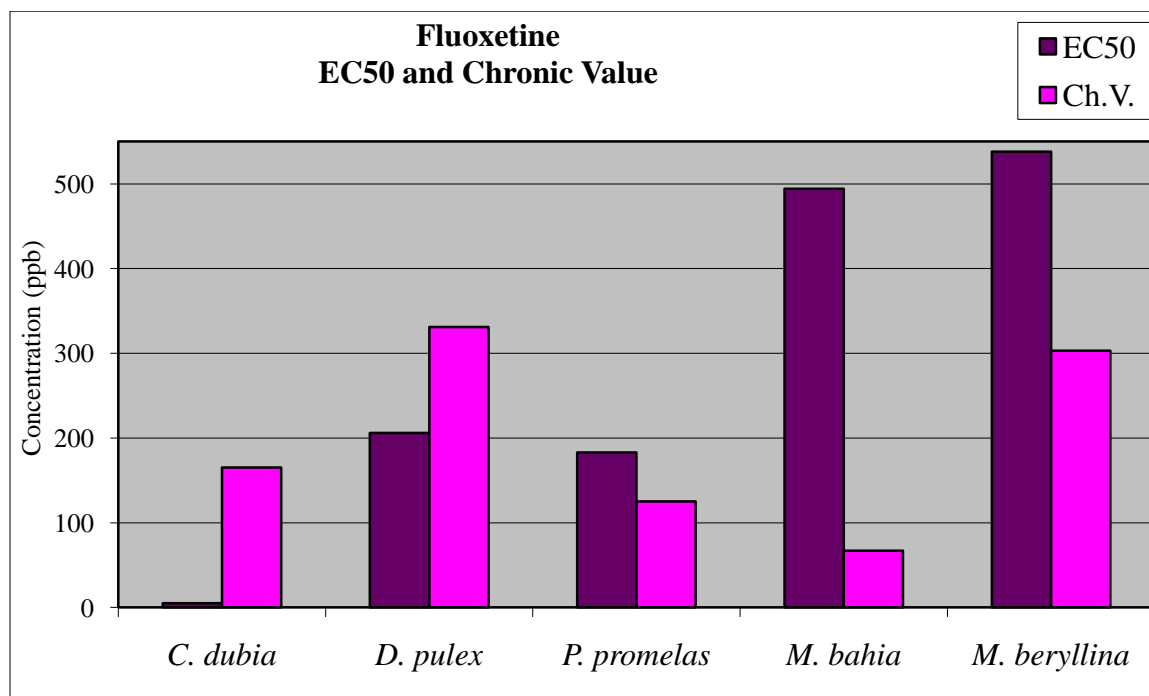


Figure 16. A comparison of the statistically analyzed EC50s and chronic values of organisms after 96 hours of exposure to fluoxetine.

### Classification of Lethal Effects

Dose-response relationships are usually very well documented in the intended target of the pharmaceutical (i.e. humans or target animals). However, the uncertainty with nontarget organisms is great due to changes in dosage, exposure time, response, pharmacokinetic action, and external interferences (O’Conner et al. 2002). Based on classification by Annex VI to the European Community legislation (Directive 67/548/EEC) pharmaceuticals can be categorized by the expected toxicity on the ecosystem based on LC50, log  $K_{ow}$ , and solubility in water.

R50: “Very toxic to aquatic organisms.” – Acute toxicity [LC50 (fish) or EC50 (daphnia)]  $\leq$  1000 ppb.

R50 and R53: “Very toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment.”  
 – Acute toxicity  $\leq$  1000 ppb, and the compound is not readily degradable, or log  $K_{ow} \geq 3$  (unless experimentally derived BCF  $\leq 100$ ).

R51: "Toxic to aquatic organisms."  
 – Acute toxicity > 1000 ppb and ≤ 10000 ppb.

R51 and 53: "Toxic to aquatic organisms and may cause long-term adverse effects in the aquatic environment."  
 – Acute toxicity > 1000 ppb and ≤ 10000 ppb and the compound is not readily degradable, or log K<sub>ow</sub> ≥ 3 (unless experimentally derived BCF ≤ 100).

R52: "Harmful to aquatic organisms."  
 – Acute toxicity > 10000 and ≤ 100,000 ppb.

R52 and 53: "Harmful to aquatic organisms and may cause long-term, adverse effects in the aquatic environment."  
 – Acute toxicity >10000 ppb and ≤ 100000 ppb and the compound is not readily degradable, or log K<sub>ow</sub> ≥ 3(unless experimentally derived BCF ≤100).

R53: "May cause long-term adverse effects in the aquatic environment."  
 – Solubility less than 1000 ppb, not readily degradable, or log K<sub>ow</sub> ≥3 (unless experimentally derived BCF ≤ 100).

Simply based on the EC50s ethinyl estradiol was very toxic (R50) in *C. dubia* (400 ppb) and in *M. bahia* (600 ppb). Ethinyl estradiol is toxic (R51) to *D. pulex* (6000 ppb) and *M. beryllina* (2000 ppb) and harmful (R52) to *P. promelas* (10000 ppb).

Simply based on the acute toxicity fluoxetine was very toxic (R50) to all test organisms: *C. dubia* (5 ppb), *P. promelas* (200 ppb), *D. pulex* (200 ppb), *M. bahia* (500 ppb), and *M. beryllina* (500 ppb).

