HAZARD ASSESSMENT OF A MIXTURE OF PHARMACEUTICALS DETECTED IN THE UPPER TENNESSEE RIVER ON *DAPHNIA MAGNA* AND THE DETERMINATION OF RIVERINE ZOOPLANKTON ASSEMBLAGE AS IT RELATES TO CHITOBIASE ACTIVITY

By

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ABSTRACT

In order to respond to the need for hazard assessment of environmentally relevant pharmaceutical mixtures, 21-d life cycle tests were performed on a mixture of pharmaceuticals found in the Tennessee River using *Daphnia- magna* as a surrogate. Endpoints measured were time to first brood, reproduction, mortality, and number of neonates produced per adult reproduction day. Reproduction was the only endpoint that showed a significant effect. The LOEC of the pharmaceutical mixture was found at 100 times (100x) greater concentration ($p=0.005$) than what was detected in the Tennessee River. Similarly, the NOEC was 75x ($p=0.150$). Single concentrations of pharmaceuticals within the mixture up to the LOEC were found to have no effect. Thus, no single pharmaceutical was responsible for the mixture LOEC. To determine if chitobiase can be used to predict secondary production, chitobiase activity and zooplankton density and biomass samples were measured in six Tennessee River tributaries.

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LIST OF ABBREVIATIONS

(ANOVA), Analysis of Variance

(ATSM), American Society for Testing Materials

(EA) Environmental Assessment

(EC_{50}), Effect concentration to 50% of the test population

(EU) European Union

(FDA) Food and Drug Administration

(HQ), Hazard Quotient

(ind./L), Individuals per liter

(LC_{50}), Lethal concentration to 50% of the test population

(Lit. MEC) Literature Maximum Environmental Concentration

(Lit. NOEC) Literature No Observed Effect Concentration

(LOEC), Lowest Observed Effect Concentration

(MEC) Maximum Environmental Concentration

(MUF), Methylumbelliferyl Acetate

(MUF-NAG), Methlumbelliferyl-2-acetamido-2-acetamido-2-deoxy-β-D-glucopyranoside

($\mu mol \cdot L^{-1} \cdot h^{-1}$), Micromoles per liter per hour

((µg d.w.)/L), Micrograms of dry weight per liter

(µg/L) micrograms per liter

(NAG) N-acetylglucasamine

(ng/L) nanograms per liter

(NOEC), No Observed Effect Concentration

(RHW) Reconstituted Hard Water

(SSRIs) Selective Serotonin Reuptake Inhibitors

(STPs), Sewage Treatment Plants

(TN. MEC) Tennessee River Maximum Environmental Concentration

(TN. NOEC) Tennessee River No Observed Effect Concentration

CHAPTER I

INTRODUCTION

Pharmaceuticals in the Environment

Awareness of pharmaceuticals in the environment has grown over the past years due to the frequent detection in surface waters worldwide. Studies have been conducted in several countries including, Australia, Brazil, England, and the United States. Through these studies, there have been more than 160 drugs detected in aquatic environments at concentrations in the ng/L to μg/L range (Costanzo, Murby, & Bates, 2005; Heberer, 2002; Kolpin et al., 2002; Stumpf, Ternes, Wilken, Rodrigues, & Baumann, 1999; Ternes, 1998; Ternes, Andersen, Gilberg, & Bonerz, 2002) Note: possibly use semicolons between authors and arrange citations by date throughout. Because pharmaceuticals are designed to be biologically active, they have the potential to affect a large variety of non-target organisms through a variety of physiological consequences.

Pharmaceutical production in most developed countries continues to increase. Some individual drugs are used in quantities of 100 tons or more per year; per capita estimates range from between 50 and 150 grams per year (Nikolaou et al., 2007). In the United States $220,388,509,960 was spent on prescription drugs in 2010. Among all 50 states, Tennessee was ranked 4th for total retail sales of prescription drugs in 2010 (Kaiser Family, 2011). Unless sewage treatment plants (STPs) are improved, increased pharmaceutical use will result in an increase in pharmaceuticals and their metabolites entering surface waters. Because

pharmaceuticals are persistent organic compounds, they are also appearing in ground water (Ankley, Brooks, Huggett, & Sumpter, 2007; Nikolaou, Meric, & Fatta, 2007).

The major pathway for human pharmaceuticals entering the natural environment is STPs (Kummerer, 2001). Human excreta (excreta includes both urine and excrement – the latter refers to waste eliminated from the bowels) is the primary source of pharmaceuticals entering STPs. Many of the pharmaceuticals consumed by humans are not completely metabolized in the body. For example, 90 % atenolol and 15% of sulfamethoxale was shown to be excreted unchanged. (Hirsch, Ternes, Haberer, & Kratz, 1999; Huschek, Hansen, Maurer, Krengel, & Kayser, 2004). Sometimes the pharmaceutical is only slightly transformed into conjugates with endogenous molecules. Conjugates such as glucuronides, the most common conjugate pharmaceutical form, can easily be cleaved during sewage treatment and the original pharmaceutical can then be released into the aquatic environment (Cunningham, 2008; Ternes, 1998). The disposal of unused medication via the toilet or sink is common practice and thus another pathway for pharmaceutical to reaching STPs. About one third of pharmaceuticals sold in Germany are disposed down the drain (Greiner & Ronnefahart, 2003).

At the STP, sewage is treated by a variety of methods. Following sufficient treatment (per environmental laws), the sewage effluent is directly discharge into surface waters. Current environmental laws address many pollutants (e.g., turbidity, pH, thermal, and dissolved oxygen), but pharmaceutical parent compounds or metabolites are not addressed. Hydrophilic, polar pharmaceuticals tend to pass through STPs - typically designed to remove lipophilic pollutants. Horsing et al. (2011) investigated the sorption of 75 pharmaceuticals in STP sludge and found that only 15 of the active pharmaceutical ingredients show a high affinity for the sludge. This creates a continuous release of pharmaceuticals into the aquatic environment. Consequently,

pharmaceuticals are found in the aquatic environment at ng/L and µg/L concentrations (Ashton, Hilton, & Thomas, 2004; Kolpin et al., 2002). For example, Kasprzyk-Hordern (2008) detected 47 pharmaceuticals in two English rivers. The concentration of each pharmaceutical was found to increase at sampling points located downstream of the STP's effluent discharge site. Conversely, STPs have also been shown to dilute the water column and as result pharmaceuticals were found at lower concentrations directly downstream (Conley, Symes, Schorr, & Richards, 2008; Ellis, 2006).

Hazard to Aquatic Organisms

The occurrence of pharmaceutical mixtures in surface water is well documented but studies are scarce regarding the potential effects on aquatic life. Most of toxicity tests have been conducted on single pharmaceuticals. Such tests on zooplankton and fish, have found that effects generally occur at concentrations above those found in surface waters (Brooks et al., 2003; Cleuvers, 2003; Flaherty & Dodson, 2005; Henry, Kwon, Armbrust, & Black, 2004; Marques, Abrantes, & Goncalves, 2004; McKinley & Hazel, 1993; Metcalfe, Koenig, et al., 2003).

Pharmaceutical effects on aquatic organisms vary according to the compound and organism. Pharmaceuticals have been reported to correlate with reduced brood sizes of the cladoceran *Daphnia magna*, a freshwater crustacean, as well as increase mortality at concentrations in the mg/L range for acute tests (Ferrari, Paxeus, Lo Giudice, Pollio, & Garric, 2003; Henry et al., 2004; Henschel, Wenzel, Diedrich, & Fliedner, 1997; Isidori, Lavorgna, Nardelli, Pascarella, & Parrella, 2005; Kim et al., 2007). When *D. magna* were exposed to pharmaceuticals for a 21-day period, effects on reproduction were seen in the mg/L and µg/L ranges (Minagh, Hernan, O'Rourke, Lyng, & Davoren, 2009; Park & Choi, 2008; Stanley,

Ramirez, Chambliss, & Brooks, 2007; Yamashita et al., 2006). Fish such as the *Pimephales promelas* exposed to pharmaceuticals, have experienced a decrease growth at a concentration of 51µg/L of fluoxetine (Payan & Girard, 1977; Stanley et al., 2007). Concentrations of ibuprofen at 1µg/L have shown to change reproduction patterns in *Oryzias latipes* (Flippin, Huggett, & Foran, 2007). Most ranges are magnitudes higher than the ng/L concentrations of pharmaceuticals observed in surface water (Ashton et al., 2004; Conley et al., 2008; Kolpin et al., 2002). Thus, although eco-toxicity tests on pharmaceuticals have increased significantly in recent years, much more research is needed on individual pharmaceuticals and environmentally relevant mixtures of pharmaceuticals (Kolpin et al., 2002).

The global detection of pharmaceuticals in surface water indicates the realistic hazard to aquatic organisms and food web dynamics by these substances must be studied. Since multiple pharmaceuticals are being detected in surface water, there is a need to study the combined or synergistic effect of environmental relevant mixtures and concentrations to determine toxic thresholds to aquatic organisms. By determining the threshold of environmentally relevant mixtures, the risk that these pharmaceutical mixtures may pose to the environment will be determined more accurately.

In 2008, thirteen pharmaceuticals were quantified in the Tennessee River and its tributaries, these pharmaceuticals ranged from from 0.0028 to 0.1757 µg/l (Conley et al., 2008). Chapter II investigates the hazard of these ratios at increased concentrations by performing chronic life cycle toxicity test on the cladoceran *Daphnia magna.*

Chitobiase as a Biomarker for Toxic Effect in Aquatic Environments

It is common practice to assess a stream's health by quantifying samples of benthic invertebrate populations. However, this process takes many months to complete and is costly

due to the amount of labor that is involved in the process (Bailey, Norris, & Reynoldson, 2001; Hanson & Lagadic, 2005; Lenat & Resh, 2001). A fast and inexpensive way to estimate the aquatic invertebrate population would create a way to gauge the arthropod community in a matter of hours or days (Hanson & Lagadic, 2005). This method would allow for community changes to be observed almost instantaneously. Chitobiase is one of two chitinolytic enzymes that are involved in the process of molting by invertebrates (Muzzarelli, 1977). This enzyme is released into the water column and can be measured. Ambient concentration of this enzyme has the potential to symbolize total molting activity or biomass production of arthropod (Hanson & Lagadic, 2005; Sastri & Dower, 2009). Laboratory and field studies have shown a correlation between chitobiase activity within the water column and aquatic invertebrate assemblages (Espie & Roff, 1995; Oosterhuis, Baars, & Breteler, 2000; Sastri & Dower, 2009).

Ambient chitobiase has been measured in the Tennessee River and has been found to fluctuate with seasons (Conley, Schorr, Hanson, Symes, & Richards, 2009). Seasonal variation in zooplankton populations have been recorded in temperate rivers (Tans, Mathoux, Grandjean, & Kestemont, 1998; Threlkeld, 1983). However, correlations between zooplankton populations and chitobiase concentrations in riverine ecosystems have yet to be confirmed. To investigate a possible relationship between chitobiase activity in a riverine environment and zooplankton assemblage, six tributaries to the Tennessee River six creeks were sampled for three seasons; four of six tributaries were sampled for four seasons. Chapter III reports the findings of this study.

CHAPTER II

HAZARD ASSESSMENT OF A MIXTURE OF PHARMACEUTICALS FOUND IN THE UPPERTENNESSEE RIVER ON *DAPHNIA MAGNA*

Introduction

Pharmaceuticals found in aquatic environments over the past years have increasingly become a point of environmental concern. Pharmaceuticals can be human or veterinary therapeutics (Glassmeyer et al., 2005). Due to their physicochemical and biological properties, there is concern about the potential for their impacts on non-target species (Park & Choi, 2008). STPs are a major point source of these compounds. As a result, pharmaceuticals reach surface water and sediments, causing concentrations ranging from ng/L to μg/L (Kummerer, 2001). The natural environment has the potential to degrade pharmaceuticals by biotic and abiotic processes, but STPs may overwhelm these processes by continuously discharging pharmaceutical-laden effluent on a daily basis (Castiglioni et al., 2006; Vieno, Tuhkanen, & Kronberg, 2007). The potential ecological significance of this continual discharge remains largely unknown (Sanderson et al., 2004).

Pharmaceuticals have been detected in surface water around the world including the Tennessee River (Buser, Muller, & Theobald, 1998; Conley et al., 2008; Kolpin et al., 2002; Kummerer, 2001). In the UK, most STPs were found to routinely discharge low quantities of pharmaceuticals into lakes and rivers (Ashton et al., 2004). Kolpin et al. (2002) sampled 139

streams and rivers around the U.S. and detected pharmaceuticals in 80% of those surface waters. A recent study examined a 295 km portion of the Tennessee River from Knoxville, TN to Chattanooga, TN, encompassing three STPs (Conley et al., 2008). That study found thirteen pharmaceuticals in the water column, with concentrations ranging from 0.0013 μg/L to 0.1757 μg/L (Table 1). The Tennessee River watershed is ideal to study given that Tennessee ranks 4th in the nation for prescription drug use (Kaiser Family, 2011) and the river passes many of the most populated regions of Tennessee.

Table 1

Thirteen pharmaceuticals measured in the Tennessee River, listed by frequency of detection (Conley et al., 2008).

	Range (μg/L)	Median (μg/L)	Frequency (%)
Caffeine	0.081-0.176	0.288	92.2
Sulfamethoxazole	0.003-0.033	0.0079	85.9
Carbamazepine	0.002-0.023	0.005	79.7
Trimethoprim	0.002-0.006	0.0056	32.0
Acetaminophen	0.002-0.012	0.0029	13.3
Diltiazem	0.001-0.01	0.019	10.2
Ciprofloxacin	0.004-0.054	0.0069	10.2
Levofloxacin	0.006-0.059	0.0119	6.3
Atorvastatin	0.003-0.101	0.0068	4.7
Lovastatin	0.010-0.1029	0.0035	3.1
Sertraline	0.002-0.012	0.0183	2.3
Fluoxetine	0.003-0.0101	0.007	1.6
Norfluoxetine	0.0028	-	-

Ecological impacts of pharmaceuticals on non-target aquatic organisms have been investigated in the laboratory and to a lesser degree in the field. Current literature on effective concentration (EC_{50}), lethal concentration (LC_{50}), no observed effect concentration (NOEC), and lowest observed effect concentration (LOEC) for individual pharmaceuticals found in the

Tennessee River on aquatic invertebrates are summarized in Table 2. The pharmaceutical that has received the most attention is fluoxetine ((±)-*N*-methyl-3-phenyl-3-[4- (trifluoromethyl) phenoxy]propan-1-amine). Fluoxetine is a serotonin re–uptake inhibitor (SSRI) and is one of the most acutely toxic pharmaceuticals reported for benthic invertebrates (Fent, Weston, & Caminada, 2006). Kolpin et al. (2002) reported that fluoxetine concentrations averaged 0.012 μg/L in US streams. Fluoxetine is primarily excreted by the human body as a glucuronide conjugate and as a result may be cleaved back to fluoxetine during treatment in STPs (Cunningham, 2008). Brooks et al. (2003) found bioaccumulation of fluoxetine in fish tissue. Pery et al. (2008) observed significant effects on growth of *D. magna* at a concentration of 241 μg/L during a 21-day assay. At the same concentration, reproduction was reduced by 32% and mortality was increased by 40%. In the same study, newborns from the 5^{th} brood were exposed to the same treatment as their mothers. Reproduction was reduced significantly at 31 μg/L for that second generation – almost a 10-fold lower dose than what inhibited reproduction in their mother. A 30-d chronic toxicity test conducted by Flaherty and Dodson (2005) showed an increase in the reproduction of *D. magna* exposed to 36 μg/L of fluoxetine. The study also found that when *D. magna* were exposed to fluoxetine and clorfibric acid *individually,* the LC_{50} was 580 μg/L and 106 mg/L, respectively (Henry et al., 2004; Henschel et al., 1997). However, when *D. magna* were exposed to fluoxetine and clorfibric acid *in combination,* lower amounts of fluoxetine and clofibric acid, 36 μg/L and 100 μg/L (respectively) resulted in a 62.5% mortality rate.

The aforementioned studies indicate the need for the investigation of environmentally relevant mixtures in order to adequately assess environmental risk. Indeed, while the above studies indicate a potential hazard to aquatic organisms, more endpoints and long-term studies

are essential to fully comprehend the ecological risks and impacts of compounds on aquatic organisms and their communities (Brooks et al., 2003; Dussault, Balakrishnan, Sverko, Solomon, & Sibley, 2008; Sanderson et al., 2004). Specifically, chronic exposure tests and the potential synergistic or antagonistic effects have been under investigated.

Table 2

Summary of EC_{50}, LC_{50}, NOEC and LOEC on Daphnia species for individual pharmaceuticals found in the Tennessee River.

Pharmaceutical/ species	Group	Endpoint/duration	Conc. (mg/L)	(Reference)
Caffeine	Stimulant			
D. magna		reproduction/EC_{50}/17-19-d		(Olmstead and LeBlanc, 2005)
Sulfamethoxazole	Antibiotic			
D. magna		LC_{50}/24-h	25.2	(Isidori et al., 2005)
D. magna		LC_{50}/48-h	189.2	(Kim et al., 2007)
D. magna		LC_{50}/48-h	>100.0	(Ferrari et al., 2003)
D. magna		LC_{50}/96-h	177.3	(Kim et al., 2007)
Carbamazepine	Anticonvulsant			
D. magna		Immobilization/EC_{50}/48-h	>100.0	(Cleuvers, 2003)
D. magna		LC_{50}/48-h	>100.0	(Kim et al., 2007)
D. magna		LC_{50}/48-h	111.0	(Han et al., 2006)
D. magna		LC_{50}/48-h	>100.0	(Cleuvers, 2003)
D. magna		LC_{50}/48-h	111.0	(Sanderson et al., 2003)
D. magna		LC_{50}/96-h	76.3	(Kim et al., 2007)
D. pulex		time to first brood/LOEC/21-d	0.2	(Lurling et al., 2006)
D. pulex		reproduction/NOEC/21-d	0.2	(Lurling et al., 2006)
Trimethoprim	Antibiotic			
D. magna		LC_{50}/48-h	167.4	(Kim et al., 2007)
D. magna		LC_{50}/48-h	123.0	(Halling-Sorensen et al., 2000)
D. magna		LC_{50}/48-h	92.0	(Park and Choi, 2008)
D. magna		LC_{50}/48-h	>123.0	(Stuer-Lauridsen et al., 2000)
D. magna		LC_{50}/96-h	120.7	(Kim et al., 2007)
D. magna		reproduction/NOEC/21-d	6.0	(Park and Choi, 2008)

Table 2 cont.