Table 17. Expected toxicity classification based on the Annex VI to the European Community legislation of EE2 and FLX according to EC50 results of the current study.

Organism	EE2 EC50 (ppb)	Classification	Toxic Potential	FLX EC50 (ppb)	Classification	Toxic Potential
<i>C. dubia</i>	400	R50	very toxic	5	R50	very toxic
<i>D. pulex</i>	6000	R51	toxic	200	R50	very toxic
<i>P. promelas</i>	10000	R52	harmful	200	R50	very toxic
<i>M. bahia</i>	600	R50	very toxic	500	R50	very toxic
<i>M. beryllina</i>	2000	R51	toxic	600	R50	very toxic

The solubility of ethinyl estradiol in water was reported as  $11.3 \times 10^4$  ppb (Yalkowsky and Dannenfelser 1992). The maximum solubility of fluoxetine in water was noted as  $14 \times 10^6$  ppb (O'Neil 2001). Additional solubilities were reported as and  $15.0 \times 10^6$ ,  $6.84 \times 10^6$ , and  $5.47 \times 10^6$  in the respective pHs of 5, 7, and 9 (Eli Lilly and Company 2005). Therefore, both drugs “may cause long-term adverse effects in the aquatic environment” (R53) based on the fact that their solubilities are less than 1000 ppb and not readily degradable.

The drugs may also be classified as R53 if the  $\log K_{ow} \geq 3$ . The reported  $\log K_{ow}$  for ethinyl estradiol was 3.67 (Hansch et al. 1995) with a predicted  $\log K_{ow}$  of 4.12 using the Ecological Structure Activity Relationships program (ECOSAR) (Sanderson et al. 2004). Depending on which stereoisomer is used for fluoxetine, the  $\log K_{ow}$  may differ slightly. For example, Eli Lilly and Company (2005) reported the  $\log K_{ow}$  for fluoxetine as 1.0, 1.8, and 2.6 at pH 5, 7, and 9 respectively. Brooks et al. (2003a) calculated values using ACD/Labs software version 5.0 in Toronto, Ontario, Canada which included 1.25, 1.57, and 4.30 at pH 2, 7, and 11 respectively. ECOSAR predicted the  $\log K_{ow}$  of fluoxetine HCl as 4.65 (Sanderson et al. 2004). Consequently, EE2 is classified as R53 based on the  $\log K_{ow}$  as is FLX at pHs greater than 9. However, according to ECOSAR the  $\log K_{ow}$  of FLX is greater than 3 at any concentration.

The higher the  $\log K_{ow}$  (octanol-water coefficient) the more hydrophobic the compound is and the greater probability for bioaccumulation. Lipophilic xenobiotics ( $\log K_{ow} > 3$ ) are absorbed into fish bloodstream primarily across the gills (Kreke and Dietrich 2008).

### **Sublethal Effects of Ethinyl Estradiol and Fluoxetine**

This study extended beyond lethality through the observation of sublethal effects which are not required for acute test methods. Abnormalities observed during the test were documented and concentration boundaries were established for sublethal effects. EE2 and FLX induced effects on these standard EPA organisms.

**Alteration of serotonin, dopamine, and estrogenic hormones.** Ethinyl estradiol and fluoxetine have the potential to alter serotonin, dopamine, and estrogenic hormones. Therefore, biological activities encompassing these chemicals could explain the various and in some cases, similar behaviors/abnormalities observed in the current study. Estrogens also have the potential increase serotonergic activity in Tilapia (Tsai and Wang 1997) and extracellular dopamine in Rats (Disshon et al. 1998). Estrogens stimulate sensitivity of dopamine receptors in vertebrate central nervous systems resulting in an increase of the catecholamine (Kelly et al. 2003; Mermelstein et al. 1996). Knoll et al. (2000) demonstrated that in estrone (E1), dopamine release is inhibited resulting in low levels of dopamine. However, Kelly and Wagner (1999) determined that the natural estrogen E2 estradiol stimulates dopamine release resulting in more lethargic activity.

Bymaster et al. (2002) state that norepinephrine and dopamine levels in mammals are also increased after acute dosing with fluoxetine. Most SSRIs only increase the amount of extracellular concentrations serotonin (Bymaster et al. 2002; Kreke and Dietrich 2008), thus making FLX a unique pharmaceutical. Fluoxetine increased extracellular concentrations of serotonin, norepinephrine and dopamine after acute dosing. The 242 nM concentration of monoamines were sufficient to block 5-HT receptors which could explain the increase of catecholamines (Bymaster et al. 2002).

Fluoxetine has significantly increased female circulating steroid concentrations in aquatic organisms (Foran et al. 2004) perhaps causing organisms exposed to FLX to exhibit abnormalities also seen after EE2 exposure.

**Control of the circadian system.** Under control of a number of neurotransmitters including but not limited to serotonin and dopamine (Fingerman et al. 1994) are distal retinal pigment hormones which modulate the amount of pigment present in the compound eyes. This

enables organisms to adapt and differentiate between light and dark intensities (Beltz 1988). If animals are unable to and measure and interpret day duration, necessary activities/functions modified by the circadian system will be disrupted. Changes in normal body chemicals can modify signaling pathways resulting in variations in behavior (Kreke and Dietrich 2008). As evident by their swimming behavior, *D. pulex* and *P. promelas* appeared confused and disoriented after EE2 exposure. This behavior was monitored in all test organisms exposed to FLX. In this case it appears that there was a disruption in the organisms' sensory perception due to variations in neuromodulators.

**Observed changes in behavior.** Abnormal behaviors observed in all exposed organisms included unresponsiveness, irregular swim patterns, erratic activity, convulsions, and changes in aggression. Changes in locomotor activity could be the result of neuroendocrine effects resulting from the dopaminergic levels following estrogenic administration (McGee et al. 2009).

Generally speaking organisms exposed to ethinyl estradiol and fluoxetine demonstrated lethargic behavior which became more pronounced as time and concentration increased. The decrease in activity was correlated with the approaching mortality of the organisms. *C. dubia* and *P. promelas* became lethargic during the first 24 hours of exposure with activity decreasing as time and concentrations of EE2 increased. *D. pulex* and *M. beryllina* also displayed a decrease in activity correlating with the increased time and concentrations with immobilization becoming extremely prominent at 72 hours. All in all, as concentration increase immobilization became more prominent. Studies by Jaser et al. (2003) observed swim inhibition in *Ceriodaphnia reticulata* and *Sida crystallina* (water fleas) after 24 hours of exposure to ethinyl estradiol yielding EC50s of 2000 ppb and less than 4000 ppb respectively.

Table 18. Effects on catatonic behavior observed in test organisms resulting from ethinyl estradiol or fluoxetine exposure.

Organism	Chemical	Catatonic behavior	Dose (ppb)
<i>Ceriodaphnia dubia</i>	EE2	More prominent as time and concentrations increased	200 -7000
<i>Ceriodaphnia dubia.</i>	FLX	More prominent as time and concentrations increased	20 – 200
<i>Daphnia pulex</i>	EE2	More prominent as time and concentrations increased	> 300
<i>Daphnia pulex</i>	FLX	Immobilization	200
		More prominent as time and concentrations increased	500
<i>Pimephales promelas</i>	EE2	More prominent as time and concentrations increased	400 – 3000
<i>Pimephales promelas</i>	FLX	More prominent as time and concentrations increased	
		Stationary vertical positioning	20 - 40
<i>Menidia beryllina</i>	EE2	More prominent as time and concentrations increased	100 – 3000
<i>Mysidopsis bahia</i>	FLX	More prominent as time and concentrations increased	
<i>Menidia beryllina</i>	FLX	More prominent as time and concentrations increased	

Stanley et al. (2007) documented similar activity in which FLX caused immobilization in *Daphnia magna* (water flea). *P. promelas* and *M. beryllina* exposed to fluoxetine were hovering at the surface demonstrating very little activity beginning at test initiation. All crustaceans (water fleas and *M. bahia*) were statically positioned around the edge of the beaker. In the current study, *P. promelas* and *M. beryllina* demonstrated catatonic behavior and vertical positioning which became more common in elevated concentrations as the test transpired. Gaworecki and Klaine (2008) documented similar tendencies in hybrid striped bass exposed to 200 ppb of FLX. In addition, elevated serotonin amounts, after exposure to SSRIs, have been connected with submissive behavior in rainbow trout (Øverli et al. 2004) and in Japanese medaka which also displayed nonresponsiveness (Foran et al. 2004). Western mosquitofish remained at the surface



and lay on their sides with little or no swimming movement after acute exposure with lesser concentrations producing less severe effects (Henry and Black 2008).

Initial increases of dopamine can cause agitation and aggression followed by depression. Increased dopamine in the motor circuits will tend to produce random, involuntary movements and muscle twitching along with possible abnormal posturing. After EE2 regiments, the restless behavior of *P. promelas* included shaking and trembling which decreased with time and usually ceased after 24 hours. The more severe tremors noted after larger dosing of EE2 and FLX in *M. beryllina* correlated with fatality. These organisms exhibited apparent CNS toxicity characterized by convulsing and seizing.

Impulsive and inconsistent activity was observed in *D. pulex* and *P. promelas* at the beginning of the FLX test. Given the nature of dopamine, it would be assumed that activity would increase as shown by the rotations and spiraling observed in *D. pulex* exposed to FLX and *M. bahia* administered both drugs. Coiled swimming was associated with the warped tails and/or spines of *P. promelas* subjected to both drugs and *M. bahia* administered FLX.

All species exhibited fits of agitation in higher concentrations of FLX which could be due to increased dopamine levels during first 24 hours. Jumpiness was observed in all species at lesser concentrations of FLX and ceased quickly with time. Lower concentrations of FLX launched restless behavior of marine organisms in just 24 hours. For water fleas, restless behavior in lower concentrations of FLX began at 96 hours.

Table 19. Effects on abnormal behavior observed in test organisms resulting from ethinyl estradiol or fluoxetine exposure.