Pharmaceutical/ species	Group	Endpoint/duration	Conc. (mg/L)	(Reference)
D. magna		reproduction/LOEC/21-d	20.0	(Park and Choi, 2008)
D. magna		time to first brood/NOEC/21-d	6.0	(Park and Choi, 2008)
D. magna		time to first brood/LOEC/21-d	20.0	(Park and Choi, 2008)
Acetaminophen	Analgesic			
D. magna		LC_{50}/48-h	30.1	(Kim et al., 2007)
D. magna		LC_{50}/48-h	9.2	(Kuhn et al., 1989)
D. magna		LC_{50}/48-h	50.0	(Henschel et al., 1997)
D. magna		LC_{50}/48-h	20.0	(Han et al., 2006)
D. magna		LC_{50}/48-h	42.0	(Sanderson et al., 2003)
D. magna		LC_{50}/96-h	26.6	(Kim et al., 2007)
Diltiazem	Calcium Channel Blocker			
D. magna		LC_{50}/48-h	28.0	(Kim et al., 2007)
D. magna		LC_{50}/96-h	26.6	(Kim et al., 2007)
Ciprofloxacin	Fluoroquinolone Antibiotic			
D. magna		NOEC/4- h	10.0	(Robinson et al., 2005)
D. magna		NOEC/48-h	60.0	(Halling-Sorensen et al., 2000)
Levofloxacin	Fluoroquinolone Antibiotic			
D. magna		NOEC/48-h	10.0	(Robinson et al., 2005)
D. magna		reproduction/EC_{50}/21-d	0.34	(Yamashita et al., 2006)
D. magna		reproduction/NOEC/21-d	0.31	(Yamashita et al., 2006)
D. magna		reproduction/LOEC/21-d	0.63	(Yamashita et al., 2006)
Sertraline	SSRI			
D. magna		LC_{50}/24-h	3.1	(Minagh et al., 2009)
D. magna		LC_{50}/48-h	1.3	(Minagh et al., 2009)
C. dubia		LC_{50}/48-h	0.12	(Henry et al., 2004)

Table 2 cont.

Pharmaceutical/ species	Group	Endpoint/duration	Conc. (mg/L)	(Reference)
D. magna		Immobilization/EC_{50}/48-h	0.92	(Christensen et al., 2007)

Table 2 cont.

C. dubia		reproduction/NOEC/8-d	0.045	(Henry et al., 2004)
D. magna		reproduction/LOEC/8-d	0.009	(Henry et al., 2004)
D. magna		LC_{50}/21-d	0.12	(Minagh et al., 2009)
D. magna		Mortality/NOEC/21-d	0.032	(Minagh et al., 2009)
D. magna		Mortality/LOEC/21-d	0.1	(Minagh et al., 2009)
D. magna		reproduction/EC_{50}/21-d	0.066	(Minagh et al., 2009)
D. magna		reproduction/NOEC/21-d	0.032	(Minagh et al., 2009)
D. magna		reproduction/LOEC/21-d	0.1	(Minagh et al., 2009)
Fluoxetine	SSRI			
C. dubia		LC_{50}/48-h	0.58	(Henry et al., 2004)
D. magna		Immobilization/EC_{50}/48-h	13.0	(Christensen et al., 2007)
C. dubia		reproduction/NOEC/8-d	0.447	(Henry et al., 2004)
C. dubia		reproduction/LOEC/8-d	1.789	(Henry et al., 2004)
D. magna		reproduction/LOEC/30-d	0.032	(Flaherty and Dodson, 2005)
S-fluoxetine				
D. magna		reproduction/NOEC/21-d	0.195	(Stanley et al., 2007)
D. magna		reproduction/LOEC/21-d	0.444	(Stanley et al., 2007)
R-fluoxetine				
D. magna		reproduction/NOEC/21-d	0.17	(Stanley et al., 2007)
D. magna		reproduction/LOEC/21-d	0.429	(Stanley et al., 2007)

[1]*NOEC, no observed effect concentration.*
[2]*LOEC, lowest observed effect concentration.*

Aquatic organisms are routinely exposed to complex mixtures of pharmaceuticals at low concentrations (Castiglioni et al., 2006; Kolpin et al., 2002). However, as indicated above, most researchers perform bioassays using single pharmaceutical exposures. For example,

sulfamethoxazole, carbamazepine, trimethoprim, and acetaminophen have been tested individually on daphnids (Cleuvers, 2003; Dussault et al., 2008; Grung, Kallqvist, Sakshaug, Skurtveit, & Thomas, 2008; Kim et al., 2007). In reality, these compounds occur in combination in surface waters (Castiglioni et al., 2006; Conley et al., 2008; Kolpin et al., 2002), and mixtures should be analyzed to determine the potential effects of these pharmaceutical mixtures as they occur in the environment on aquatic organisms and investigate sub-lethal effects. Currently, the Food and Drug Administration (FDA, 1998) examines a drug for toxic effects at several levels. If the compound is found to have properties that suggest it will be degraded at a high rate in the environment and does not harm microbes in STPs, then no further examination is undertaken. Otherwise, the FDA employs a three tier environmental assessment (EA) to asses the impact of the drug. The first two tiers of the EA measure acute toxicity by performing three assays on three types of species, typically a fish, an invertebrate, and an alga. As long as no sub-lethal effects occur, the LC50 for the most sensitive species is divided by the maximum estimated environmental concentration. If this value is greater than or equal to 1000 in the first tier or 100 in the second tier, no further toxicity assays are performed (FDA, 1998). It is therefore possible for a pharmaceutical to pass FDA guidelines without undergoing a chronic toxicity evaluation, which is the last tier in the guideline. At this time, the US Environmental Protection Agency does not have any environmental testing requirements regarding human consumption or excretion of pharmaceuticals.

In this study D. magna was selected for the aquatic toxicology bioassay. *D. magna* are freshwater zooplankton in inland water ecosystems around the world and play a significant role in aquatic food webs (Ternes et al., 2002). Moreover, they are sensitive to foreign chemicals and undergo rapid reproduction (Flaherty and Dodson, 2005). Indeed, they have been showed to be

more susceptible to effects from pharmaceutical exposure than other aquatic organisms such as the (*Oryzias latipes*) a Japanese killifish (Kim et al., 2007).

Materials and Methods

To evaluate the aquatic toxicity of the pharmaceutical mixture detected by Conley et al. (2008), a single species 21-d chronic laboratory toxicity test with *D. magna* was conducted (American Society for Testing, 2002). After the NOEC and LOEC were determined, single pharmaceuticals were then tested at the concentration in which they appeared during the mixture LOEC toxicity test. These pharmaceuticals in the LOEC mixture were tested individually in order to determine if one of the pharmaceuticals was solely contributing to the results of the mixture assay. Sub-lethal endpoints of toxicity were chosen to evaluate effects on the normal life cycle of *D. magna.* Hazard quotients (HQs) were then performed on the NOEC for the mixture and single pharmaceutical. Safety factors were applied to HQs values to account for additional stressful interactions that occur in the environment that are not addressed in a laboratory setting. A safety factor of 10 was applied (as recommended by the FDA) and a safety factor of 100 was also applied as recommended by European standards (EMEA, 2003; FDA, 1998).

D. magna Cultures

D. magna individuals were obtained from Aquatic BioSystems, Fort Collins, Co. in August of 2009. An in-laboratory mother culture was created from those individuals and maintained in an incubator set at 23^{o}C with a 16:8 light to dark cycle. Mother cultures were housed in 1L beakers with approximately 40 adults per beaker. Culture media was renewed twice a week and if neonates were produced they were removed from the mother culture. All

culture water was tested to meet physiochemical requirements set in American Society for Testing and Materials (ASTM) 2002 guidelines (e.g., pH and hardness).

Chronic Life Cycle Assay

In order to assess the effects of an 11- pharmaceutical mixture to *D. magna,* life-cycle toxicity assays were performed according to ASTM guidelines (ATSM, 2002). When a life cycle assay was ready to begin, seven or eight *D. magna* (less than a week old) were removed from the rest of the mother culture and were placed in individual chambers and allowed to reproduce (parthenogenically). Neonates from the 3rd through 7th brood were then used from these *D. magna* to start chronic toxicity assays. Eleven of those neonates less than 24 hours old were randomly transferred to individual test chambers containing 160 ml of test solution. Five endpoints of toxic effect were measured in the present study: (1) length of *D. magna* at the conclusion of the assay, (2) survival of the first generation, (3) total number of neonates produced (reproduction), (4) time to the 1st brood, and (5) number of young produced per adult female reproduction day. These five endpoints were used to calculate the Lowest Observable Effect Concentration (LOEC) and No Observable Effect Concentration (NOEC).

The test solutions were identical in ratio to the mixture of pharmaceuticals (at maximum concentrations) that were detected in the Tennessee River (Table 1) (Conley et al., 2008). Nominal test solutions were 10x, 25x, 50x, 75x, 100x and 1000x (the maximum concentration) of each pharmaceutical detected in the Tennessee River. These concentrations ranged from a single pharmaceutical concentration of .01 μg/L (Diltiazem/Fluoxetine) to 176 μg/L (Caffeine) and a total concentration of 4.96 μg/L to 496 μg/L, respectively (Table 3). For example, the maximum concentration of caffeine detected by Conley et al. (2008) was 0.176 μg/L . The 10x mixture concentration contained 1.76 μg/L of caffeine and the 10x maximal values of the other

10 pharmaceuticals found in the Tennessee River Conley et al. (2008) resulting in 4.96 µg/L of total pharmaceuticals in the 10x test solution (Table 3). After the 21-d lifecycle LOEC of the mixture was determined, 21-d life cycle tests on single pharmaceuticals were conducted.

Table 3

Maximum concentration of 11 pharmaceuticals detected in the Tennessee River by Conley et al., (2008) and the corresponding test concentrations used in the present study. The 10x mixture contained 10 times the maximum concentration of each pharmaceutical detected. Accordingly, the 25x contained 25 times the maximum concentration of each pharmaceutical detected, and so forth.

	Detected (µg/L)	10x (µg/L)	25x (µg/L)	50x (µg/L)	75x (µg/L)	100x (µg/L)	1000x (µg/L)
Caffeine	0.176	1.760	4.400	8.800	13.200	17.600	176.000
Sulfamethoxazole	0.033	0.330	0.825	1.650	2.475	3.300	33.000
Carbamazepine	0.023	0.230	0.575	1.150	1.725	2.300	23.000
Trimethoprim	0.006	0.060	0.150	0.300	0.450	0.600	6.000
Acetaminophen	0.012	0.120	0.300	0.600	0.900	1.200	12.000
Diltiazem	0.01	0.100	0.250	0.500	0.750	1.000	10.000
Ciprofloxacin	0.054	0.540	1.350	2.700	4.050	5.400	54.000
Levofloxacin	0.059	0.590	1.475	2.950	4.425	5.900	59.000
Atorvastatin	0.101	1.010	2.525	5.050	7.575	10.100	101.000
Sertraline	0.012	0.120	0.300	0.600	0.900	1.200	12.000
Fluoxetine	0.010	0.100	0.250	0.500	0.750	1.000	10.000
Total Concentration (µg/L)	0.496	4.960	12.400	24.800	37.200	49.600	496.000

Maintenance and Monitoring

D. magna (n=11) were individually exposed (each in its own chamber) for 21 days to a mixture of pharmaceuticals or single concentrations of pharmaceuticals (Table 3). The control

organisms were treated identically (n=11, housed individually), but exposed to only reconstituted hard water (RHW). A solvent control was applied in the same manner only with the exception of a solvent added to the medium. Methanol was used as a solvent for the pharmaceuticals that were not water soluble with a maximum concentration of 0.03 µl/L used during the 1000x test. Test and control chambers were 80 x 65 (w x h) mm glass wide-mouth jars (Jarden Home Brands; Daleville, IN). Each toxicity assay was a static-renewal exposure lasting for 21 days wherein the pharmaceuticals were renewed three times a week. Solutions were prepared the day of renewal by serial dilution with RHW, solvent (if necessary), and pharmaceuticals. Feeding consisted of suspensions of green alga (*Selenatrum capricornutm*) and 50 µl TetraMin fish food. Depending on the age of the *D. magna,* the algal ration varied from 4.8×10^4 cells/ml for days 0 to 3, 5.1×10^4 cells/ml for days 4 to 5, 5.8×10^4 cell/ml for days 6 to 7, 7.7×10^4 cell/ml, for days 8 to 9, and 9.6×10^4 cell/ml for days 10 to 21 (Phillips, Dinglasan-Panlilio, Mabury, Solomon, & Sibley, 2010). Reproduction (number of neonates) was monitored every day but neonates were only removed when solution was renewed. Neonates were removed via a large-mouth pipet and concentrated on filter paper where they could be accurately counted. After the conclusion of the experiment adult body size was measured from top of the head to base of tail Lopes et al. (2009), using a Peak glass scale under an Olympic CX-31 compound microscope. *D. magna* were measured to the nearest mm. Mortality was monitored each day until the conclusion of the experiment.

Hazard Assessment

Environmental hazard posed by the mixture and single pharmaceuticals found in the Tennessee River was assessed by the calculation of hazard quotients (HQs). HQs are designed to show the potential effects that the mixture and single pharmaceuticals potentially have on the

aquatic environment (FDA, 1998, EPA, 2008). The HQ was mathematically calculated by using the following equation:

$$HQ = \frac{MEC}{NOEC} \quad 2.1$$

Where MEC represents the maximum environmental concentration in the Tennessee River and NOEC represents the no observed effect concentration for the most sensitive endpoint. The MEC found in the Tennessee River was used for single pharmaceuticals and mixture HQ determination along with the highest NOEC to ensure a conservative estimate of hazard. An HQ value <1 indicates toxicity is not likely to occur where as an HQ value >1 indicates an apparent hazard to *D. magna* (Han, Hur, & Kim, 2006). Safety factors of 10 and 100 were applied to the HQ values to account for interspecies variability and interaction that may cause stress on organisms in the environment that are not assessed in a laboratory setting (Robinson, Belden, & Lydy, 2005).

Statistics

Effects of various pharmaceuticals concentrations on different endpoints were compared using one-way repeated-measures analysis of variance (ANOVA) followed by a post-hoc Tukey test ($\alpha = 0.05$). Normality of data was checked using a Shapiro-Wilk test. Reproduction data (raw values) were normalized by converting to percent of control. This allowed for direct comparisons between life cycle assays that were performed at different times. All statistical analyses were conducted using Statistical Analysis System (SAS Inc., 2008).

Results

Pharmaceutical Mixture Chronic Assay

Of the five endpoints evaluated (length, survival of the first generation, reproduction, time to the first brood, and number of young produced per adult female reproduction day), only reproduction was significantly affected in the mixture life cycle assay. Criteria for acceptable *D. magna* control survival (>70%) and reproduction (>60 neonates per female) were met during all assays (ASTM, 2002). The number of young produced per female reproduction day stayed moderately similar at nine to ten for control and lower concentrations. At the 100x concentration, number of young produced per female reproduction day dropped slightly to 8.6 neonates (88% of control, $p > 0.05$). In the 1000x concentration, number of young produced per female reproduction day dropped significantly to 2.1 (28% of control, $p < 0.0001$) neonates per female reproduction day (Fig. 1). Fecundity was significantly reduced at 100x (49.6 μg/L total concentration; $p = 0.005$) and 1000x (496.0 μg/L total concentration; $p > 0.0001$). No observed effects were seen in mixture treatments that were 75x and lower, resulting in a NOEC of 75x (37.2 μg/L total concentration of pharmaceuticals) (Fig. 2).

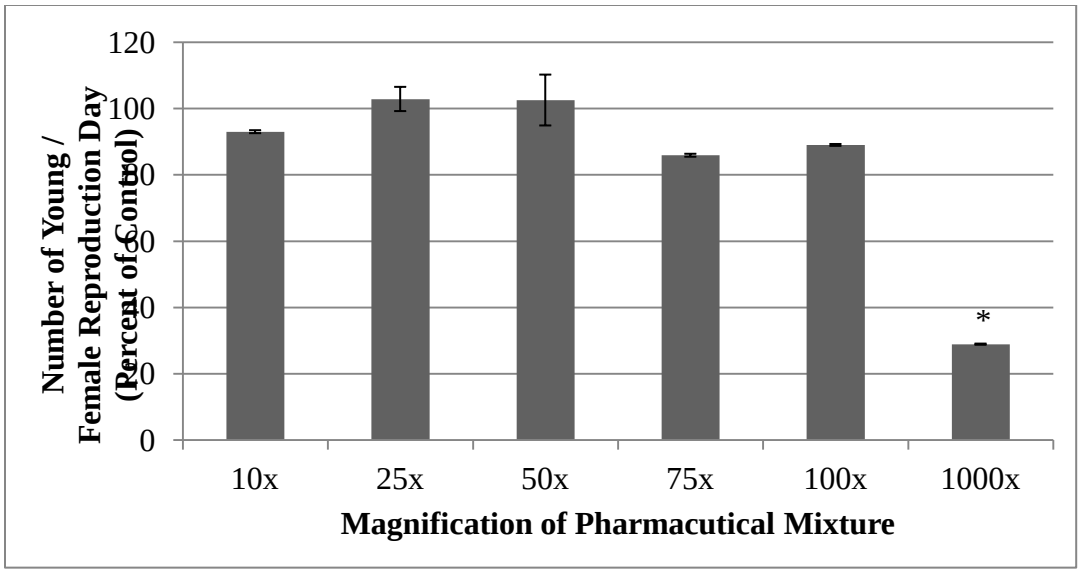

Figure 1

Mean numbers of young produced per female reproduction day are express as the percent of the control value. An asterisk indicates that the mean response was significantly different from that of the control ($p \leq 0.05$). Error bars represent standard errors.