Organism	Chemical	Abnormal Behavior	Dose (ppb)
<i>Ceriodaphnia dubia</i>	FLX	Very active and anxious	7
<i>Daphnia pulex</i>	EE2	Swimming around the parameter of the beaker	300 and 1000
		Crashed into the sides of the beakers and attempted escape	5000

(Table 19 continued)

Organism	Chemical	Abnormal Behavior	Dose (ppb)
<i>Daphnia pulex</i>	FLX	Jerky swimming motions	60
		Combating and/or mating	
		Rotating backwards	100
<i>Pimephales promelas</i>	EE2	Swimming in circles around beaker	
		Frantic	800
		Swimming inconsistently throughout the water column	400 and 2000
		Jumpy	
<i>Pimephales promelas</i>	FLX	Disoriented	
		Stationary vertical positioning	20 – 40
		Disoriented	10 – 90
		Restless behavior	
<i>Mysidopsis bahia</i>	FLX	Corkscrew swimming pattern	90 – 200
		More active	190
		Tumbling	100 and 800
<i>Menidia beryllina</i>	EE2	Crooked tails	100
		Extremely restless behavior	400
		Flipping backwards	2000
<i>Menidia beryllina</i>	FLX	Convulsions and Seizures	2000 and 3000
		Agitation	Higher concentrations
		Convulsions and seizures	900 and 2000

**Observed changes in feeding.** The neurotransmitter serotonin additionally functions as a hormone which can directly impact the immune system and alter appetite (Fent et al. 2006; Barton et al. 2002). Goldfish showed a significant reduction in food intake when injected with 5-HT (serotonin) (de Pedro et al. 1998). Exposure to FLX decreased growth and feeding rates in fathead minnow (Stanley et al. 2007). In the current study, *M. bahia* decreased feeding in 1000-2000 ppb EE2 and at concentrations above 200 ppb FLX.

Table 20. Effects on feeding habits observed in test organisms resulting from ethinyl estradiol or fluoxetine exposure.

Organism	Chemical	Response	Dose (ppb)
<i>Mysidopsis bahia</i>	EE2	Decreased feeding	1000-2000
		Artemia, were alive and very active	1000-2000
<i>Mysidopsis bahia</i>	FLX	Decreased feeding	> 200

**Observed changes in aggression.** Metcalfe et al. (2001) deduce that serotonin is the antiaggressive agent in fish. The increased concentrations of EE2 and FLX caused a decrease in probing and hostility in *P. promelas* and *M. bahia*. Those allotted higher concentrations of these drugs ceased this disruptive behavior usually within 24 hours. However, antagonism documented in lower concentrations occurred closer to test termination. In accordance with submissive activity, cannibalistic casualties also subsided. *M. bahia* are naturally cannibalistic; these tendencies were observed in both tests. After EE2 exposure the absence of *D. pulex* and the apparent mutilation of deceased *P. promelas* were assumed to be prompted by the surviving organisms.

Some aquatic species require aggressive behaviors to define hierarchical status and dominance in reproduction. By omitting these behaviors during sensitive periods of reduction, genetically less desirable organisms would mate arguably producing less desirable or unhealthy offspring thus upsetting the balance of natural selection (Kreke and Dietrich 2008).

Table 21. Effects on aggressive behavior observed in test organisms resulting from ethinyl estradiol or fluoxetine exposure.

Organism	Chemical	Response	Dose (ppb)
<i>Daphnia pulex</i>	EE2	Missing organisms	300
		Eaten by survivors	300 and 1000
<i>Pimephales promelas</i>	EE2	Aggressive and probing actions of inactive organisms	800 and 2000
		Damage and mutilation of deceased	100 and 200
		Injured underbelly	3000

(Table 21 continued)

Organism	Chemical	Response	Dose (ppb)
<i>Pimephales promelas</i>	FLX	Probing of inactive organisms	90 – 200
<i>Mysidopsis bahia</i>	EE2	Aggressive behaviors and hostility	80 and 1000
		Cannibalism	≤ 300 and ≥ 600
<i>Mysidopsis bahia</i>	FLX	Cannibalism	> 50 <

**Observed changes in fecundity.** *C. dubia* and *D. pulex* exhibited increase in fecundity though sexual maturity is not expected to occur this early. Therefore, it can be assumed that ethinyl estradiol stimulates egg production. A minute number of *D. pulex* were observed mating in both EE2 and FLX; however this behavior cannot be confirmed. It is important to note that although the *Daphnia* population is usually made up entirely of females, under times of stress male daphnids are produced in relatively small numbers with the propriety to mate (EPA 2002). As a response to stress, species may produce offspring as early as possible in order to ensure survival (Jaser et al. 2003).

Table 22. Effects on fecundity or reproduction observed in test organisms resulting from ethinyl estradiol exposure.

Organism	Response	Dose (ppb)
<i>Ceriodaphnia dubia</i>	1 offspring in each container	200
	Larvae in the brood chamber of deceased organisms	
	Offspring present	400
<i>Daphnia pulex</i>	Offspring	600 and 3000
	Eggs in brood chamber	1000
	Ephippia	> 3000
	Eggs not released	10000

**Observed changes in growth and development.** Accelerated maturation was observed in *C. dubia*, *D. pulex*, *P. promelas*, and *M. bahia* by evidence of coloration and/or larval classification exposed to EE2. The growth increase of *C. dubia*, *D. pulex*, and *M. bahia* assigned lower concentrations can possibly be attributed to femization since females of these species are

larger (EPA 2002). It was assumed that molting and growth was inhibited in these species at higher concentrations based on size. Additionally, ephippia, typically released during the molt cycle, were first observed within 24 hours to 10000 ppb on the tails of *D. pulex*. The process of development and molting is managed by constituents of the endocrine system. Simazine, a known endocrine disruptor, was shown to interfere with the molt cycle of *D. pulex* (Fitzmayer et al. 1982).