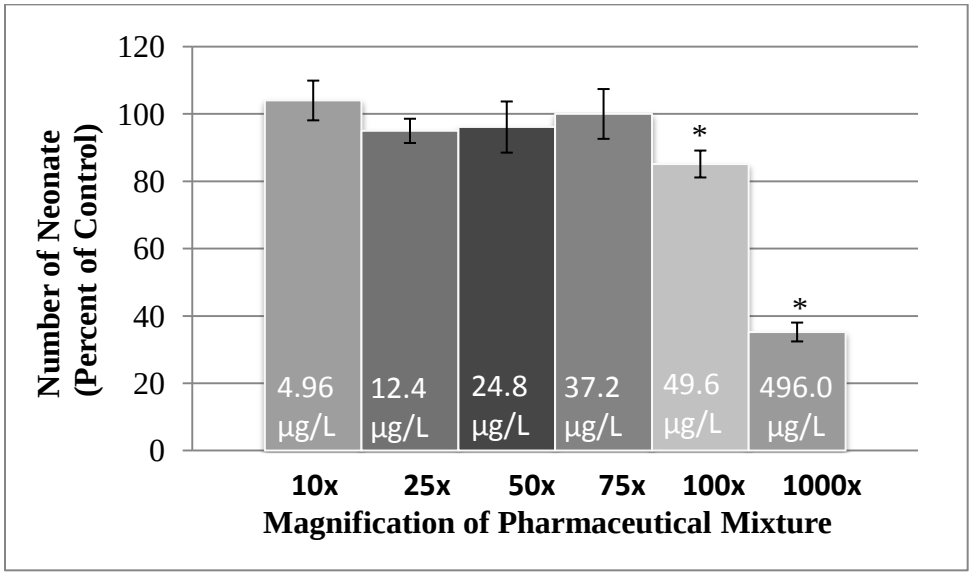

Figure 2

Effects of pharmaceutical mixtures on the reproduction of *D. magna*. Mean numbers of *D. magna* neonates produced are expressed as the percent of the control during a 21-day life cycle assay. An asterisk indicates that the mean response was significantly different from that of the control ($p \leq 0.05$). Error bars represent standard errors.

Individual Chronic Assay

When the LOEC (100x) was observed for the mixture of pharmaceuticals, life cycle test were preformed on individual pharmaceuticals to determine if one or more pharmaceutical might be driving the decrease in reproduction. Assays were performed using the individual pharmaceutical concentrations that were present in the LOEC for the mixture assays. All single pharmaceuticals were statistically similar to their control for all endpoints ($p > 0.05$). However, ciprofloxacin and levofloxacin showed a non-significant decrease in reproduction with 87.57% and 88.27% of control, respectively. Caffeine, sulfamethoxazole, carbamazepine, trimethoprim, sertraline and fluoxetine showed a slight increase in reproduction when compared to control treatments (Fig. 3)

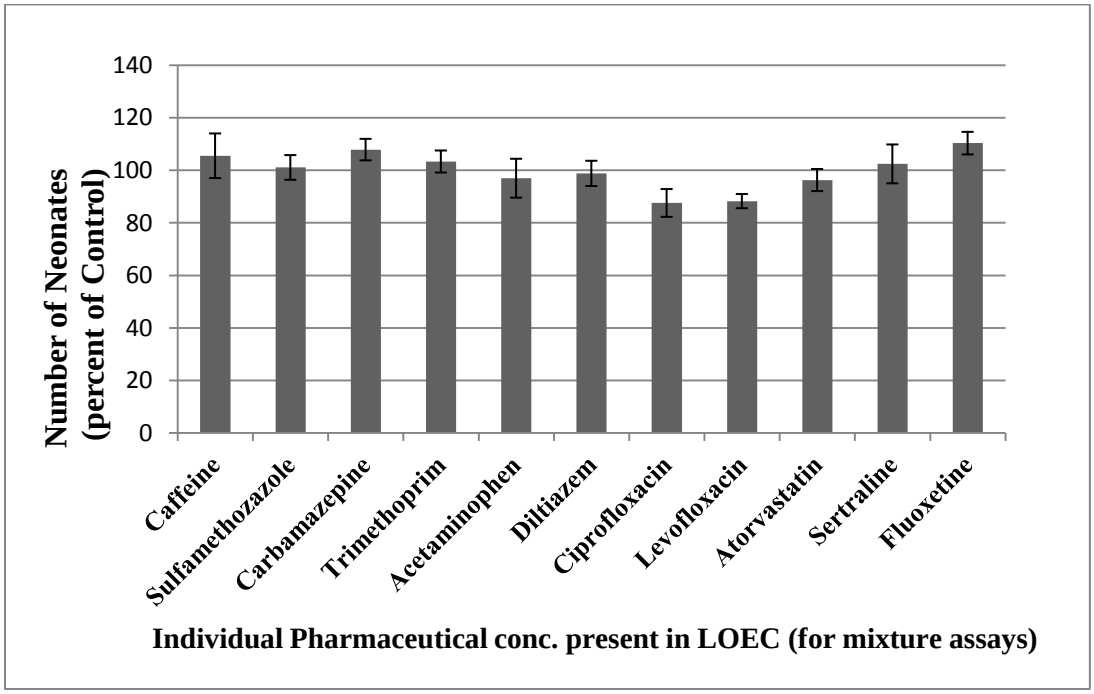

Figure 3

Single pharmaceutical assays results for *D. magna* reproduction. Numbers of *D. magna* neonates produced are expressed as percent of control values during a 21-day life cycle assay. No statistical significance was observed for any of the single pharmaceuticals at their concentration in the LOEC for the mixture. Error bars represent standard error.

Hazard Assessment

Hazard quotients (HQs) were calculated in order to estimate the hazard that individual pharmaceuticals and a mixture of pharmaceuticals may pose for daphnids in the Tennessee River. When all pharmaceuticals were considered as a mixture, the HQ, with a safety factor of 100, was >1 (Table 4). Moreover, the individual pharmaceuticals caffeine, sulfamethoxazole, and diltiazem also had HQ values > 1 when a safety factor of 100 was applied to the pharmaceutical maximum environmental concentration found in the Tennessee River (TN. MEC) (Table 4). This is due to the conservative NOEC numbers for the single pharmaceuticals used in the HQ calculation. The actual NOEC for single pharmaceuticals may be orders of magnitude higher then what was estimated in the current study. Four of the 11 pharmaceuticals in the present study have been studied previously for effects on *D. magna* 21-day reproduction. In those studies, carbamazepine, trimethoprim, levofloxacin, and sertraline produced NOEC at 200 µg/L, 6,000 µg/L, 310 µg/L, and 32 µg/L, respectively (Lurling et al., 2006, Minagh et al., 2009, Park and Choi, 2008, Yamahita et al., 2006). Those values are orders of magnitude higher than the present study estimates of NOEC. When these NOEC values for the four pharmaceuticals were used to calculate HQ, a more realistic value was produced. In this study the HQs for all four pharmaceuticals was < 1 even *after* a safety factor of a 100 was applied (Table 2). When MECs from around the world were combined with the NOEC values observed herein, an HQ >1 was produced (1.9) – *without* the addition of a safety factor (Table 4).

Table 4

Hazard quotients (HQs) for *D. magna* when calculated using the highest Tennessee River measured environmental concentration (TN. MEC) with the no observed effect concentration observed for reproduction in the present 21-day study (TN. NOEC). Values >1 indicate an apparent hazard to *D. magna.* Literature MEC (Lit. MEC) are maximum concentrations detected globally. The Lit. NOEC is the NOEC value taken from the literature (except for the Mixture value).

Pharmaceutical	TN. MEC/ TN. NOEC	Safety factor of 10	Safety factor of 100	TN.MEC/Lit. NOEC	Lit. MEC/ Lit. NOEC
Caffeine	0.0100	0.1002	1.0017		0.3415[e]
Sulfamethoxazole	0.0100	0.1000	1.0000		1.5758[f]
Carbamazepine	0.0100	0.0996	0.9957	0.0001[a]	1.0823[g]
Trimethoprim	0.0095	0.0952	0.9524	$1x10^{-6}$ [b]	1.1270[e]
Acetaminophen	0.0098	0.0976	0.9756		8.1301[e]
Diltiazem	0.0103	0.1031	1.0309		0.0103[k]
Ciprofloxacin	0.0100	0.0996	0.9963		0.0066[e]
Levofloxacin	0.0099	0.0995	0.9949	0.0002[c]	0.0017[i]
Atorvastatin	0.0100	0.0997	0.9970		0.059[j]
Sertraline	0.0097	0.0968	0.9677	0.0004[d]	0.4597[h]
Fluoxetine	0.0099	0.0990	0.9901		0.0337[g]
Mixture	0.0133	0.1329	1.3294		1.9171[l]

[a] NOEC Lurling et al. (2006)
[b] NOEC Park and Choi (2008)
[c] NOEC Yamashita et al. (2006)
[d] NOEC Minagh et al. (2009)
[e] MEC Kolpin et al. (2002)
[f] MEC Cahill et al. (2004)
[g] MEC Sadezky et al. (2010)
[h] MEC Thomas and Hilton (2004)
[i] MEC Metcalfe et al. (2003)
[j] MEC Lee et al. (2009)
[k] MEC Conley et al. (2008)
[l] MEC present study

Discussion

Pharmaceutical Mixture Toxicity

When assessing hazard of a compound, the more conservative estimate is safest for the environment. Therefore, the present experiment and HQ calculations were designed to represent the worst-case scenario of the pharmaceutical mixture found in the Tennessee River. All pharmaceuticals tested in the present mixture were not detected in every surface water sample, nor was every sample detected at the maximum concentration (Conley et al., 2008). The results from the present study indicate that the mixture of pharmaceuticals has a lower LOEC than any of the individual pharmaceuticals separately, when considering reproduction as the endpoint (Fig.2). This is likely due to the fact that the total amount of xenobiotic the *D. magna* were exposed to in the mixture study was 49.6 µg/L (in the LOEC) (Fig. 2) versus the single pharmaceutical study which ranged from 0.6 µg/L to 17.6 µg/L. This indicates that there is some type of additive effect. It is not known if there is some antagonistic effect occurring. For example, 10 of the pharmaceuticals could work in an additive manner, while one works in an antagonistic manner, thus reducing the total additive effect of the 10 pharmaceuticals. Thus, it is possible that fewer pharmaceuticals may have a greater effect (not likely), but this could not be determined in the present study. The fact that reproduction was the only endpoint significantly reduced is not unexpected because reproduction has been shown to be one of the most sensitive endpoints in *D. magna* (Minagh et al., 2009). In the 100x concentration, reproduction was reduced to 85% of control. The 1000x concentration caused reproduction to be reduced to 35% of control indicating reproduction was affected in a dose dependent manner by the mixture. This drop in the total number of neonates is proportional to the number of young per adult female reproduction days observed in 100x and 1000x concentrations (Fig.2). The results here support

previous studies reported by Park and Choi (2008) that have shown that reproduction is dose dependent. These workers found a dose dependent response on population effects when investigating the aquatic toxicities of eleven antibiotics on *D. magna*. Accordingly, Cleuvers (2008) conducted a study investigating the chronic effects of naproxen, ibuprofen, and diclofenac mixture on *D. magna* and found that reproduction was also reduced in a dose dependent manner by the mixture. That study also showed that the highest concentrations of 22.62 mg/L, 22.97 mg/L, and 64.18 mg/L of diclofenac, ibuprofen, and naproxen, respectively, reduced reproduction by 100%. This is noteworthy due to the fact that mortality was not observed at this concentration. Data such as these reaffirm the need to use sub-lethal parameters as endpoints to address the pharmaceuticals' potential environmental risk.

The HQ for the pharmaceutical mixture of this study (calculated using Tennessee River MECs) was 0.013. When a safety factor of 10 was applied the mixture HQ was still below 1. This indicates that the mixture of pharmaceuticals currently detected in the Tennessee River poses an insignificant hazard for *D. magna* at concentrations 10x greater than what is currently maximally detected – according to FDA guidelines (FDA, 1998). Furthermore, a safety factor of 100 had to be applied before the HQ value exceeded 1. European Union (EU) surface water hazard assessments commonly apply a safety factor of 100 to HQs (EMEA, 2003). Thus, the mixture of pharmaceuticals currently detected in the Tennessee River poses an apparent hazard for *D. magna* at 100x if EU standards are applied. The fundamental purpose of the addition of safety factors is to account for stressors in the environment that cannot be duplicated or accounted for in the laboratory – especially in lower-tier hazard assessments such as the present study (Solomon et al., 2008). While safety factors (also referred to as uncertainty factors) are

somewhat arbitrary in their numerical value, they have utility in presenting a conservative estimate.

Because pharmaceutical use occurs worldwide, it is appropriate to use MECs found outside of the Tennessee River. If the worldwide MEC (where measurements have been taken) is applied to the NOEC found in the current study for the mixture, the HQ would be greater than 1 with no safety factor applied (Table 4). Thus, in surface waters that approach the worldwide MEC for all pharmaceutical in the present mixture, there is a hazard to *D. magna*. This is a very conservative assessment. However, this HQ does not account for any of the uncertainties associated with laboratory to field extrapolation. Nor does this HQ consider the increasing human population, the increasing age of the human population, increasing pharmaceutical use, and additive effects of potentially hundreds of pharmaceuticals. Therefore, while the scenario of combined MECs co-occurring is unlikely, one still needs to consider all of the present and future variables before discounting the hazard illustrated in the present mixture HQ calculations.

Individual Pharmaceutical Toxicity

The main purpose of conducting the studies on the singular pharmaceuticals was to determine if one or more of the single pharmaceuticals were solely responsible for reduced reproduction. No single pharmaceutical was the driving factor (Fig. 3) (i.e., no singular pharmaceutical was as toxic as the mixture) (Table 4). All HQs values were <1 for MECs - even when a safety factor of 10 was applied. It should be noted that further tests to define a more accurate NOEC or LOEC were not performed, as this was beyond the scope of the present study. As such, the NOECs for single pharmaceuticals could be much lower than what is reported in the present study. This would result in HQs to be much lower than what is presented herein for the individual pharmaceutical hazard potential.

Two single pharmaceutical tests did show a drop (statistically insignificant) in reproduction for ciprofloxacin and levofloxacin of 87.5% and 88.2%, respectively ($p > 0.05$) (Fig. 3). Both of these compounds are antibiotics and belong to the fluoroquinolone class. Fluoroquinolones, have a fluorine atom added to their structure to enhance the antibiotic action against gram negative and positive bacteria (Robinson et al., 2005). This class of antibiotics is commonly used and because it is an antibiotic, it is not readily biodegradable (Al-Ahmad, Daschner, & Kummerer, 1999). Most antibiotics are developed to have a specific metabolic pathway in humans and/or domestic animals but when exposed to non-target organisms, they often have various and unknown effects (Daughton & Ternes, 1999).

Neither levofloxacin or ciprofloxacin was found to pose a significant risk when tested in an acute 48-hour survival test of *D. magna* with a NOEC at 10 mg/L (Robinson et al., 2005). However that study's sole endpoint was survival and only lasted two days. In the present study, exposure concentrations for levofloxacin and ciprofloxacin were 5.9 µg/L and 5.4 µg/L, respectively, and exposure lasted for 21 days. Halling-Sorensen et al. (2000) reported a NOEC of 60 mg/L when *D. magna* were exposed to ciprofloxacin during a 48-h assay. Data for pharmaceutical life cycle toxicity assays are sparse. Levofloxacin was one of four pharmaceuticals in the current study for which NOEC and LOEC values for a single pharmaceutical 21-d reproduction were available. Yamashita et al. (2006) found reproduction NOEC and LOEC for levofloxacin in *D. magna* to be 310 µg/L and 630 µg/L, respectively (Table 2). In the present study, the concentration of levofloxacin was 5.9 µg/L in the mixture LOEC. In the present study, both ciprofloxacin and levofloxacin were found to have NOEC at 5.4 µg/L and 5.9 µg/L, respectively. Future studies should try to find the NOEC and LOEC for ciprofloxacin in order to better assess this fluoroquinolone chronic exposures hazard.

The antibiotic trimethoprim was found to have a LOEC of 20 mg/L and a NOEC of 6 mg/L in a 21-day assay with *D. magna* (Park & Choi, 2008). These results tend to suggest that trimethoprim is not one of the driving compounds involved in decreased reproduction found in the mixture because the LOEC observed associated with a significant decrease in reproduction is 1000x greater than what was tested in the current study. The longest assay that could be found in the literature for sulfamethoxazole lasted 96 h which calculated an LC_{50} of 177.3 mg/L (Kim et al., 2007). Sulfamethoxazole was found to have a NOEC of 3.3 µg/L. Since no attempt was made to pinpoint the LOEC for sulfamethoxazole this NOEC is a conservative estimate.

The antiepileptic drug, carbamazepine, has been shown to significantly stimulate *D. pulex* reproduction when exposed to 1 µg/L. This concentration produced more neonates than the controls or any other higher treatment (Lurling, Sargant, & Roessink, 2006). However, at higher concentrations of 100 and 200 µg/L, the rate of population growth was 9% and 32%, respectively (not statistically significant). Results from Lurling et al. (2006) suggest that carbamazepine has stimulatory effects at the environmental relative concentration and a NOEC at 200 µg/L. In the current experiment, the NOEC for carbamazepine was 2.3µg/L; although, there was an insignificant increase in reproduction that resulted in 107 percent of control (p=0.11) (Table 2). A significant increase in reproduction would indicate that carbamazepine was acting as an antagonist in the mixture LOEC. Cleuvers (2003) found that carbamazepine, when combined with clofibric acid, followed the concept of addition and as a result had a much stronger effect than when tested individually. Admittedly, our results, as well as those of Lurling et al. (2006) and Cleuvers (2003), are not enough to make definite statements regarding mixture toxicity, but it does illustrate how some pharmaceuticals in the mixture could be working against the reproductive inhibitory effects of the rest of the mixture.

Fluoxetine and sertraline are in a class of pharmaceuticals known as selective serotonin reuptake inhibitors (SSRIs). These drugs are developed to inhibit the reuptake of serotonin in the postsynaptic cleft of mammals but in non-target organisms, serotonin may be responsible for mechanisms that may alter appetite or influence behavior and sexual function (Fent et al., 2006; Richards & Cole, 2006; Schloss & Williams, 1998). As mentioned previously, fluoxetine has been shown to have stimulatory effects on reproduction when acting alone but also decreased reproduction in mixtures (Flaherty & Dodson, 2005). Richards et al. (2004) exposed aquatic microcosms to ibuprofen, fluoxetine and ciprofloxacin at concentrations of 60 µg/L, 100 µg/L, and 100 µg/L, respectively, and found that zooplankton abundance increased but diversity decreased directly in proportion to the dose. The other SSRI tested in that mixture, sertraline, has been shown to have a LOEC at 100 µg/L in a 21-day assay as well as a NOEC on reproduction at 32 µg/L when *D. magna* were exposed (Minagh et al., 2009). This is the lowest observed LOEC found for any of the individual pharmaceuticals in a 21-d life cycle assay on *D. magna*. A study conducted by Henry et al. (2004) investigated the 8-d chronic toxicity of five SSRI on *Ceriodaphnia dubia*, a water flea similar to *D. magna*. The LOECs for fluoxetine and sertraline were 146 µg/L and 45 µg/L, respectively. The fact that SSRIs used in this study have been found to reduce reproduction in *D. magna* at µg/L levels could suggest that they may have had a role in the reduced number of neonates in the current mixture study.

Data Gaps and Uncertainties

Out of the 11 pharmaceuticals that made up the mixture in the current study only four could be found in the literature with NOEC values for 21-day assays that evaluated reproduction of *D. magna* as an endpoint (Table 1). Trimethoprim's NOEC was the highest reported at a concentration of 6 mg/L (Park & Choi, 2008). Sertraline had the lowest NOEC at 32 µg/L

(Minagh et al., 2009). The lack of data available for these individual pharmaceuticals need to be addressed in order to know their potential hazard in chronic exposure scenarios. When the LOEC was observed for the mixture at 100x, 21-d assays were conducted on the individual pharmaceuticals in order to determine if one was responsible for the reduction in neonates produced. As a result NOECs were found for each pharmaceutical at their concentration within the mixture LOEC. No attempt was made to find the LOEC for any of the individual pharmaceuticals as this was outside the scope of the current study. As a result, our NOEC for the single pharmaceuticals may be orders of magnitude lower than the actual NOEC. As mentioned earlier, HQs for the individual pharmaceuticals calculated herein must be seen as highly conservative estimates. For example, the NOEC found for trimethoprim in the current study was 0.6µg/L whereas the NOEC reported in the literature is 6 mg/L (Park & Choi, 2008). Our NOEC is > 1,000x lower than what is reported in the literature causing our HQ for trimethoprim to be highly conservative. The NOEC in the present study illustrates how little hazard trimethoprim poses. Indeed, even though an extremely conservative NOEC estimate was used to calculate the HQ, no hazard is predicted for trimethoprim – *with* a safety factor of 100 applied. In the least, the estimated trimethoprim HQ of this study provides only a relative estimate of hazard.

A similar situation exists for sertraline. Sertraline's literature NOEC is 32 µg/L (Minagh et al., 2009). The present study estimated a NOEC of 1.24 µg/L. This is also a conservative estimate (25x lower than the reported NOEC) and no hazard is predicted for sertraline, even with a safety factor of 100 applied. Seven of the pharmaceuticals do not have data in the literature for 21-day reproduction assays; therefore, similar comparisons of literature NOEC and present-study NOEC cannot be made.

Conclusions

The occurrence of pharmaceuticals as complex mixtures and at low concentrations is well documented around the globe. Most studies that address the acute ecological hazard of pharmaceuticals only account for the toxicity of single pharmaceutical exposure and do not take into account chronic additive or synergistic affects that can occur in mixtures. This is concerning given the fact that low-level combinations of pharmaceuticals are continually released into the aquatic environment with aquatic species being exposed over the course of their life cycles. Herein, we attempted to determine the hazard of environmentally relevant mixtures of pharmaceuticals to *D. magna*. My results indicate that the LOEC for such a mixture was below the NOEC for any single pharmaceutical, indicating that interactions or cumulative effect of the mixture resulted in greater toxicity. When these data were used to calculate a conservative HQ, no hazard was indicated. When a safety factor of 10 was applied to the HQ, as recommended by the FDA, the predicted hazard for *D. magna* exposed to the pharmaceutical mixture – at maximum environmental concentrations – is low.

My experimental data suggest that current hazard of the 11 pharmaceutical mixture in the environment is low. However, some consideration needs to be given to future hazard due to the increasing size and age of human populations and associated subsequent increases in pharmaceutical use. In addition, the present research (and that of others) indicates that as the number of pharmaceuticals is added to the system, toxicity is likely increase as well. Conley et al. (2008) measured for 14 pharmaceuticals in the Tennessee River and found 13. Which raises the questions: what if they measured for 140 pharmaceuticals? Would Conley et al. (2008) have found 130 pharmaceuticals? With over 3000 active ingredients in use today, the possibility of many more pharmaceuticals in the environment cannot be ignored. The use of safety factors will

compensate for much of this uncertainty, but it is difficult to determine how much. Indeed, the present study has illustrated how toxicity increases (relative to individual concentrations) when ultra-low concentrations are combined. In the present study we have shown that if *D. magna* are exposed to 11 pharmaceuticals simultaneously at the maximum environmental concentration that were detected in the Tennessee River, the threshold for significant reproductive hazard would not be reached unless concentrations increased by a factor of 100. While this is indeed orders of magnitude away from a perceived hazard, it is difficult to determine how long, or if, this 100-fold safety could be diminished as human populations grow.

CHAPTER III

DETERMINATION OF RIVERINE ZOOPLANKTON ASSEMBLAGE RELATION TO CHITOBIASE ACTIVITY

Introduction

Invertebrate diversity and biomass are standard indicators of stream health (Reynoldson & Metcalfe-Smith, 1992). The diversity of invertebrate taxa found in lotic systems can be incredibly complex. In the southern Appalachian Mountains, over 293 invertebrate taxa have been recorded in headwater streams (Wallace & Eggert, 2009). Zooplankton are a vital part of the aquatic ecosystem. In a riverine food web, zooplankton provide a vital link between phytoplankton and fish (Jack & Thorp, 2002; Thorp & Delong, 2002). Zooplankton are a significant biotic component of the carbon cycle in larger riverine ecosystems (Gliwicz, 2002; Gosselain et al., 1998; Thorp & Delong, 2002).

Methods that are currently used by researchers to measure aquatic invertebrate diversity are expensive, due to the amount of time and labor it takes to collect and process invertebrate samples (Bailey et al., 2001; Lenat & Resh, 2001). There is a need for alternative methods that have the potential of assessing aquatic invertebrate populations rapidly and at low cost. One such method that has shown promise for invertebrate assessment is the monitoring of chitobiase activity in the water column (Hanson & Lagadic, 2005; Sastri & Dower, 2009). Chitobiase along with chitinase are two chitinolytic enzymes that partially digest the chitin exoskeleton of crustaceans in a process called apolysis. Chitin consists of a polymer of β-(1-4)-linked sugar

derivatives. The chitinolytic enzymes break down chitin with chitinase, cleaving the chitin to oligomers and dimers. Chitobiase then degrades the products to the monomer *N-acetylglucosamine* (NAG) (Espie & Roff, 1995; Oosterhuis et al., 2000). Both enzymes are released into the ambient water during the pre-molt stage of arthropods (Zou & Fingerman, 1999). Since chitinase and chitobiase are essential for molting to take place and are released in the water column, ambient concentrations of these enzymes have the potential to represent total molting activity or biomass production of arthropod populations (Conley et al., 2009; Sastri & Dower, 2009). Chitobiase is a good choice as a natural tracer, due to the established and simple assay used to calculate its presence in the water column (Espie & Roff, 1995).

Vrba and Machacek (1994) observed that chitobiase is liberated into the surrounding aquatic environment after apolysis. Oosterhuis et al. (2000) measured the rate of chitobiase released by *T. longicornis* and found that chitobiase activity provided a good natural indicator for body weight increase. Most recently, Sastri and Dower (2009) found a significant positive relationship between total zooplankton biomass and liberated chitobiase (r^2=0.41) off the coast of British Columbia. These studies showed the potential of monitoring chitobiase activity and indirectly measure the total invertebrate biomass of an aquatic system in an extremely short amount of time (Hanson & Lagadic, 2005).

To date, no research has examined the relationship between chitobiase activity and zooplankton assemblages in freshwater river systems. To achieve this, zooplankton and associated water samples were collected over the course of 2010-2011 from 4^{th}-6^{th} order Tennessee River tributaries, with one sampling period during each season. This allowed for a snapshot of zooplankton assemblages and the concentration of chitobiase activity in lotic ecosystems (Dodson et al., 2009). By measuring zooplankton and chitobiase parameters at the

same point and time, I aimed to determine the relationship between zooplankton and ambient chitobiase activity concentration.

Materials and Methods

Study Sites

Study creeks include Wolftever Creek (N 35˚ 08’02.62’’, W 85˚ 04’39.88’’), Soddy Creek (N 35˚ 17’47.24’’, W 85˚ 08’57.03’’), North Chickamauga Creek (N 35˚ 06’49.78’’, W 85˚ 13’45.38’’) and South Chickamauga Creek (N 35˚ 05’17.87’’, W 85˚ 15’43.73’’) were sampled during four seasons. These creeks were sampled once per season in the Spring (5/25/2010), Summer (8/25/2010), Fall (10/27/2010), and Winter (2/11/2011). Sequatchie River (N 35˚ 02’52.97’’, W 85˚ 37’34.07’’), and Candies Creek (N 35˚ 18’36.75’’, W 84˚ 50’50.06’’) were added later and samples were only taken during the Summer, Fall, and Winter. All creeks are located in the Tennessee River watershed and are classified as 4th order or higher.

Sampling

Zooplankton sampling was carried out using the procedure from Havel et al. (2009). This method provided an accurate volume measurement between sample sites and collected via a Guzzler hand-pump (Model 400h) and then filtered through plankton nets. In order to accurately estimate the density of all major groups of zooplankton, two plankton net sizes were used. Macrozooplankton were collected by pumping a total of 180 L of water through a 63 μm mesh plankton net. This allowed for a proper estimation of crustacean zooplankton (Chick, Levchuk, Madley, & Havel, 2010). This large amount of water that was pumped through the net allowed for a better estimate of macrozooplankton that are most scarce. Microzooplankton were collected by pumping a total of 18 L through a 20 μm mesh plankton net (Angradi, 2006; Havel

et al., 2009). Micro samples were used to estimate rotifers and copepod nauplii that would not be captured in the larger net size. By using this dual net system the most accurate estimates were obtained for all of the aforementioned zooplankton taxa (Chick et al., 2010). Depth of samples ranged according to the size of the creek channel (Fig. 4). Viroux (1999) conducted a study that indicated zooplankton density is not evenly distributed in the water column of lotic systems. To insure accurate measurement of the river zooplankton assemblage, samples were collected from three vertical points and three horizontal points when possible. Vertical samples were collected 0.5 m below the surface, at the mid-channel depth, and at 0.5 m above channel bed (a, b, and c, respectively; Fig. 4) (Angradi, 2006). Horizontal samples were collected at the center of the channel and at half the distance from the shore on either side of the center (Fig. 4). A 20 L sample of water was collected from each point for macrozooplankton and 2 L for microzooplankton; samples were then combined for a total volume of 180 L and 18 L. All samples were preserved in 95% ethanol for processing (Angradi, 2006; Havel et al., 2009). At the time of zooplankton sampling, a water sample was collected from the vertical mid-channel depths to determine ambient chitobiase activity within the water column.

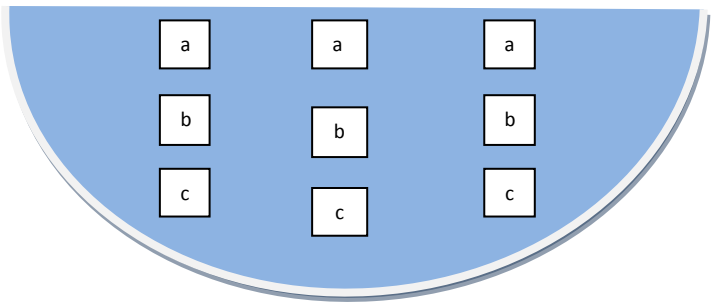

Figure 4

Simulated cross section of a river channel. Letters indicate zooplankton sampling points. Samples were collected at (a) 0.5 m below surface, (b) mid depth and (c) 0.5 m above river bed. Horizontal samples were collected at the center of the channel and at half the distance from the shore on either side of the center A 20 L sample was collected from each point for macrozooplankton and 2 L form each point for microzooplankton.

Zooplankton analysis

Zooplankton samples were processed by BSA Environmental Services Cleveland, OH. Briefly, concentrated samples were measured in three 1 ml aliquots under a Wilovert inverted microscope outfitted with a phase contrast until 400 specimens were counted. Density was then expressed in number of individuals per liter (ind./L) based on the volume of water pumped through the net (Beaver et al., 2010; Havens et al., 2011). Taxonomic identification followed methods from Ruttner-Kolisko (1974), Stemberger (1979), and Edmundson (1959). Biomass estimates were based on established length-width relationships (Lawrence, Malley, Findlay, Maclver, & Delbaere, 1987; McCauley, 1984). Biomass was estimated for each location and season with a species biomass calculated for each sample.

Chitobiase Activity Analysis

The method to fluorometrically measure chitobiase activity is well established and has been used in previous studies by Hanson and Lagadic (2005) and Conley et al. (2009). This assay

indirectly measures chitobiase activity by quantifying liberated methylumbelliferyl acetate (MUF) from a 4-Methylumbelliferyl-2-acetamido-2-acetamido-2-deoxy-β-D-glucopyranoside (MUF-NAG) substrate. When samples are introduced to the MUF-NAG substrate, chitobiase within the water sample cleaves the substrate into MUF and NAG. The fluorescent MUF is then quantified with a spectrophotometer set at 360 nm excitation and 450 nm emission (Hanson & Lagadic, 2005). Samples first were filtered using a 0.7 μm glass fiber filter and then through a 0.45 μm membrane filter. This process removed free-floating particulates in the sample. Samples were next filtered with 0.2 μm filter to remove any bacteria that may interfere with the test. Samples were stored in a -80^{o} C freezer until analysis. On the day of quantitation, samples were thawed and then incubated with 50 μl of 0.31 mmol$\cdot$L^{-1}MUF-NAG in 0.15 mol$\cdot$L^{-1} 5.5 citrate phosphate buffer for 1 hour at 25^{o}C. After an hour, 50 μl of 0.25 M NaOH was added to stop the reaction. The ambient chitobiase activity was analyzed using a BIO-TEK synergy HT spectrophotometer and was expressed as fluorescence of freed MUF in micromoles per liter per hr (μmol$\cdot$L$^{-1}\cdot$h^{-1}) at 360 nm excitation and 450 nm emission. To measure the fluorescence absorbance, a standard curve was produced using stock solutions of MUF (Conley et al., 2009, Hanson and Lagadic, 2005).

Statistics

All statistics where preformed with Statistical Analysis System (SAS Inc., 2008). Two factor repeated-measures ANOVA without replication on ranks was used to determine significance between seasons for chitobiase activity and biotic factors ($\alpha = 0.1$). These biotic factors included biomass value for total zooplankton, macro zooplankton, micro zooplankton, cladocerans, copepods, nauplii, macro sample combined with nauplii, and copepods with nauplii

biomass. Zooplankton abundance was analyzed for the above mentioned groups as well. Data did not meet normality assumptions so ranked values were used in all ANOVAs. To evaluate possible differences between means, pairwise comparisons were performed using Tukey's test. To compare the effect of season, separate tests were run on four creeks over four seasons (Wolftever Creek, Soddy Creek, North Chickamauga Creek and South Chickamauga Creek) and six creeks over three seasons (Wolftever Creek, Soddy Creek, North Chickamauga Creek, South Chickamauga Creek Sequatchie River and Candies Creek). Data that underwent ANOVA tests were chitobiase activity taken during each season along with the aforementioned groups of biotic factors.

Results

Seasonal analysis on chitobiase activity showed no significant difference for chitobiase activity among the four creeks over four seasons (Two factor repeated measure ANOVA without replication on ranks; $p > 0.1$) (Table 5). Although there was no significant difference, Fall had the highest mean chitobiase activity followed by Summer, Spring, and then Winter. There was no significant difference in biomass between seasons for the four creeks over four seasons ($p > 0.1$) (Table 6). However, abundance did show significant differences (Table 5). For total abundance, pairwise comparison indicate Summer zooplankton abundance was significantly greater than Winter abundance ($p = 0.062$), but Fall and Spring samples were similar to both Summer and Winter. Samples of Copepoda combined with napulii, macro combined with napulii, and napulii individuals were found to be significantly different for Summer and Fall when compared to Winter. Fall samples were found to be similar to all seasons (Table 5).

Table 5

Chitobiase activity and zooplankton abundance mean ranks in regard to season for four creeks over four seasons. Letters indicate a significant difference in Tukey's test groupings ($\alpha = 0.1$). All significant difference in groupings follow the pattern of Summer having the largest mean followed by Spring, Fall, Winter for biotic factors.