Previously documented research shows that EE2 can have an effect on growth; however, these organisms were exposed for longer periods of time than in the current study. Still, it appeared that acute exposure to EE2 can affect growth. *C. dubia* in 7 ppb (NOEC for FLX mortality) were larger than control organisms or those exposed to other concentrations. This low dose may stimulate growth while higher doses may inhibit growth. It is difficult to make this assumption because so many of the organisms died at elevated concentrations. *D. pulex* demonstrated a different effect in which higher concentrations appeared to stimulate growth.

Table 23. Effects on growth and development observed in test organisms resulting from ethinyl estradiol or fluoxetine exposure.

Organism	Chemical	Response	Dose (ppb)
<i>Ceriodaphnia dubia</i>	FLX	Larger size	7
<i>Daphnia pulex</i>	EE2	Larger size	1000
<i>Daphnia pulex</i>	FLX	Larger size	900 and 1000
<i>Pimephales promelas</i>	EE2	Accelerated maturation	
<i>Mysidopsis bahia</i>	EE2	Larger size	200
<i>Mysidopsis bahia</i>	FLX	Larger size	190
<i>Menidia beryllina</i>	EE2	Accelerated maturation	100-200
		Mature pigmentation	2000

**Observed changes in color.** A number of species exhibiting decrease or absence in color after EE2 exposure was documented in *Danio rerio* (zebrafish) (Bogers et al. 2006), *Poecilia reticulata* (guppy) (Nielsen and Baatrup 2006), and *Pomatoschistus minutes* (sand goby) (Robinson et al. 2003). The dispersion or accumulation of pigment hormones determines the

coloration of organisms. These chromatophores are under control of a number of neurotransmitters including but not limited to serotonin and dopamine (Fingerman et al. 1994). As concentrations of EE2 and FLX increased, pigmentation decreased in *D. pulex*, *P. promelas*, *M. bahia*, and *M. beryllina*. Larval classification in EPA methods suggested that *P. promelas* in levels above 800 ppb EE2 matured faster; however, they remained pale in color. Their apparent transparency could stem from deprivation of hemoglobin (USEPA 2002). Henderson et al. (1981) noted a hemoglobin deficiency in minnows exposed to Hydrazine, an endocrine disruptor widely used in industrial manufacturing.

Table 24. Effects on pigmentation observed in test organisms resulting from ethinyl estradiol or fluoxetine exposure.

Organism	Chemical	Response	Dose (ppb)
<i>Pimephales promelas</i>	EE2	Opaque pigmentation	> 800
<i>Mysidopsis bahia</i>	EE2	Denser coloration	200
<i>Mysidopsis bahia</i>	FLX	Decreased pigmentation	> 200
<i>Menidia beryllina</i>	EE2	Denser pigmentation at the head	100-200
		Mature pigmentation	2000

**Observed abnormalities.** After EE2 exposure *P. promelas* had spinal deformities. Deformation of the vertebrae after estrogen dosing was reported in *Fundulus heteroclitus* (mummichog) and *Pimephales promelas* (fathead minnow) (Lange et al. 2001; Warner and Jenkins 2007). Lange et al. (2001) also reported the occurrence of distended abdomens. Protruding abdomens occurred in *D. pulex*, *P. promelas*, and *M. beryllina* and became more pronounced at elevated concentrations of EE2. Interestingly, swollen abdomens in these species were also triggered with FLX dosing. Fluoxetine also caused warped vertebrae in *P. promelas*

and *M. bahia*. Foran et al. (2004) reported that Japanese medaka exposed to FLX also possessed spinal curvatures.

Table 25. Abnormalities observed in test organisms resulting from ethinyl estradiol or fluoxetine exposure.

Organism	Chemical	Response	Dose (ppb)
<i>Daphnia pulex</i>	EE2	Cloudy water	10000
<i>Daphnia pulex</i>	FLX	Engorged abdomens	900 and 1000
<i>Pimephales promelas</i>	EE2	Engorged swim bladders Injured underbelly	3000
<i>Pimephales promelas</i>	FLX	Engorged abdomens Crooked spines Larger and lighter swim bladders	90 – 200
<i>Menidia beryllina</i>	EE2	Engorged abdomens	200-400
<i>Menidia beryllina</i>	FLX	Engorged abdomens	

A rationalization of the abdominal protrusion occurring in the two fish species could be due to possible overinflation of swim bladders. A swim bladder or air bladder is a large, thin-walled sac located in the dorsal portion of the body cavity that functions as a buoyant float, a sound producer and receptor, and a respiratory organ. It allows the fish to move up and down through the water column and remain at a specific water depth without wasting energy swimming. The fish exposed to both drugs had lighter colored swim bladders thus enforcing the idea that hemoglobin is lacking. Hemoglobin allows fish to fill or empty the swim bladder. This organ is mostly inflated at night when organisms trying to escape predators rise to the surface of the water and take in air (Büke et al. 2005). The circadian rhythmic offset due to changes in serotonin amounts could explain why the organisms were floating on the surface. Overinflation of the swim bladder causes organisms to float to the surface of the water and in turn they die of starvation because they stop eating (Chatain 1994; Planas and Cunha 1999) as observed after EE2 and FLX exposure. High levels of catecholamines have been closely linked to Swim

bladder Stress Syndrome (SBSS), characterized by overinflation of the swim bladder, in which no other anatomical alterations are apparent (Kolbeinshavn & Wallace 1985). Generally, observed swim bladder size increased as concentrations increased.