Parameters	Spring	Summer	Fall	Winter
Chitobiase	6.75	10.00	10.50	6.75
Total	9.50[a,b]	12.75[a]	7.75[a,b]	4.00[b]
Macro	9.25	11.00	8.87	4.88
Micro	9.50[a,b]	12.75[a]	7.75[a,b]	4.00[b]
Cladocera	9.12	11.25	8.37	5.20
Copepoda	9.12	10.50	8.88	5.50
Copepoda + Napulii	11.25[a]	12.00[a]	6.75[a,b]	4.00[b]
Macro + Napulii	11.75[a]	11.75[a]	6.75[a,b]	3.75[b]
Napulii	10.87[a]	12.00[a]	7.12[a,b]	4.00[b]

Table 6

Zooplankton biomass mean ranks for four creeks in four seasons. There was no significant difference between any season ($\alpha = 0.1$).

Parameters	Spring	Summer	Fall	Winter
Total	9.75	11.75	8.00	4.50
Macro	8.75	10.75	8.75	5.70
Micro	10.00	12.50	6.75	4.75
Cladocera	8.37	11.75	7.62	6.25
Copepoda	9.50	9.75	8.75	6.00
Copepoda + Napulii	10.00	10.75	8.50	4.75
Macro + Napulii	10.25	10.75	8.25	4.75
Napulii	10.75	11.00	7.50	4.75

In the ANOVA of the six creek dataset, season had a significant effect on chitobiase activity ($p = 0.086$). Although Tukey's test, a fairly conservative multiple comparison procedure, did not indicate any significant difference among the mean ranks (Table 7). Chitobiase activity

ranked means appeared greatest in the Fall (12.83) followed by Summer (8.16), Spring (8.16), and Winter (7.5). Biotic factor for six creeks over three seasons showed significant differences when biomass and abundance was analyzed ($p < 0.1$) (Table 7, 8). Biomass was significantly different for macro, micro, copepoda combined with napulii, macro combined with napulii, and napulii individual samples ($p < 0.1$). The trend of Summer samples being significantly different then winter but Fall being similar to both Summer and Winter was observed for all of these biotic groups (Table 7). This same trend was observed for biotic grouping of total abundance and micro abundance (Table 8)

Table 7

Chitobiase activity and zooplankton abundance mean ranks in (by season) for six creeks over three seasons. Letters indicate significant difference in Tukey's test groupings ($\alpha = 0.1$). All significant difference in groupings follow the pattern of Summer, Spring, Fall, Winter for biotic factors.

Parameters	Summer	Fall	Winter
Chitobiase	8.16	12.83	7.50
Total	13.83	7.83	6.83
Macro	13.67^{a}	$9.83^{a,b}$	5.00^{b}
Micro	14.00^{a}	$8.50^{a,b}$	6.00^{b}
Cladocera	13.33^{a}	$9.5^{a,b}$	5.66^{b}
Copepoda	12.08	9.83	6.58
Copepoda + Napulii	13.33^{a}	$9.33^{a,b}$	5.83^{b}
Macro + Napulii	13.50^{a}	$9.83^{a,b}$	5.16^{b}
Napulii	13.25^{a}	$9.08^{a,b}$	6.16^{b}

Table 8

Zooplankton biomass in regard to season for six creeks over three seasons. Letters indicate significant difference in Tukey's test groupings of ranked means ($\alpha = 0.1$). All significant difference in groupings follow the pattern of Summer, Spring, Fall, Winter for biotic factors.

Parameters	Summer	Fall	Winter
Chitobiase	8.16	12.83	7.50
Total	13.16[a]	8.83[a,b]	6.50[b]
Macro	12.66	9.33	6.50
Micro	13.33[a]	8.33[a,b]	6.83[b]
Cladocera	12.83	8.33	7.33
Copepoda	11.33	9.66	7.50
Copeopda + Napulii	12.00	9.66	6.83
Macro + Napulii	12.50	9.33	6.66
Napulii	12.30	9.00	7.16

Ambient chitobiase activity ranged from a low of 0.517 $\mu mol \cdot L^{-1} \cdot h^{-1}$ in the Soddy Creek Fall sample to a high of 1.515 $\mu mol \cdot L^{-1} \cdot h^{-1}$ in the Candies Creek fall sample (Table 9). Total zooplankton abundance ranged from 0.95 ind./L to 4927.51 ind./L throughout the sampling period (Table 10). Macro zooplankton abundance were low for all creeks during all seasons with the largest sample taken at Wolftever during the spring, containing 4.51 ind./L (Table 10). Micro zooplankton abundance ranged from 0.94 ind./L taken during the winter on Soddy Creek to 1589.88 ind./L taken from the same creek during the Summer (Table 10). A partial list of the most abundant taxa can be found in Table 11. A complete list of taxa is found in Appendix A. The most common species found throughout the samples was the rotifer *Polyartha vulgaris*, observed a total of 646 times. In the samples collected, rotifers were found to outnumber copepods 17.7:1 and cladocerans 13529.9:1. *Cyclopoid copepodid* was the most common copepod observed, a total of 648 times. Appendix A includes a complete list of taxa.

Table 9

Chitobiase activity detected for all creeks and seasons. Chitobiase activity is expressed as $\mu mol \cdot L^{-1} \cdot h^{-1}$. Sequatchie and Candies Creek were not sampled in the Spring.

	Spring	Summer	Fall	Winter
North Chickamauga	1.233	0.670	0.980	0.568
South Chickamauga	0.609	0.517	1.067	1.284
Soddy	0.786	0.850	0.587	0.301
Wolftever	0.991	0.808	1.024	0.736
Sequatchie	n/a	0.507	0.930	0.446
Candies	n/a	1.107	1.515	0.938

Table 10

Abundance and biomass for zooplankton samples collected over one year for creeks. Rotifers showed the largest abundance among groups. Biomass is presented as µg of dry weight per liter.

		Total Abundance (Ind./L)	Total Biomass (µg/L)	Macro Abundance (Ind./L)	Macro Biomass (µg/L)	Micro Abundance (Ind./L)	Micro Biomass (µg/L)
North	Spring	6.367	0.253	0.033	0.025	6.333	0.228
Chickamauga	Summer	37.678	0.726	0.511	0.284	37.167	0.441
	Fall	51.151	2.027	1.373	1.191	49.778	0.836
	Winter	1.033	0.063	0.033	0.047	1.000	0.016
South	Spring	1.944	0.053	0.000	0.000	1.944	0.053
Chickamauga	Summer	145.033	2.088	0.200	0.080	144.833	2.008
	Fall	6.922	0.332	0.311	0.195	6.611	0.137
	Winter	2.656	0.157	0.044	0.043	2.611	0.114
Soddy	Spring	418.919	31.015	3.641	27.842	415.278	3.173
	Summer	1590.744	17.040	0.856	2.516	1589.889	14.524
	Fall	6.494	0.131	0.106	0.027	6.389	0.105
	Winter	0.950	0.029	0.006	0.008	0.944	0.021
Wolftever	Spring	4927.512	52.183	4.512	3.051	4923.000	49.133
	Summer	902.856	14.743	0.856	2.516	902.000	12.227
	Fall	3.522	1.271	0.078	1.175	3.444	0.096
	Winter	26.733	1.152	0.244	0.419	26.489	0.733
Sequatchie	Summer	421.106	18.905	2.578	1.507	418.528	17.398
	Fall	2.117	0.901	0.506	0.633	1.611	0.268
	Winter	26.500	0.777	0.011	0.045	26.489	0.733
Candies	Summer	2.550	0.105	0.161	0.050	2.389	0.056
	Fall	5.861	0.096	0.028	0.011	5.833	0.085
	Winter	10.589	0.501	0.089	0.408	10.500	0.093

Table 11

List of most abundant zooplankton found in six 4th-6th order tributaries of the Tennessee River in southeast Tennessee.

Creek	Division	Genus species	ind/L
Candies	Cladocera	*Alona rectangula*	0.6
	Rotifera	*Anuraeopsis fissa*	697.8
	Rotifera	*Bdelloid*	4.4
North	Cladocera	*Bosmina longirostris*	2.5
Chickamauga	Rotifera	*Collotheca*	7.7
	Rotifera	*Collurella*	665.3
	Rotifera	*Conochiloides dossuarius*	66.2
	Rotifera	*Conochilus unicornis*	149.8
Sequatchie	Copepoda	*Calanoid copepodid*	1.0
	Rotifera	*Keratella cochlearis*	118.2
Soddy	Copepoda	*Cyclopoid copepodid*	13.2
	Rotifera	*Keratella cochlearis*	892
	Rotifera	*Limnias*	133.8
South	Cladocera	*Diaphanosoma brachyurum*	0.6
Chickamauga	Copepoda	*Epischura fluviatilis*	2.4
	Cladocera	*Cladoceran immature*	0.9
	Copepoda	*Napulii*	441.5
	Rotifera	*Polyarthra vulgaris*	697.5
Wolftever	Ostracoda	*Ostracod*	0.3
	Rotifera	*Polyarthra vulgaris*	567.2

Discussion

In lotic systems, such as those in the present study, water residence time is lower than that in lentic systems; therefore riverine zooplankton populations are typically dominated by rotifers and other taxa that require shorter generation times (i.e. time to reach sexually maturity) (Basu &

Pick, 1996). Zooplankton with longer generation times, such as cladocerans and to some extent copepods, are susceptible to advection-related declines in local population density in riverine systems due to the short water residence time (Pace, Findlay, & Lints, 1992). The abundance of zooplankton observed in the current study (rotifers > nauplii > copepods > cladocerans) corresponds with previous literature on river zooplankton community structure (Basu & Pick, 1996; Pace et al., 1992; Thorp, Black, Haag, & Wehr, 1994).

The creeks in the current study are large enough to support a zooplankton population (Gliwicz, 2002; Gosselain et al., 1998; Thorp & Delong, 2002). However, benthic invertebrates generally make up a large proportion of aquatic invertebrate assemblages in 4^{th}-6^{th} order creeks and were not accounted for in this study. Indeed, a majority of biodiversity in lotic systems can be attributed to benthic macroinvertebrates, most of which release chitobiase (Heino, 2005). Because no benthic macroinvertebrates were assessed, the effect of that population on chitobiase concentrations in the water column is unknown.

Lentic systems have been shown to support larger populations of zooplankton than lotic systems (Bradt, Urban, Goodman, Bissell, & Spiegel, 1999). Which may contribute more significantly to total ambient chitobiase activity future studies should be conducted to evaluate possible correlations between zooplankton population dynamics and ambient chitobiase activity.

There was no statistical between season differences in the mean ranks of ambient chitobiase activity; however, the amount of chitobiase activity appeared to be relatively high in the Fall and low in the Winter. Conley et al. (2009) found the same seasonal pattern of chitobiase activity levels in the upper Tennessee River ($p<0.05$). The fact that no significant difference was found in the present study may be due to several factors. First, the sample size in the present study (n=6) is half that of Conley et al. (2009). Second, the Tennessee River is a 8^{th}

order river that is regulated by dams that control the volume of water throughout all seasons. Seasonal fluctuation of creek volume created by river stage manipulation and natural rainfall patterns was observed for all creeks in the study. For example, Candies Creek and Wolftever Creek both had drastic fluctuation in volume throughout the sampling periods. Ambient chitobiase activity in the water column is susceptible to seasonal water level dilution as well as the amount of arthropods molting. Therefore, lower amounts of water could contribute to higher measurements of chitobiase activity even though there is a lower arthropod population. Lastly, chitobiase degradation rates were not measured. Oosterhuis et al. (2000) found that decay of chitobiase caused by bacteria varied with seasons and also was not necessarily dependent on the amount of bacteria present culture in a in situ study. These degradation rates are important when trying to link chitobiase activity to secondary production (Oosterhuis et al., 2000).

Arthropod communities have shown an increase in abundance for more tolerant species and decrease in less tolerant species when certain pollutants have contaminated the aquatic environment (Bradt et al., 1999; Reice, 1994; Richards et al., 2004). In these cases chitobiase activity would not be useful in reflecting this type of change in the arthropod community. In addition, long term monitoring is needed to determine averages and inconsistency in aquatic environments (Likens & Lambert, 1998). Accordingly, for ambient chitobiase activity to be used as a monitoring tool, chitobiase samples would have to be taken over a long period of time. This would allow for a pattern to emerge that would indicate anomalies. However, data from the present study do not support the use of ambient chitobiase as an indicator of water-column zooplankton community health, growth, or abundance.

CHAPTER IV

CONCLUSION

This thesis presented the concerns and alternatives to aquatic toxicology and monitoring. In the first study, *D. magna* were exposed to a mixture of pharmaceuticals that was detected in the Tennessee River in order to investigate what concentration would cause a toxic effect. The second study attempted to find a correlation between zooplankton population dynamics and a molting enzyme, called chitobiase, to develop a quick and simple assay to monitor invertebrate production in freshwater environments. Both studies were preformed in order to assist in closing current knowledge gaps concerning relevant ecotoxicological issues.

The chronic 21-d assays was chosen to evaluate the effects of the pharmaceutical cocktail found in the Tennessee River on *D. magna* to asses toxic effects over the course of the organism's life cycle. These types of tests are the most relevant to exposure scenarios in the environment. Indeed, pharmaceuticals are in the aquatic environment in complex mixtures and organisms are exposed to these pollutants over the course of their entire life. That study found that the mixture would have to be 100 times greater than what is currently detected, with all pharmaceuticals being at their maximum concentration, before reproduction would be significantly reduced. Currently, the FDA does not require pharmaceuticals to undergo chronic mixture toxicity tests before approval and some may be exempt from chronic assays altogether. Studies, such as the one presented in Chapter I, illustrated that mixtures of pharmaceuticals can result in toxic effects that are below the NOEC for any single pharmaceutical. Future studies should continue to focus on environmentally relative mixtures and chronic exposures to evaluate the hazard.

The monitoring of aquatic environments can be very time consuming and costly. Current practices of assessing invertebrates to evaluate aquatic system health often take months and are labor intensive. The second study presented examined whether the monitoring of ambient aquatic chitobiase could determine zooplankton population health in 4^{th}-6^{th} order streams. The results from this study showed no relationship. This is due in part because no significant difference in chitobiase activity between seasons was found. However, other work indicates that chitobiase still shows promise as an indicator of secondary production when degradation rates of the enzyme are known.

Increased awareness and research on pharmaceuticals released into the environment is the first step to truly understanding the impact that such compounds have on the aquatic environment. The development of novel endpoints that can be used on a river system level would aid in the monitoring of the potential ecological consequences of these compounds.

REFERENCES

Al-Ahmad, A., Daschner, F. D., & Kummerer, K. (1999). Biodegradability of cefotiam, ciprofloxacin, meropenem, penicillin G, and sulfamethoxazole and inhibition of waste water bacteria. *Archives of Environmental Contamination and Toxicology, 37*(2), 158-163.

American Society for Testing, M. (2002). Standard guide for conducting *Daphnia magna* life-cycle toxicity test (E 1193-97) *Annual book of ASTM standards* (Vol. 11.05, pp. 430-447). West Conshohocken PA: ATSM International.

Angradi, T. R. (2006). *Environmental monitoring and assessment program, Great River ecosystems field operations manual*. Washington, DC.

Ankley, G. T., Brooks, B. W., Huggett, D. B., & Sumpter, J. P. (2007). Repeating history: Pharmaceuticals in the environment. *Environmental Science & Technology, 41*(24), 8211-8217. doi: 10.1021/es072658j

Ashton, D., Hilton, M., & Thomas, K. V. (2004). Investigating the environmental transport of human pharmaceuticals to streams in the United Kingdom. *Science of the Total Environment, 333*(1-3), 167-184. doi: 10.1016/j.scitotenv.2004.04.062

Bailey, R. C., Norris, R. H., & Reynoldson, T. B. (2001). Taxonomic resolution of benthic macroinvertebrate communities in bioassessments. *Journal of the North American Benthological Society, 20*(2), 280-286. doi: 10.2307/1468322

Basu, B. K., & Pick, F. R. (1996). Factors regulating phytoplankton and zooplankton biomass in temperate rivers. *Limnology and Oceanography*, 1572-1577.

Beaver, J. R., Tietjen, T. E., Blasius-Wert, B. J., Kirsch, J. E., Rosati, T. C., Holdren, G. C., . . . Evans, S. K. (2010). Persistence of Daphnia in the epilimnion of Lake Mead, Arizona-Nevada, during extreme drought and expansion of invasive quagga mussels (2000-2009). *Lake and Reservoir Management, 26*(4), 273-282. doi: 10.1080/07438141.2010.519858

Bradt, P., Urban, M., Goodman, N., Bissell, S., & Spiegel, I. (1999). Stability and resilience in benthic macroinvertebrate assemblages - Impact of physical disturbance over twenty-five years. *Hydrobiologia, 403*, 123-133. doi: 10.1023/a:1003711928980

Brooks, B. W., Foran, C. M., Richards, S. M., Weston, J., Turner, P. K., Stanley, J. K., La Point, T. W. (2003). Aquatic ecotoxicology of fluoxetine. *Toxicology Letters, 142*(3), 169-183. doi: 10.1016/s0378-4274(03)00066-3

Buser, H. R., Muller, M. D., & Theobald, N. (1998). Occurrence of the pharmaceutical drug clofibric acid and the herbicide mecoprop in various Swiss lakes and in the North Sea. *Environmental Science & Technology, 32*(1), 188-192. doi: 10.1021/es9705811

Cahill, J. D., Furlong, E. T., Burkhardt, M. R., Kolpin, D., & Anderson, L. G. (2004). Determination of pharmaceutical compounds in surface- and ground-water samples by solid-phase extraction and high-performance liquid chromatography-electrospray ionization mass spectrometry. *Journal of Chromatography A, 1041*(1-2), 171-180. doi: 10.1016/j.chroma.2004.04.005

Castiglioni, S., Bagnati, R., Fanelli, R., Pomati, F., Calamari, D., & Zuccato, E. (2006). Removal of pharmaceuticals in sewage treatment plants in Italy. *Environmental Science & Technology, 40*(1), 357-363. doi: 10.1021/es050991m

Chick, J. H., Levchuk, A. P., Madley, K. A., & Havel, J. H. (2010). Underestimation of rotifer abundance a much greater problem than previously appreciated. *Limnology and Oceanography: Mehtods*, 79-87.

Cleuvers, M. (2003). Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. *Toxicology Letters, 142*(3), 185-194. doi: 10.1016/s0378-4274(03)00068-7

Cleuvers, M. (2008). Chronic mixture toxicity of pharmaceuticals to Daphnia - the example of non-steroidal anti-infammatory drugs. In K. Kummerer (Ed.), *Pharmaceuticals in the Environment. Sources Fate Effects and Risk* (3 ed., pp. 277-284). Berlin, Heidelberg: Springer.

Conley, J. M., Schorr, M. S., Hanson, M. L., Symes, S. J., & Richards, S. M. (2009). Is ambient chitobiase activity a monitoring tool for impacts on secondary production in lotic systems? *Canadian Journal of Fisheries and Aquatic Sciences, 66*(8), 1274-1281. doi: 10.1139/f09-092

Conley, J. M., Symes, S. J., Schorr, M. S., & Richards, S. M. (2008). Spatial and temporal analysis of pharmaceutical concentrations in the upper Tennessee River basin. *Chemosphere, 73*(8), 1178-1187. doi: 10.1016/j.chemosphere.2008.07.062

Costanzo, S. D., Murby, J., & Bates, J. (2005). Ecosystem response to antibiotics entering the aquatic environment. *Marine Pollution Bulletin, 51*(1-4), 218-223. doi: 10.1016/j.marpolbul.2004.10.038

Cunningham, V. L. (2008). Special characteristics of pharmaceuticals related to environmental fate. In K. Kummerer (Ed.), *Pharmaceuticals in the Environment. Sources Fate Effects and Risk* (3 ed., pp. 23-34). Berlin Heidelberg: Springer.

Daughton, C. G., & Ternes, T. A. (1999). Pharmaceuticals and personal care products in the environment: Agents of subtle change? *Environmental Health Perspectives, 107*, 907-938. doi: 10.2307/3434573

Dodson, S. I., Newman, A. L., Will-Wolf, S., Alexander, M. L., Woodford, M. P., & Egeren, S. (2009). The relationship between zooplankton community structure and lake characteristics in temperate lakes (Northern Wisconsin, USA). *Journal of Plankton Research, 31*(1), 93-100. doi: 10.1093/plankt/fbn095

Dussault, E. B., Balakrishnan, V. K., Sverko, E., Solomon, K. R., & Sibley, P. K. (2008). Toxicity of human pharmaceuticals and personal care products to benthic invertebrates. *Environmental Toxicology and Chemistry, 27*(2), 425-432. doi: 10.1897/07-354r.1

Edmundson, W. T. (1959). *Freshwater Biology, 2nd Edition*: Wiley-Interscience.

Ellis, J. B. (2006). Pharmaceutical and personal care products (PPCPs) in urban receiving waters. *Environmental Pollution, 144*(1), 184-189. doi: 10.1016/j.envpol.2005.12.018

EMEA. (2003). Note for guidance on environmental risk assessment of medicinal products for human use. CPMP/SWP/44447/00 drage corr. Available from <http:/www.emea.eu.int/pdfs/human/swp/444700en.pdf>.

Espie, P. J., & Roff, J. C. (1995). A biochemical index of duration of the molt cycle for planktonic crustacean based on the chitin-degrading enzyme, chitobiase. *Limnology and Oceanography, 40*(6), 1028-1034.

FDA. (1998). *Guidance for industry-Environmental assessment of human drugs and biologics applications, Revision1*. Rockville: FDA Center for Drug Evaluation and Research.

Fent, K., Weston, A. A., & Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology, 76*(2), 122-159. doi: 10.1016/j.aquatox.2005.09.009

Ferrari, B., Paxeus, N., Lo Giudice, R., Pollio, A., & Garric, J. (2003). Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibric acid, and diclofenac (vol 55, pg 359, 2003). *Ecotoxicology and Environmental Safety, 56*(3), 450-450. doi: 10.1016/s0147-6513(03)00111-8

Flaherty, C. M., & Dodson, S. I. (2005). Effects of pharmaceuticals on Daphnia survival, growth, and reproduction. *Chemosphere, 61*(2), 200-207. doi: 10.1016/j.chemosphere.2005.02.016

Flippin, J. L., Huggett, D., & Foran, C. M. (2007). Changes in the timing of reproduction following chronic exposure to ibuprofen in Japanese medaka, Oryzias latipes. *Aquatic Toxicology, 81*(1), 73-78. doi: 10.1016/j.aquatox.2006.11.002

Glassmeyer, S. T., Furlong, E. T., Kolpin, D. W., Cahill, J. D., Zaugg, S. D., Werner, S. L., . . . Kryak, D. D. (2005). Transport of chemical and microbial compounds from known wastewater discharges: Potential for use as indicators of human fecal contamination. *Environmental Science & Technology, 39*(14), 5157-5169. doi: 10.1021/es048120k

Gliwicz, Z. M. (2002). On the different nature of top-down and bottom-up effects in pelagic food webs. *Freshwater Biology, 47*(12), 2296-2312.

Gosselain, V., Descy, J. P., Viroux, L., Joaquim-Justo, C., Hammer, A., Metens, A., & Schweitzer, S. (1998). Grazing by large river zooplankton: a key to summer potamoplankton decline? The case of the Meuse and Moselle rivers in 1994 and 1995. *Hydrobiologia, 370,* 199-216.

Greiner, P., & Ronnefahart, I. (2003). *Management of environmental risks in the life cycle of pharmaceuticals*. Lyon, April 14-16 2003.

Grung, M., Kallqvist, T., Sakshaug, S., Skurtveit, S., & Thomas, K. V. (2008). Environmental assessment of Norwegian priority pharmaceuticals based on the EMEA guideline. *Ecotoxicology and Environmental Safety, 71*(2), 328-340. doi: 10.1016/j.ecoenv.2007.10.015

Halling-Sorensen, B., Lutzhoft, H. C. H., Andersen, H. R., & Ingerslev, F. (2000). Environmental risk assessment of antibiotics: comparison of mecillinam, trimethoprim and ciprofloxacin. *Journal of Antimicrobial Chemotherapy, 46,* 53-58. doi: 10.1093/jac/46.suppl_1.53

Han, G. H., Hur, H. G., & Kim, S. D. (2006). Ecotoxicological risk of pharmaceuticals from wastewater treatment plants in Korea: Occurrence and toxicity to Daphnia magna. *Environmental Toxicology and Chemistry, 25*(1), 265-271. doi: 10.1897/05-193r.1

Hanson, M., & Lagadic, L. (2005). Chitobiase activity as an indicator of aquatic ecosystem health. *Aquatic Ecosystem Health & Management, Volume 8, Number 4, Number 4/October-December 2005 , pp. 441-450(10)*(4), 441-450.

Havel, J. E., Medley, K. A., Dickerson, K. D., Angradi, T. R., Bolgrien, D. W., Bukaveckas, P. A., & Jicha, T. M. (2009). Effect of main-stem dams on zooplankton communities of the Missouri River (USA). *Hydrobiologia, 628*(1), 121-135. doi: 10.1007/s10750-009-97508

Havens, K. E., Beaver, J. R., Casamatta, D. A., East, T. L., James, R. T., McCormick, P., . . . Rodusky, A. J. (2011). Hurricane effects on the planktonic food web of a large subtropical lake. *Journal of Plankton Research, 33*(7), 1081-1094. doi: 10.1093/plankt/fbr002

Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. *Toxicology Letters, 131*(1-2), 5-17. doi: 10.1016/s0378-4274(02)00041-3

Heino, J. (2005). Functional biodiversity of macroinvertebrate assemblages along major ecological gradients of boreal headwater streams. *Freshwater Biology, 50*(9), 1578-1587. doi: 10.1111/j.1365-2427.2005.01418.x

Henry, T. B., Kwon, J. W., Armbrust, K. L., & Black, M. C. (2004). Acute and chronic toxicity of five selective serotonin reuptake inhibitors in Ceriodaphnia dubia. *Environmental Toxicology and Chemistry, 23*(9), 2229-2233. doi: 10.1897/03-278

Henschel, K. P., Wenzel, A., Diedrich, M., & Fliedner, A. (1997). Environmental hazard assessment of pharmaceuticals. *Regulatory Toxicology and Pharmacology, 25*(3), 220-225. doi: 10.1006/rtph.1997.1102

Hirsch, R., Ternes, T., Haberer, K., & Kratz, K. L. (1999). Occurrence of antibiotics in the aquatic environment. *Science of the Total Environment, 225*(1-2), 109-118. doi: 10.1016/s0048-9697(98)00337-4

Huschek, G., Hansen, P. D., Maurer, H. H., Krengel, D., & Kayser, A. (2004). Environmental risk assessment of medicinal products for human use according to European commission recommendations. *Environmental Toxicology, 19*(3), 226-240. doi: 10.1002/tox.20015

Isidori, M., Lavorgna, M., Nardelli, A., Pascarella, L., & Parrella, A. (2005). Toxic and genotoxic evaluation of six antibiotics on non-target organisms. *Science of the Total Environment, 346*(1-3), 87-98. doi: 10.1016/j.scitotenv.2004.11.017

Jack, J. D., & Thorp, J. H. (2002). Impacts of fish predation on an Ohio River zooplankton community. *Journal of Plankton Research, 24*(2), 119-127. doi: 10.1093/plankt/24.2.119

Kaiser Family, F. (2011). 50 state comparison: retail prescription drugs filled at pharmacies (per capita). *http://www.statehealthfacts.org/* Retrieved 10/24/11, 2011

Kasprzyk-Hordern, B., Dinsdale, R. M., & Guwy, A. J. (2008). The occurrence of pharmaceuticals, personal care products, endocrine disruptors and illicit drugs in surface water in South Wales, UK. *Water Research, 42*(13), 3498-3518. doi: 10.1016/j.watres.2008.04.026

Kim, Y., Choi, K., Jung, J. Y., Park, S., Kim, P. G., & Park, J. (2007). Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea. *Environment International, 33*(3), 370-375. doi: 10.1016/j.envint.2006.11.017

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. (2002). Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environmental Science & Technology, 36*(6), 1202-1211. doi: 10.1021/es011055j

Kummerer, K. (2001). Drugs in the environment: emission of drugs, diagnostic aids and disinfectants into wastewater by hospitals in relation to other sources - a review. *Chemosphere, 45*(6-7), 957-969. doi: 10.1016/s0045-6535(01)00144-8

Lawrence, S. G., Malley, D. F., Findlay, W. J., Maclver, M. A., & Delbaere, I. L. (1987). Method for Estimating Dry Weight of Freshwater Planktonic Crustaceans from Measures of Length and Shape. *Canadian Journal of Fisheries and Aquatic Sciences, 44*(S1), s264-s274. doi: 10.1139/f87-301

Lee, H. B., Peart, T. E., Svoboda, M. L., & Backus, S. (2009). Occurrence and fate of rosuvastatin, rosuvastatin lactone, and atorvastatin in Canadian sewage and surface water samples. *Chemosphere, 77*(10), 1285-1291. doi: 10.1016/j.chemosphere.2009.09.068

Lenat, D. R., & Resh, V. H. (2001). Taxonomy and stream ecology - The benefits of genus- and species-level identifications. *Journal of the North American Benthological Society, 20*(2), 287-298. doi: 10.2307/1468323

Likens, G. E., & Lambert, K. (1998). The importance of long-term data in addressing regional environmental issues. *Northeastern Naturalist, 5*(2), 127-136.

Lopes, C., Charles, S., Vollat, B., & Garric, J. (2009). Toxicity of ivermectin on cladocerans: comparison of toxic effects on daphnia and ceriodaphnia species. *Environmental Toxicology and Chemistry, 28*(10), 2160-2166.

Lurling, M., Sargant, E., & Roessink, I. (2006). Life-history consequences for Daphnia pulex exposed to pharmaceutical carbamazepine. *Environmental Toxicology, 21*(2), 172-180. doi: 10.1002/tox.20171

Marques, C. R., Abrantes, N., & Goncalves, F. (2004). Life-history traits of standard and autochthonous cladocerans: I. Acute and chronic effects of acetylsalicylic acid. *Environmental Toxicology, 19*(5), 518-526. doi: 10.1002/tox.20059

McCauley, E. (1984). *The estimation of the abundance and biomass of zooplankton in samples*: Blackwell Scientific Publishers.

McKinley, S. J., & Hazel, J. R. (1993). Epinephrine stimulation of glucose-release from perfused trout liver - effects of assay and acclimation temperature. *Journal of Experimental Biology, 177*, 51-62.

Metcalfe, C. D., Koenig, B. G., Bennie, D. T., Servos, M., Ternes, T. A., & Hirsch, R. (2003). Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. *Environmental Toxicology and Chemistry, 22*(12), 2872-2880. doi: 10.1897/02-469

Metcalfe, C. D., Miao, X. S., Koenig, B. G., & Struger, J. (2003). Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower Great Lakes, Canada. *Environmental Toxicology and Chemistry, 22*(12), 2881-2889. doi: 10.1897/02-627

Minagh, E., Hernan, R., O'Rourke, K., Lyng, F. M., & Davoren, M. (2009). Aquatic ecotoxicity of the selective serotonin reuptake inhibitor sertraline hydrochloride in a battery of freshwater test species. *Ecotoxicology and Environmental Safety, 72*(2), 434-440. doi: 10.1016/j.ecoenv.2008.05.002

Muzzarelli, R. A. A. (1977). *Chitinases and related enzymes*. UK: Oxford.

Nikolaou, A., Meric, S., & Fatta, D. (2007). Occurrence patterns of pharmaceuticals in water and wastewater environments. *Analytical and Bioanalytical Chemistry, 387*(4), 1225-1234. doi: 10.1007/s00216-006-1035-8

Oosterhuis, S. S., Baars, M. A., & Breteler, W. (2000). Release of the enzyme chitobiase by the copepod Temora longicornis: characteristics and potential tool for estimating crustacean biomass production in the sea. *Marine Ecology-Progress Series, 196*, 195-206. doi: 10.3354/meps196195

Pace, M. L., Findlay, S. E. G., & Lints, D. (1992). Zooplankton in advective environments - the Hudson River community and a comparative-analysis. *Canadian Journal of Fisheries and Aquatic Sciences, 49*(5), 1060-1069. doi: 10.1139/f92-117

Park, S., & Choi, K. (2008). Hazard assessment of commonly used agricultural antibiotics on aquatic ecosystems. *Ecotoxicology, 17*(6), 526-538. doi: 10.1007/s10646-008-0209-x

Payan, P., & Girard, J. P. (1977). Adrenetgic receptors regulating patterns of blood flow through gills of trout *American Journal of Physiology 232* (1), H18-H23.

Pery, A. R. R., Gust, M., Vollat, B., Mons, R., Ramil, M., Fink, G., . . . Garric, J. (2008). Fluoxetine effects assessment on the life cycle of aquatic invertebrates. *Chemosphere, 73*(3), 300-304. doi: 10.1016/j.chemosphere.2008.06.029

Phillips, M. M., Dinglasan-Panlilio, M. J. A., Mabury, S. A., Solomon, K. R., & Sibley, P. K. (2010). Chronic toxicity of fluorotelomer acids to daphnia magna and chironomus dilutus. *Environmental Toxicology and Chemistry, 29*(5), 1123-1131. doi: 10.1002/etc.141

Reice, S. R. (1994). Nonequilibrium determinants of biological community structure - biological communities are always recovering from the last disturbance - disturbance and heterogeneity, not equilibrium, generate biodiversity. *American Scientist, 82*(5), 424-435.

Reynoldson, T. B., & Metcalfe-Smith, J. L. (1992). An overview of the assessment of aquatic ecosystem health using benthic invertebrates. *JOURNAL OF AQUATIC ECOSYSTEM STRESS AND RECOVERY, 1*(4), 295-308. doi: 10.1007/BF00044171

Richards, S. M., & Cole, S. E. (2006). A toxicity and hazard assessment of fourteen pharmaceuticals to Xenopus laevis larvae. *Ecotoxicology, 15*(8), 647-656. doi: 10.1007/s10646-006-0102-4

Richards, S. M., Wilson, C. J., Johnson, D. J., Castle, D. M., Lam, M., Mabury, S. A., . . . Solomon, K. R. (2004). Effects of pharmaceutical mixtures in aquatic microcosms. *Environmental Toxicology and Chemistry, 23*(4), 1035-1042. doi: 10.1897/02-616

Robinson, A. A., Belden, J. B., & Lydy, M. J. (2005). Toxicity of fluoroquinolone antibiotics to aquatic organisms. *Environmental Toxicology and Chemistry, 24*(2), 423-430. doi: 10.1897/04-210r.1

Ruttner-Kolisko, A. (1974). Plankton rotifers, biology and taxonomy. *Die Binnengewasser, 26*, 1-146.

Sadezky, A., Loffler, D., Schlusener, M., Roig, B., & Ternes, T. (2010). Real situation: occurrence of the main investigated PPs in water bodies. In B. Roig (Ed.), *Pharmaceuticals in the environment* (pp. 31-40). London: IWA Publishing.

Sanderson, H., Johnson, D. J., Reitsma, T., Brain, R. A., Wilson, C. J., & Solomon, K. R. (2004). Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. *Regulatory Toxicology and Pharmacology, 39*(2), 158-183. doi: 10.1016/j.yrtph.2003.12.006

SAS. Institute Inc. 2008. SAS Version 9.2. Cary, North Carolina.

Sastri, A. R., & Dower, J. F. (2009). Interannual variability in chitobiase-based production rates of the crustacean zooplankton community in the Strait of Georgia. *Marine Ecology-Progress Series, 388*, 147-157. doi: 10.3354/meps08111

Schloss, P., & Williams, D. C. (1998). The serotonin transporter: A primary target for antidepressant drugs. *Journal of Psychopharmacology, 12*(2), 115-121. doi: 10.1177/026988119801200201

Solomon, K., Brock, T., De Zwart, D., Dyer, S., Posthuma, L., Richards, S., . . . van den Brink, P. (2008). Conclusions *Extrapolation practice for ecotoxicological effect characterization of chemicals* (pp. 257-267). New York: CRC press.