## CONCLUSIONS

Every year people use thousands of tons of drugs to treat illnesses, to prevent unwanted pregnancy, or to face the stresses of modern life. This appraisal is steadily increasing at an exponential rate. The actual amounts consumed are based on estimates and are probably much more abundant than reported because annual sales data does not include internet sales. Even after extensive metabolism these reasonably stable active pharmaceutical ingredients are typically unaltered with large portions of the parent compound being excreted. The metabolites may return to the original parent compound either during sewage treatment or through environmental transformations. Sewage treatment plants are not designed to remove pharmaceuticals and the active pharmaceutical ingredients are conceived to withstand degradation. These oppositions increase the potential for these compounds to remain in animal tissue and bioaccumulate enforcing biomagnifications and bioconcentrations. Moreover, the pharmaceutical industry is producing more effective active APIs by increasing potency, bioavailability, and degradation resistance. For these reasons they should be regarded as contaminants of concern.

In terms of other man-made pollutants environmental pollutants, pharmaceuticals exemplify a minuscule fraction of harmful contaminants present in the aquatic environment. Ethinyl estradiol and fluoxetine, two of the most popular pharmaceuticals in the United States have been available for decades, making them present in the environment since their introduction to the market. Because APIs are typically present in extremely low concentrations it is through recent advances in analytical instrumentation and techniques that their incidence has been validated. This does not belittle APIs potential to cause harm to the environment because pharmaceuticals are formulated to generate maximum responses at minimal amounts.

Environmental concern is not exclusively based on high production rates. Risk assessment focuses on the chemicals' properties and behavior in the environment such as

degradation resistance, accumulation potential, toxicity potential, and effectiveness on biological key functions such as hormone interference, reproduction, growth disturbance, etc. which are all properties of EE2 and FLX.

After test termination, mortality curves were analyzed to quantify 96 hour EC50s, NOECs, LOECs, and Ch.V.s for each of exposed to ethinyl estradiol and fluoxetine. Additionally the acute toxic responses were assessed, compared, and contrasted for the five standard EPA test organisms.

A broad range of effects and the ability for bodily chemicals to neutralize and return to normal after exposure suggests that a traditional dose-response relationship may not be expected. Higher mortality did not always correlate with increased concentration as evident by the hormetic responses of organisms: *P. promelas* and *M. beryllina* and a slightly in *D. pulex* and *M. bahia* after EE2 exposure and in *C. dubia* at 48 hours (causing 100% mortality at 96 hours) and in *M. bahia* after FLX exposure and slightly in *D. pulex*. A normal dose-response was observed in dose-response plots of *C. dubia* exposed to ethinyl estradiol and in *D. pulex*, *P. promelas*, and *M. beryllina* exposed to fluoxetine.

At a similar EE2 concentration of 2000 ppb, mortalities included *P. promelas* (8%), *D. pulex* (30-20%), *M. beryllina* (42.5%), *M. bahia* (95-100%) and *C. dubia* (100%).

The mortalities at 100 ppb of FLX included *D. pulex* (10%), *P. promelas* (3-40%), *M. beryllina* (20%), *M. bahia* (47.5 – 45%), and *C. dubia* (60%).

The lowest concentrations of EE2 causing 100% mortality were as follows: *C. dubia* (900 ppb), *M. bahia* (2000 ppb), *M. beryllina* (3000 ppb), *P. promelas* (8000 ppb), and *D. pulex* (10000 ppb).

The lowest concentration of FLX causing 100% were as follows: *C. dubia* (20 ppb), *P. promelas* (400 ppb), *D. pulex* (900 ppb), *M. bahia* (1000 ppb), and *M. beryllina* (2000 ppb).

EE2 yielded higher EC50s and has been detected at levels greater than FLX which produced lower lethal concentrations. The most sensitive species depends on the endpoint. *C. dubia* is the most sensitive species based on EC50 for EE2 and FLX. Based on EC50 the most resilient species was *P. promelas* for EE2 and *M. beryllina* for FLX.

The most sensitive species based on EC50 EE2: *C. dubia* (400 ppb) > *M. bahia* (600 ppb) > *M. beryllina* (2000 ppb) > *D. pulex* (6000 ppb) > *P. promelas* (10000 ppb).

The most sensitive species based on EC50 of FLX: *C. dubia* (5 ppb) > *P. promelas* (200 ppb) > *D. pulex* (200 ppb) > *M. bahia* (500 ppb) > *M. beryllina* (500 ppb).

The most sensitive species exposed to EE2 based on chronic value: *C. dubia* (300 ppb) > *M. bahia* (400 ppb) > *M. beryllina* (1000 ppb) > *P. promelas* (2000 ppb) > *D. pulex* (4000 ppb).