Stanley, J. K., Ramirez, A. J., Chambliss, C. K., & Brooks, B. W. (2007). Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. *Chemosphere, 69*(1), 9-16. doi: 10.1016/j.chemosphere.2007.04.080

Stemberger, R. A. g. t. r. o. t. L. G. L. (1979). A guide to rotifers of the Laurentian Great Lakes.

Stumpf, M., Ternes, T. A., Wilken, R. D., Rodrigues, S. V., & Baumann, W. (1999). Polar drug residues in sewage and natural waters in the state of Rio de Janeiro, Brazil. *Science of the Total Environment, 225*(1-2), 135-141. doi: 10.1016/s0048-9697(98)00339-8

Tans, M., Mathoux, L., Grandjean, T., & Kestemont, P. (1998). Temporal variation and spatial heterogeneity of zooplankton food availability in backwaters and the main channel of the River Meuse (Belgium). *Annales De Limnologie-International Journal of Limnology, 34*(1), 49-55. doi: 10.1051/limn/1998006

Ternes, T. A. (1998). Occurrence of drugs in German sewage treatment plants and rivers. *Water Research, 32*(11), 3245-3260. doi: 10.1016/s0043-1354(98)00099-2

Ternes, T. A., Andersen, H., Gilberg, D., & Bonerz, M. (2002). Determination of estrogens in sludge and sediments by liquid extraction and GC/MS/MS. *Analytical Chemistry, 74*(14), 3498-3504. doi: 10.1021/ac015717z

Thomas, K. V., & Hilton, M. J. (2004). The occurrence of selected human pharmaceutical compounds in UK estuaries. *Marine Pollution Bulletin, 49*(5-6), 436-444. doi: 10.1016/j.marpolbul.2004.02.028

Thorp, J. H., Black, A. R., Haag, K. H., & Wehr, J. D. (1994). Zooplankton assemblages in the ohio river - seasonal, tributary, and navigation dam effects. *Canadian Journal of Fisheries and Aquatic Sciences, 51*(7), 1634-1643. doi: 10.1139/f94-164

Thorp, J. H., & Delong, A. D. (2002). Dominance of autochthonous autotrophic carbon in food webs of heterotrophic rivers. *Oikos, 96*(3), 543-550. doi: 10.1034/j.1600-0706.2002.960315.x

Threlkeld, S. T. (1983). Spatial and temporal variation in the summer zooplankton community of a riverine reservoir. *Hydrobiologia, 107*, 249-254.

Vieno, N., Tuhkanen, T., & Kronberg, L. (2007). Elimination of pharmaceuticals in sewage treatment plants in Finland. *Water Research, 41*(5), 1001-1012. doi: 10.1016/j.watres.2006.12.017

Viroux, L. (1999). Zooplankton distribution in flowing waters and its implications for sampling: case studies in the River Meuse (Belgium) and the River Moselle (France, Luxembourg). *Journal of Plankton Research, 21*(7), 1231-1248. doi: 10.1093/plankt/21.7.1231

Vrba, J., & Machacek, J. (1994). Release of dissolved extracellular beta-n-acetylglucosaminidase during crustacean molting. *Limnology and Oceanography, 39*(3), 712-716.

Wallace, J. B., & Eggert, S. L. (2009). Benthic invertebrate fauna, small strerams. *Encyclopedia of Inland Waters, 2*, 173-190.

Yamashita, N., Yasojima, M., Nakada, N., Miyajima, K., Komori, K., Suzuki, Y., & Tanaka, H. (2006). Effects of antibacterial agents, levofloxacin and clarithromycin, on aquatic organisms. *Water Science and Technology, 53*(11), 65-72. doi: 10.2166/wst.2006.338

Zou, E. M., & Fingerman, M. (1999). Effects of estrogenic agents on chitobiase activity in the epidermis and hepatopancreas of the fiddler crab, Uca pugilator. *Ecotoxicology and Environmental Safety, 42*(2), 185-190. doi: 10.1006/eesa.1998.1740

APPENDIX A

COMPLETE LIST OF ZOOPLANTION

Table 12

Complete list of taxa for each creek with number of individuals per liter (ind./L) and species biomass expressed as micrograms of dry weight per liter ((µg d.w.)/L).

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
North	Macro	Spring	Cladocera	Alona	rectangula	0.006	0.000
Chickamauga	Macro	Spring	Cladocera	Bosmina	longirostris	0.006	0.002
Creek	Macro	Spring	Copepoda	Calanoid	copepodid	0.006	0.005
	Macro	Spring	Copepoda	Cyclopoid	copepodid	0.011	0.010
	Macro	Spring	Copepoda	Paracyclops	chiltoni	0.006	0.008
North	Macro	Summer	Cladocera	Alona	rectangula	0.317	0.030
Chickamauga	Macro	Summer	Cladocera	Bosmina	longirostris	0.022	0.031
Creek	Macro	Summer	Cladocera	Bosminopsis	deitersi	0.083	0.133
	Macro	Summer	Cladocera	Pleuroxus	spp.	0.022	0.003
	Macro	Summer	Copepoda	Cyclopoid	copepodid	0.056	0.051
	Macro	Summer	Copepoda	Epischura	fluviatilis	0.006	0.031
	Macro	Summer	Copepoda	Tropocyclops	prasinus	0.006	0.006
North	Macro	Fall	Cladocera	Bosmina	longirostris	1.127	0.978
Chickamauga	Macro	Fall	Cladocera	Ceriodaphnia	spp.	0.020	0.019
Creek	Macro	Fall	Cladocera	Daphnia	ambigua	0.020	0.032
	Macro	Fall	Cladocera	Diaphanosoma	brachyurum	0.007	0.002
	Macro	Fall	Cladocera	Kurzia	latissima	0.007	0.001
	Macro	Fall	Copepoda	Calanoid	copepodid	0.127	0.113
	Macro	Fall	Copepoda	Cyclopoid	copepodid	0.060	0.026
	Macro	Fall	Copepoda	Eurytemora	affinis	0.007	0.020

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
North	Macro	Winter	Cladocera	Bosmina	longirostris	0.022	0.025
Chickamauga	Macro	Winter	Cladocera	Bosminopsis	deitersi	0.006	0.013
Creek	Macro	Winter	Cladocera	Chydorus	sphaericus	0.006	0.009
South Chickamauga	Macro	Spring	N/A	No Zooplankton Observed		N/A	N/A
Creek	Macro	Summer	Cladocera	Alona	rectangula	0.133	0.006
	Macro	Summer	Cladocera	Bosminopsis	deitersi	0.044	0.059
	Macro	Summer	Cladocera	immature	Cladoceran	0.006	0.004
	Macro	Summer	Copepoda	Cyclopoid	copepodid	0.011	0.005
	Macro	Summer	Copepoda	Tropocyclops	prasinus	0.006	0.006
South	Macro	Fall	Cladocera	Alona	guttata	0.006	0.000
Chickamauga	Macro	Fall	Cladocera	Alona	rectangula	0.011	0.001
Creek	Macro	Fall	Cladocera	Bosmina	longirostris	0.217	0.158
	Macro	Fall	Cladocera	Chydorus	sphaericus	0.011	0.006
	Macro	Fall	Cladocera	Kurzia	latissima	0.006	0.001
	Macro	Fall	Ostracoda	OSTRACOD		0.022	0.006
	Macro	Fall	Copepoda	Calanoid	copepodid	0.011	0.019
	Macro	Fall	Copepoda	Cyclopoid	copepodid	0.028	0.004
South	Macro	Winter	Cladocera	Alona	rectangula	0.011	0.001
Chickamauga	Macro	Winter	Cladocera	Bosmina	longirostris	0.006	0.004
Creek	Macro	Winter	Copepoda	Calanoid	copepodid	0.006	0.016
	Macro	Winter	Copepoda	Cyclopoid	copepodid	0.006	0.003
	Macro	Winter	Copepoda	Tropocyclops	prasinus	0.017	0.019

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
Candies	Macro	Fall	Ostracoda	OSTRACOD		0.011	0.001
Creek	Macro	Fall	Copepoda	Cyclopoid	copepodid	0.017	0.010
Candies	Macro	Summer	Cladocera	Bosmina	longirostris	0.139	0.040
Creek	Macro	Summer	Cladocera	Bosminopsis	deitersi	0.006	0.003
	Macro	Summer	Cladocera	Ilyocryptus	spp.	0.006	0.000
	Macro	Summer	Copepoda	Calanoid	copepodid	0.006	0.006
	Macro	Summer	Copepoda	Cyclopoid	copepodid	0.006	0.001
Candies	Macro	Summer	Cladocera	Bosmina	longirostris	0.139	0.040
Candies	Macro	Winter	Cladocera	Alona	rectangula	0.017	0.002
Creek	Macro	Winter	Cladocera	Bosmina	longirostris	0.006	0.004
	Macro	Winter	Cladocera	Chydorus	sphaericus	0.011	0.021
	Macro	Winter	Copepoda	Cyclopoid	copepodid	0.044	0.370
	Macro	Winter	Copepoda	Tropocyclops	prasinus	0.011	0.011
Soddy Creek	Macro	Spring	Cladocera	Bosmina	longirostris	0.145	0.042
	Macro	Spring	Cladocera	Chydorus	sphaericus	0.048	0.031
	Macro	Spring	Cladocera	Holopedium	gibberum	0.097	17.065
	Macro	Spring	Cladocera	Sida	crystallina	0.016	0.019
	Macro	Spring	Cladocera	immature	Cladoceran	0.161	0.102
	Macro	Spring	Ostracoda	OSTRACOD		0.016	0.001
	Macro	Spring	Copepoda	Calanoid	copepodid	0.628	0.452
	Macro	Spring	Copepoda	Cyclopoid	copepodid	0.129	0.049
	Macro	Spring	Copepoda	Epischura	fluviatilis	2.401	10.081

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
Soddy	Macro	Summer	Cladocera	Bosmina	longirostris	0.117	0.099
Creek	Macro	Summer	Cladocera	Bosminopsis	deitersi	0.039	0.060
	Macro	Summer	Cladocera	Macrothrix	rosea	0.117	0.018
	Macro	Summer	Cladocera	Sida	crystallina	0.078	0.267
	Macro	Summer	Cladocera	Simocephalus	serrulatus	0.039	1.757
	Macro	Summer	Ostracoda	OSTRACOD		0.078	0.042
	Macro	Summer	Copepoda	Calanoid	copepodid	0.039	0.060
	Macro	Summer	Copepoda	Cyclopoid	copepodid	0.311	0.173
	Macro	Summer	Copepoda	Tropocyclops	prasinus	0.039	0.040
Soddy	Macro	Fall	Cladocera	Alona	rectangula	0.083	0.006
Creek	Macro	Fall	Copepoda	Cyclopoid	copepodid	0.022	0.021
Soddy Creek	Macro	Winter	Cladocera	Chydorus	sphaericus	0.006	0.008
Sequatchie	Macro	Summer	Cladocera	Bosmina	longirostris	0.114	0.119
River	Macro	Summer	Cladocera	Ceriodaphnia	spp.	0.159	0.141
	Macro	Summer	Cladocera	Diaphanosoma	brachyurum	0.568	0.112
	Macro	Summer	Cladocera	Ilyocryptus	spp.	0.011	0.000
	Macro	Summer	Cladocera	Simocephalus	serrulatus	0.011	0.405
	Macro	Summer	Cladocera	immature	Cladoceran	0.659	0.166
	Macro	Summer	Copepoda	Cyclopoid	copepodid	0.988	0.441
	Macro	Summer	Copepoda	Ergasilus	spp.	0.045	0.055
	Macro	Summer	Copepoda	Macrocyclops	albidus	0.011	0.055
	Macro	Summer	Copepoda	Tropocyclops	prasinus	0.011	0.012
Sequatchie	Macro	Fall	Cladocera	Bosmina	longirostris	0.444	0.351

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
Sequatchie	Macro	Fall	Cladocera	Bosminopsis	deitersi	0.017	0.013
River	Macro	Fall	Cladocera	Simocephalus	vetulus	0.017	0.246
	Macro	Fall	Copepoda	Cyclopoid	copepodid	0.028	0.023
Sequatchie	Macro	Winter	Cladocera	Camptocercus	spp.	0.006	0.039
River	Macro	Winter	Copepoda	Tropocyclops	prasinus	0.006	0.005
Wolftever	Macro	Spring	Cladocera	Bosmina	longirostris	0.017	0.012
Creek	Macro	Spring	Cladocera	Ceriodaphnia	spp.	0.017	0.011
	Macro	Spring	Cladocera	Chydorus	sphaericus	0.086	0.024
	Macro	Spring	Cladocera	Daphnia	ambigua	0.138	0.171
	Macro	Spring	Cladocera	Diaphanosoma	brachyurum	0.017	0.021
Wolftever	Macro	Spring	Cladocera	Eurycercus	spp.	0.017	0.013
Creek	Macro	Spring	Cladocera	Simocephalus	serrulatus	0.017	0.033
	Macro	Spring	Cladocera	immature	Cladoceran	0.121	0.031
	Macro	Spring	Ostracoda	OSTRACOD		0.172	0.014
	Macro	Spring	Copepoda	Calanoid	copepodid	0.224	0.311
	Macro	Spring	Copepoda	Cyclopoid	copepodid	3.358	1.837
	Macro	Spring	Copepoda	Ergasilus	spp.	0.034	0.006
	Macro	Spring	Copepoda	Harpacticoid		0.017	0.012
	Macro	Spring	Copepoda	Mesocyclops	edax	0.155	0.259
	Macro	Spring	Copepoda	Skistodiaptomus	pallidus	0.121	0.297
Wolftever	Macro	Summer	Cladocera	Bosmina	longirostris	0.100	0.113
Creek	Macro	Summer	Cladocera	Ceriodaphnia	spp.	0.067	0.171
	Macro	Summer	Copepoda	Cyclopoid	copepodid	8.100	4.948

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
Wolftever	Macro	Fall	Copepoda	Cyclopoid	copepodid	0.022	0.014
Creek	Macro	Fall	Copepoda	Macrocyclops	albidus	0.056	1.162
Wolftever	Macro	Winter	Cladocera	Alona	guttata	0.006	0.000
Creek	Macro	Winter	Cladocera	Alona	rectangula	0.006	0.003
	Macro	Winter	Cladocera	Bosmina	longirostris	0.011	0.006
	Macro	Winter	Cladocera	Daphnia	ambigua	0.017	0.016
	Macro	Winter	Ostracoda	OSTRACOD		0.017	0.030
	Macro	Winter	Copepoda	Calanoid	copepodid	0.122	0.271
	Macro	Winter	Copepoda	Cyclopoid	copepodid	0.017	0.018
	Macro	Winter	Copepoda	Skistodiaptomus	reighardi	0.044	0.069
	Macro	Winter	Copepoda	Tropocyclops	prasinus	0.006	0.006
Candies	Micro	Summer	Copepoda	Nauplii		0.111	0.001
Creek	Micro	Summer	Rotifera	Bdelloid		0.111	0.018
	Micro	Summer	Rotifera	Brachionus	havanaensis	0.111	0.002
	Micro	Summer	Rotifera	Keratella	cochlearis	1.222	0.002
	Micro	Summer	Rotifera	Keratella	crassa	0.222	0.000
	Micro	Summer	Rotifera	Lepadella	spp.	0.111	0.000
	Micro	Summer	Rotifera	Monommata	spp.	0.111	0.001
	Micro	Summer	Rotifera	Synchaeta	spp.	0.167	0.001
	Micro	Summer	Rotifera	Trichocerca	multicrinis	0.056	0.004
	Micro	Summer	Rotifera	Trichocerca	spp.	0.056	0.000
	Micro	Summer	Rotifera	unidentified	rotifer	0.111	0.025

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
Candies	Micro	Fall	Copepoda	Nauplii		0.444	0.008
Creek	Micro	Fall	Rotifera	Bdelloid		0.278	0.013
	Micro	Fall	Rotifera	Dicranophorus	spp.	0.056	0.004
	Micro	Fall	Rotifera	Euchlanis	meneta	0.111	0.011
	Micro	Fall	Rotifera	Kellicottia	longispina	0.056	0.000
	Micro	Fall	Rotifera	Keratella	cochlearis	0.111	0.000
	Micro	Fall	Rotifera	Keratella	crassa	1.000	0.004
	Micro	Fall	Rotifera	Ploesoma	truncatum	0.056	0.002
	Micro	Fall	Rotifera	Synchaeta	spp.	3.722	0.044
Candies	Micro	Winter	Copepoda	Nauplii		0.500	0.024
Creek	Micro	Winter	Rotifera	Anuraeopsis	fissa	0.167	0.000
	Micro	Winter	Rotifera	Collurella	spp.	4.611	0.004
	Micro	Winter	Rotifera	Keratella	cochlearis	2.444	0.002
	Micro	Winter	Rotifera	Keratella	cochlearis f. tecta	0.056	0.000
	Micro	Winter	Rotifera	Keratella	crassa	0.278	0.001
	Micro	Winter	Rotifera	Lepadella	ovalis	0.111	0.001
	Micro	Winter	Rotifera	Monommata	spp.	0.056	0.000
	Micro	Winter	Rotifera	Notholca	acuminata	0.111	0.005
Candies	Micro	Winter	Rotifera	Polyarthra	major	0.056	0.003
Creek	Micro	Winter	Rotifera	Polyarthra	vulgaris	0.389	0.005
	Micro	Winter	Rotifera	Proales	spp.	0.167	0.017
	Micro	Winter	Rotifera	Synchaeta	spp.	1.556	0.030
Soddy	Micro	Spring	Copepoda	Nauplii		14.444	0.443
Creek	Micro	Spring	Rotifera	Brachionus	angularis	1.806	0.011
	Micro	Spring	Rotifera	Brachionus	havanaensis	9.028	0.060