The most sensitive species exposed to FLX based on chronic value: *M. bahia* (70 ppb) > *P. promelas* (100 ppb) > *C. dubia* (200 ppb) > *D. pulex* (200 ppb) > *M. beryllina* (300 ppb).

The Ch.V. is based on the ratio of the NOEC and LOEC; because it is the concentration that is considered “safe” it should be lower than EC50. This rule pertains to all organisms exposed to EE2. Freshwater species exposed to FLX broke this rule which can be explained on a case by case situation. While still following the rule, the marine species exhibited much higher EC50s. This can be attributed to the disruption in dose-response of *M. bahia* and *M. beryllina* and the fact that the LOEC is not the first significantly different concentration.

Target receptors and/or biomolecules are present in humans and mammals are also present a number of vertebrate and invertebrate nontarget organisms. Hence, these nontarget organisms can potentially experience similar effects documented in mammalian toxicity tests.

These two drugs presented unanticipated and in many cases similar effects to the organisms even after 96 hours. Exposure to these chemicals can potentially alter levels of serotonin and dopamine and affect hormone production consequently signaling changes in

behavior and normal development. Changes in behavior could be due to neuroendocrine effects attributable to the changes in dopamine and serotonin levels. *M. beryllina* exhibited CNS toxicity characterized by convulsing and seizing which correlated with fatality in both drugs.

Normal activity decreased with increased concentration and time (all org. all drugs). Abnormal behaviors observed in both chemicals included unresponsiveness, irregular swim patterns, erratic activity, and decreased aggression. All in all, as concentration increase immobilization became more prominent for both drugs.

Both chemicals caused distended abdomens in *P. promelas*, *M. beryllina*, and *D. pulex*. This affected swim behavior in the sense that as abdomen became more pronounced, immobilization was more prominent. Also affecting swim behavior was the spinal deformities observed in *M. bahia* exposed to FLX and *P. promelas* after exposure to both drugs.

Naturally cannibalistic *M. bahia* exhibited decreases in feeding and aggression when exposed to elevated concentrations of both pharmaceuticals. Cannibalisms occurred at higher concentrations for EE2 and FLX. Aggression decreased with concentration and time in *M. bahia* exposed to both drugs. *P. promelas* began probing less active organisms in both drugs. Organisms in lower concentrations ceased this activity but continued in elevated doses. *P. promelas* FLX aggression increased with time in lower concentrations.

Some aquatic species require aggressive behaviors to define hierarchical status and dominance in reproduction. Modifications in any essential behaviors as well as transformations in growth and development during sensitive periods of reduction could lead to genetically less desirable organisms to mate. Arguably less desirable or unhealthy offspring would be produced thus upsetting the balance of natural selection. The two species of water fleas (*C. dubia* and *D. pulex*) exhibited different mortality curves but both displayed induction of fecundity when

exposed to EE2. Although *M. bahia* demonstrated a similar in mortality curve to *C. dubia*, mysid did not exhibit any offspring after EE2.

Ethinyl estradiol accelerated maturation in lower concentrations. Because the test organisms, have relatively short life cycles, are not supposed to mature this fast. Higher concentrations increased maturity a slightly but delayed growth possibly by decreasing the ability to molt. The lower concentrations produced larger *C. dubia*, *D. pulex*, and *M. bahia*. Organisms exposed to higher concentrations of EE2 and FLX lacked pigment, except one *M. beryllina* exposed to EE2 in which case exhibited maturity by coloration.

The hypothesis was that complex organisms would exhibit less lethality at higher concentrations. As this hold true in some cases. *P. promelas* was the most resilient species based on similar concentrations and the EC50. *D. pulex* was the most resilient in terms of 100% and Ch.V. *C. dubia* was the most sensitive in terms of similar concentration, 100% mortality, EC50, and Ch.V.

The results in the present study confirm pharmaceuticals can produce lethality of non-target organisms through acute exposure. Similar mortality patterns were observed between *C. dubia* and *M. bahia* and between *P. promelas*, *D. pulex*, and *M. beryllina*.

It was expected that similar species would exhibit similar mortalities to one another. While exposure to EE2 caused *C. dubia* and *M. bahia* (both crustaceans) to exhibit similarities in terms of similar dose-response, EC50s, and Ch.V.s, the *D. pulex* (the other species of water flea) was much more resilient and in some cases the most resilient organism. The two fish species had similarities in the dose response curve. At 3000 ppb *M. beryllina* had 100% mortality while *P. promelas* had only 40% mortality making it very resilient. Interestingly at the lesser concentrations, 100 - 800 ppb both fish exhibited identical hormetic changes in mortality. It

appears that *D. pulex* sensitivities were in between these two fish in similar concentrations and EC50. The sensitivities were higher than fish in 100% and Ch.V. In all cases though, *M. beryllina* was more sensitive than *P. promelas* and *D. pulex*. In all cases *C. dubia* was most sensitive with *M. bahia* being the second most sensitive.