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
Soddy	Micro	Spring	Rotifera	Conochiloides	dossuarius	9.028	0.272
Creek	Micro	Spring	Rotifera	Conochilus	unicornis	25.278	0.409
	Micro	Spring	Rotifera	Kellicottia	longispina	3.611	0.024
	Micro	Spring	Rotifera	Keratella	cochlearis	99.306	0.087
	Micro	Spring	Rotifera	Keratella	cochlearis f. tecta	90.278	0.064
	Micro	Spring	Rotifera	Keratella	crassa	137.222	0.609
	Micro	Spring	Rotifera	Polyarthra	vulgaris	18.056	0.425
	Micro	Spring	Rotifera	Trichocerca	iernis	7.222	0.769
Soddy	Micro	Summer	Copepoda	Nauplii		59.222	3.931
Creek	Micro	Summer	Rotifera	Anuraeopsis	fissa	13.667	0.007
	Micro	Summer	Rotifera	Collurella	spp.	646.889	0.344
	Micro	Summer	Rotifera	Filinia	longiseta	9.111	0.225
	Micro	Summer	Rotifera	Hexarthra	mira	18.222	0.334
	Micro	Summer	Rotifera	Keratella	americana	27.333	0.046
	Micro	Summer	Rotifera	Keratella	cochlearis	195.889	0.162
	Micro	Summer	Rotifera	Polyarthra	vulgaris	496.556	7.466
	Micro	Summer	Rotifera	Synchaeta	spp.	95.667	1.824
	Micro	Summer	Rotifera	Trichocerca	pusilla	18.222	0.112
	Micro	Summer	Rotifera	Trichocerca	similis	9.111	0.074
Soddy	Micro	Fall	Copepoda	Nauplii		0.556	0.012
Creek	Micro	Fall	Rotifera	Bdelloid		0.556	0.004
	Micro	Fall	Rotifera	Collurella	spp.	0.833	0.001
	Micro	Fall	Rotifera	Keratella	cochlearis	1.111	0.001
	Micro	Fall	Rotifera	Keratella	cochlearis f. tecta	0.278	0.000
	Micro	Fall	Rotifera	Keratella	earlinae	0.278	0.001

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
Soddy	Micro	Fall	Rotifera	Trichocerca	similis	0.833	0.040
Creek	Micro	Fall	Rotifera	Trichotria	tetractis	0.278	0.015
	Micro	Fall	Rotifera	unidentified	rotifer	0.278	0.002
	Micro	Fall	Bivalvia	unidentified	veliger	0.278	0.006
	Micro	Fall	Rotifera	Lecane	inermis	1.111	0.021
Soddy	Micro	Winter	Copepoda	Nauplii		0.111	0.002
Creek	Micro	Winter	Rotifera	Bdelloid		0.167	0.003
	Micro	Winter	Rotifera	Cephalodella	spp.	0.167	0.004
	Micro	Winter	Rotifera	Collotheca	spp.	0.056	0.001
	Micro	Winter	Rotifera	Keratella	cochlearis	0.167	0.000
	Micro	Winter	Rotifera	Notholca	acuminata	0.111	0.003
	Micro	Winter	Rotifera	Polyarthra	vulgaris	0.056	0.002
	Micro	Winter	Rotifera	Proales	spp.	0.111	0.005
North	Micro	Spring	Copepoda	Nauplii		1.667	0.202
Chickamauga	Micro	Spring	Rotifera	Brachionus	caudatus	0.500	0.006
Creek	Micro	Spring	Rotifera	Brachionus	havanaensis	0.167	0.004
	Micro	Spring	Rotifera	Kellicottia	longispina	0.167	0.001
	Micro	Spring	Rotifera	Keratella	cochlearis	1.000	0.001
	Micro	Spring	Rotifera	Keratella	cochlearis f. tecta	1.833	0.002
	Micro	Spring	Rotifera	Keratella	crassa	0.500	0.002
	Micro	Spring	Rotifera	Synchaeta	spp.	0.500	0.011
North	Micro	Summer	Copepoda	Nauplii		3.333	0.099
Chickamauga	Micro	Summer	Rotifera	Conochiloides	dossuarius	2.833	0.069
Creek	Micro	Summer	Rotifera	Conochilus	unicornis	2.667	0.008
	Micro	Summer	Rotifera	Keratella	cochlearis	0.667	0.000

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
North	Micro	Summer	Rotifera	Platyias	quadricornus	0.333	0.005
Chickamauga	Micro	Summer	Rotifera	Polyarthra	vulgaris	23.000	0.253
	Micro	Summer	Rotifera	Keratella	cochlearis f. tecta	4.167	0.004
	Micro	Summer	Rotifera	Lecane	spp.	0.167	0.002
North	Micro	Fall	Copepoda	Nauplii		6.000	0.463
Chickamauga	Micro	Fall	Rotifera	Anuraeopsis	fissa	0.222	0.000
Creek	Micro	Fall	Rotifera	Bdelloid		2.444	0.077
	Micro	Fall	Rotifera	Cephalodella	spp.	0.444	0.006
	Micro	Fall	Rotifera	Kellicottia	longispina	0.222	0.001
	Micro	Fall	Rotifera	Keratella	cochlearis	13.111	0.011
	Micro	Fall	Rotifera	Keratella	cochlearis f. tecta	7.778	0.006
	Micro	Fall	Rotifera	Keratella	crassa	0.444	0.001
	Micro	Fall	Rotifera	Lepadella	spp.	0.222	0.001
	Micro	Fall	Rotifera	Monommata	spp.	0.222	0.001
	Micro	Fall	Rotifera	Ploesoma	truncatum	0.667	0.033
	Micro	Fall	Rotifera	Polyarthra	vulgaris	7.333	0.120
	Micro	Fall	Rotifera	Synchaeta	spp.	10.444	0.112
	Micro	Fall	Rotifera	Trichocerca	iernis	0.222	0.004
North	Micro	Winter	Copepoda	Nauplii		0.167	0.015
Chickamauga	Micro	Winter	Rotifera	Collurella	spp.	0.333	0.000
Creek	Micro	Winter	Rotifera	Keratella	cochlearis	0.333	0.001
	Micro	Winter	Rotifera	Keratella	cochlearis f. tecta	0.167	0.000

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
South	Micro	Spring	Copepoda	Nauplii		0.778	0.043
Chickamauga	Micro	Spring	Rotifera	Keratella	cochlearis f. tecta	0.611	0.001
Creek	Micro	Spring	Rotifera	Proales	spp.	0.278	0.004
	Micro	Spring	Rotifera	unidentified	rotifer	0.278	0.005
South	Micro	Spring	Copepoda	Nauplii		0.778	0.043
Chickamauga	Micro	Spring	Rotifera	Keratella	cochlearis f. tecta	0.611	0.001
South	Micro	Summer	Copepoda	Nauplii		1.222	0.026
Chickamauga	Micro	Summer	Rotifera	Anuraeopsis	fissa	14.056	0.015
Creek	Micro	Summer	Rotifera	Cephalodella	spp.	4.889	0.053
	Micro	Summer	Rotifera	Collurella	spp.	1.833	0.002
	Micro	Summer	Rotifera	Conochiloides	dossuarius	9.778	0.143
	Micro	Summer	Rotifera	Conochilus	unicornis	2.444	0.030
	Micro	Summer	Rotifera	Filinia	longiseta	3.056	0.099
	Micro	Summer	Rotifera	Keratella	cochlearis	2.444	0.003
	Micro	Summer	Rotifera	Keratella	cochlearis f. tecta	10.389	0.011
	Micro	Summer	Rotifera	Keratella	crassa	1.222	0.005
	Micro	Summer	Rotifera	Lecane	leontina	0.611	0.028
	Micro	Summer	Rotifera	Lecane	spp.	0.611	0.002
	Micro	Summer	Rotifera	Polyarthra	major	6.722	0.414
	Micro	Summer	Rotifera	Polyarthra	vulgaris	69.056	1.019
	Micro	Summer	Rotifera	Proales	spp.	0.611	0.059
	Micro	Summer	Rotifera	Trichocerca	rousseleti	13.444	0.070
	Micro	Summer	Rotifera	Trichocerca	similis	2.444	0.029
South	Micro	Fall	Copepoda	Nauplii		0.778	0.065
Chickamauga	Micro	Fall	Rotifera	Cephalodella	spp.	0.056	0.002
Creek	Micro	Fall	Rotifera	Collurella	spp.	0.056	0.000

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
South	Micro	Fall	Rotifera	Euchlanis	meneta	0.167	0.025
Chickamauga	Micro	Fall	Rotifera	Keratella	americana	0.111	0.000
Creek	Micro	Fall	Rotifera	Keratella	cochlearis	1.000	0.001
	Micro	Fall	Rotifera	Keratella	cochlearis f. tecta	0.611	0.001
	Micro	Fall	Rotifera	Keratella	crassa	2.000	0.006
	Micro	Fall	Rotifera	Lecane	luna	0.056	0.001
	Micro	Fall	Rotifera	Lepadella	spp.	0.056	0.000
	Micro	Fall	Rotifera	Monostyla	quadridentata	0.056	0.001
	Micro	Fall	Rotifera	Polyarthra	major	0.111	0.008
	Micro	Fall	Rotifera	Polyarthra	vulgaris	0.222	0.004
	Micro	Fall	Rotifera	Synchaeta	spp.	1.333	0.023
South	Micro	Winter	Copepoda	Nauplii		0.333	0.023
Chickamauga	Micro	Winter	Rotifera	Cephalodella	spp.	0.111	0.006
Creek	Micro	Winter	Rotifera	Collurella	spp.	0.389	0.000
	Micro	Winter	Rotifera	Euchlanis	spp.	0.056	0.002
	Micro	Winter	Rotifera	Kellicottia	bostoniensis	0.056	0.000
	Micro	Winter	Rotifera	Keratella	cochlearis	0.111	0.000
	Micro	Winter	Rotifera	Keratella	earlinae	0.389	0.001
	Micro	Winter	Rotifera	Notholca	acuminata	0.056	0.002
	Micro	Winter	Rotifera	Proales	spp.	0.444	0.065
	Micro	Winter	Rotifera	Trichotria	tetractis	0.056	0.001
	Micro	Winter	Rotifera	unidentified	rotifer	0.611	0.014
Sequatchie	Micro	Summer	Copepoda	Nauplii		15.250	0.330
River	Micro	Summer	Rotifera	Anuraeopsis	fissa	18.639	0.019
	Micro	Summer	Rotifera	Asplanchna	priodonta	8.472	3.500

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
Sequatchie River	Micro	Summer	Rotifera	Brachionus	angularis	27.111	0.251
	Micro	Summer	Rotifera	Brachionus	havanaensis	25.417	0.170
	Micro	Summer	Rotifera	Conochiloides	dossuarius	5.083	0.064
	Micro	Summer	Rotifera	Conochilus	unicornis	13.556	0.177
	Micro	Summer	Rotifera	Keratella	cochlearis	1.694	0.001
	Micro	Summer	Rotifera	Limnias	sp.	133.861	10.369
	Micro	Summer	Rotifera	Monostyla	quadridentata	13.556	0.258
	Micro	Summer	Rotifera	Polyarthra	vulgaris	83.028	1.463
	Micro	Summer	Rotifera	Synchaeta	spp.	52.528	0.538
	Micro	Summer	Rotifera	Trichocerca	pusilla	5.083	0.018
	Micro	Summer	Rotifera	Trichocerca	similis	15.250	0.240
Sequatchie River	Micro	Fall	Copepoda	Nauplii		0.111	0.013
	Micro	Fall	Rotifera	Bdelloid		0.389	0.006
	Micro	Fall	Rotifera	Euchlanis	alata	0.056	0.001
	Micro	Fall	Rotifera	Euchlanis	meneta	0.278	0.231
	Micro	Fall	Rotifera	Euchlanis	spp.	0.278	0.013
	Micro	Fall	Rotifera	Keratella	cochlearis	0.111	0.000
	Micro	Fall	Rotifera	Monostyla	spp.	0.111	0.001
	Micro	Fall	Rotifera	Synchaeta	spp.	0.278	0.003
Sequatchie River	Micro	Winter	Copepoda	Nauplii		0.111	0.002
	Micro	Winter	Rotifera	Bdelloid		0.222	0.010
	Micro	Winter	Rotifera	Brachionus	angularis	0.111	0.003
	Micro	Winter	Rotifera	Collurella	spp.	0.222	0.000
	Micro	Winter	Rotifera	Keratella	cochlearis	0.222	0.001
	Micro	Winter	Rotifera	Keratella	cochlearis f. tecta	0.111	0.000

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
Sequatchie River	Micro	Winter	Rotifera	c.f. Lecane	flexilis	0.111	0.001
	Micro	Winter	Rotifera	Lecane	inermis	0.111	0.001
	Micro	Winter	Rotifera	Proales	spp.	0.667	0.093
	Micro	Winter	Rotifera	unidentified	rotifer	0.222	0.001
Wolftever Creek	Micro	Spring	Copepoda	Nauplii		270.000	24.684
	Micro	Spring	Rotifera	Anuraeopsis	fissa	387.000	0.457
	Micro	Spring	Rotifera	Brachionus	budapestinensis	18.000	0.335
	Micro	Spring	Rotifera	Brachionus	forficula	99.000	0.918
	Micro	Spring	Rotifera	Gastropus	hyptopus	9.000	0.125
	Micro	Spring	Rotifera	Keratella	cochlearis	675.000	0.906
	Micro	Spring	Rotifera	Keratella	cochlearis f. tecta	2601.000	3.278
	Micro	Spring	Rotifera	Lecane	spp.	9.000	0.076
	Micro	Spring	Rotifera	Polyarthra	vulgaris	567.000	11.763
	Micro	Spring	Rotifera	Proales	spp.	9.000	1.217
	Micro	Spring	Rotifera	Synchaeta	spp.	108.000	3.716
	Micro	Spring	Rotifera	Trichocerca	spp.	54.000	0.367
	Micro	Spring	Rotifera	unidentified	rotifer	117.000	1.291
Wolftever Creek	Micro	Summer	Copepoda	Nauplii		63.556	5.830
	Micro	Summer	Rotifera	Anuraeopsis	fissa	264.000	0.246
	Micro	Summer	Rotifera	Brachionus	bidentata	2.444	0.053
	Micro	Summer	Rotifera	Brachionus	calyciflorus	2.444	0.116
	Micro	Summer	Rotifera	Brachionus	forficula	17.111	0.102
	Micro	Summer	Rotifera	Collotheca	spp.	2.444	0.051
	Micro	Summer	Rotifera	Conochiloides	dossuarius	39.111	0.771
	Micro	Summer	Rotifera	Conochilus	unicornis	105.111	1.009

Table 12 cont.

Site Name	Sample Type	Season	Division	Genus	species	ind. / L	species biomass ((µg d.w.)/L)
Wolftever	Micro	Summer	Rotifera	Filinia	longiseta	2.444	0.060
Creek	Micro	Summer	Rotifera	Keratella	cochlearis	12.222	0.011
	Micro	Summer	Rotifera	Monostyla	quadridentata	4.889	0.122
	Micro	Summer	Rotifera	Polyarthra	vulgaris	312.889	3.392
	Micro	Summer	Rotifera	Synchaeta	spp.	22.000	0.124
	Micro	Summer	Rotifera	Trichocerca	rousseleti	14.667	0.030
	Micro	Summer	Rotifera	Trichocerca	similis	36.667	0.309
Wolftever	Micro	Fall	Copepoda	Nauplii		1.000	0.071
Creek	Micro	Fall	Rotifera	Bdelloid		0.222	0.016
	Micro	Fall	Rotifera	Keratella	cochlearis	0.333	0.000
	Micro	Fall	Rotifera	Keratella	cochlearis f. tecta	0.222	0.000
	Micro	Fall	Rotifera	Keratella	crassa	1.333	0.004
	Micro	Fall	Rotifera	unidentified	rotifer	0.333	0.005
Wolftever	Micro	Winter	Copepoda	Nauplii		1.778	0.252
Creek	Micro	Winter	Rotifera	Cephalodella	spp.	0.356	0.001
	Micro	Winter	Rotifera	Collurella	spp.	2.133	0.003
	Micro	Winter	Rotifera	Conochiloides	dossuarius	0.356	0.011
	Micro	Winter	Rotifera	Encentrum	spp.	0.444	0.043
	Micro	Winter	Rotifera	Euchlanis	alata	0.356	0.071
	Micro	Winter	Rotifera	Keratella	cochlearis	4.178	0.007
	Micro	Winter	Rotifera	Lecane	spp.	0.711	0.005
	Micro	Winter	Rotifera	Lepadella	spp.	0.356	0.002
	Micro	Winter	Rotifera	Monostyla	spp.	0.356	0.001
	Micro	Winter	Rotifera	Notholca	acuminata	0.711	0.018
	Micro	Winter	Rotifera	Proales	spp.	2.933	0.139
	Micro	Winter	Rotifera	Synchaeta	spp.	11.822	0.180

VITA

D. Neil Wolfe was born in Abingdon, Virginia, to parents Susan and Dan Wolfe. When he was 13, his family relocated to Kearney, Nebraska, where he attended Kearney High School. Upon graduating high school, he started his post-secondary education at Doane College in Crete, Nebraska. As an undergraduate, Neil was a member of the football team and co-founded a Roots and Shoots conservation club chapter at Doane. During the fall semester of 2006 he was given the opportunity to study abroad in Africa, where he backpacked from Ethiopia to South Africa. Upon returning from his semester abroad he graduated in May of 2007 with his B.S. in Biology and took a job as an arborist in Kansas City for two years. In August of 2009, Neil decided to continue his post-secondary education at the University of Tennessee at Chattanooga. While at UTC Neil has worked in the Biology department as a Teacher Assistant teaching biology labs. His research interests are in the toxicology of emerging mico-pollutants and novel methods for monitoring aquatic ecosystem health.