Exposure to FLX caused *C. dubia* to be the most sensitive in 100%, similar concentration, and EC50. In Ch.V. *M. bahia* was the most sensitive with *C. dubia* being 3<sup>rd</sup>. The most resilient species in 100% and EC50, and Ch.V. was *M. beryllina*, The most resilient based on similar concentrations was *D. pulex*. In 100% *D. pulex* and *M. bahia* (crustaceans) were similar along with *M. beryllina*. All these were more resilient than *C. dubia* and *P. promelas*. In similar concentrations, *D. pulex* had similar mortalities to the two fish which were more sensitive than the water flea. The *C. dubia* and *M. bahia* were similar. *D. pulex* and *P. promelas* were similar in EC50 with the other freshwater species being most sensitive. However, *C. dubia* had an EC50 closer to *P. promelas*. The two marine species, *M. bahia* and *M. beryllina* were similar in EC50s.

It has been apparent that these pharmaceuticals not only elicit lethality but also abnormal behaviors that have a number of possible explanations or in some cases, cannot be explained. Simply observing lethality alone is not a sufficient enough endpoint to determine potential effects on environment. It is the unpredicted effects that are causing harm to ecosystems. Ethinyl estradiol and fluoxetine have the potential to alter serotonin, dopamine, and estrogenic hormones. Therefore, biological activities encompassing these chemicals could explain the various and in some cases, similar behaviors/abnormalities observed in the current study. Abnormal behaviors observed in all exposed organisms included unresponsiveness, irregular swim patterns, erratic activity, convulsions, and changes in aggression. Generally speaking organisms exposed to ethinyl estradiol and fluoxetine demonstrated lethargic behavior which

became more pronounced as time and concentration increased. All in all, as concentration increase immobilization became more prominent. Generally, observed swim bladder size increased as concentrations increased.

Traditional toxicity tests are designed usually designated specific mortality endpoints to assess. More specific toxicity analysis should be developed implementing the available knowledge that is generated during the pharmaceutical drug development process (e.g. mechanisms of action, pharmacokinetic behavior and metabolism, target organs and side effects in mammals).

Aside from the major mode of action, which in many cases are completely understood for humans, pharmaceuticals have multiple minor modes of actions. However, given what we do know about the drugs, experiments should be targeted towards known target modes of action based on the assumption of similar modes of action in different species. Although a pharmaceutical is projected to execute a specific pathway does not mean it will; the present study has confirmed this idea. Nontarget tissues and organs can become disrupted complicating the strategy to analyze potential effects.

Although animals are exposed to a multitude of chemical combinations, studies rarely give effects on mixtures because it is difficult to evaluate which drug may cause which unanticipated effect. It is important to understand how these drugs act as single exposure before taking into account mixtures. These time consuming tests are much more complex, extensive, and expensive and usually only justifiable at environmentally present concentrations. Also mixture effects are most often anticipated by the idea of concentration addition in which the combined toxicity is derived from the addition of the individual concentrations of each compound. With this said, in the current study, species exhibited similar sublethal endpoints in

both drugs. With this in mind, hypothetically concentrations that do not illicit lethality can cause sublethal effects and based on additions can occur at environmentally detected levels.

It is difficult to anticipate outcomes based solely upon the drug's intentional function or an organism's classification. Simple observations during acute tests can aid in analysis for environmental risk and eventually aid in quantifying/development for new test regulations. Even though some effects may not be statistically relevant this does not lessen their importance. Even subtle alterations in normal life progressions, control the promise to directly or indirectly affect entire populations or species. Disturbances in the environmental balance can bring about catastrophes or unanticipated deteriorations of entire ecosystems.



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## VITA

Julia Kaye Daigle was born and raised in Lake Arthur, Louisiana. She graduated from Lake Arthur High School in 2002 where she served as valedictorian and class president. She earned her degree in environmental management systems with a concentration in environmental sciences and a minor in chemistry in the fall of 2006 from Louisiana State University. During her undergraduate years she belonged to a number of societies including Alpha Zeta Honor Fraternity, Environmental Management Society for which she served as vice president, Air and Waste Management Association for which she served as vice chair, and the Agriculture Student Association for which she served as a council member and the environmental management systems representative. She was also awarded the Fritz Lang Scholarship and the Undergraduate Research Grant in which she studied “The Effect of Arsenic Upon, *Eichhornia crassipes*, the Common Water Hyacinth” under Dr. Maud Walsh. She contributed her time in various community services including the Baton Rouge Hazardous Waste Day where she served as a Louisiana State University representative, Court Appointed Special Advocates where she served as a fundraising collaborator and mentor, and donated her time to Habitat for Humanity and Volunteers of America.

Julia began working on her Master of Science in the spring of 2007 at Louisiana State University in the School of Plant, Environmental, and Soil Sciences under the direction of Dr. Gary Barbee. Her degree program focused on environmental toxicology and risk assessment to aid in the completion of her thesis project, “The Acute Responses of Aquatic Species to Ethinyl Estradiol and Fluoxetine.”

During her graduate school career she served as a graduate assistant to Dr. Gary Barbee and a teaching assistant in the School of Plant, Environmental, and Soil Sciences. She was awarded Fritz Lang Scholarship for graduate students and membership in the Golden Key Honor

Society. Julia presented her thesis in New Orleans, Louisiana, during the annual Society of Environmental Toxicologists and Chemists convention in the fall of 2009. She plans to graduate in August of 2010 with a Master of Science.