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Hazard assessment for a pharmaceutical mixture detected in the upper Tennessee River using *Daphnia magna*

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ABSTRACT: Widespread use of pharmaceuticals has resulted in mixture concentrations ranging from mg/L in effluent to µg/L concentrations in surface water. In a 2008 study, 13 pharmaceuticals, ranging in amounts from 0.0028 to 0.1757 µg/l, were identified in the Tennessee River, USA and its tributaries. In order to address the need for risk assessment of environmentally relevant pharmaceutical mixtures, *Daphnia magna* 21-d life cycle tests were performed on a mixture of 11 of the 13 pharmaceuticals as well as on the individual components of the mixture. Mixture exposures were based on the same initial ratios of individual compounds, up to 1000x the initial mixture concentrations. The endpoints of mortality, time to first brood, size, and fecundity were assessed. The LOEC of the 11- pharmaceutical mixture was determined to be 100x greater than the measured mixture concentration detected in the Tennessee River, with the NOEC being 75x that of the measured mixture. Single concentrations of pharmaceuticals within the mixture up to the 100x LOEC were not statistically different from control for any of the assessed endpoints. Thus, no single pharmaceutical was deemed predominately responsible for the mixture toxicity at the concentrations tested. While mixtures of pharmaceuticals are common in many systems, based on the findings of the present study, they may not pose a significant acute or chronic hazard to aquatic invertebrates at current concentrations.

Keywords: *Pharmaceuticals, Mixture toxicity, Hazard assessment, Daphnia magna*

INTRODUCTION

Human or veterinary therapeutics are commonly found in the environment (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 and Choi, 2008). Sewage treatment plants (STPs) are a major point source of these compounds. As a result, pharmaceuticals reach surface water and sediments, resulting in concentrations typically ranging from ng/L to µg/L (Kummerer, 2001). The natural aquatic environment has the potential to degrade pharmaceuticals by biotic and abiotic processes, but the continuous discharge of pharmaceutical-contaminated effluent on a daily basis results in pseudo-persistence (Castiglioni *et al*., 2006; Vieno *et al*., 2007). The potential long-term 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 (Ashton *et al*., 2004; Buser *et al*., 1998; Conley *et al*., 2008; Kolpin *et al*., 2002; Kummerer, 2001). 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 by Conley *et al*., (2008) examined a 295 km portion of the Tennessee River from Knoxville, TN to Chattanooga, TN, encompassing three STPs. That study detected thirteen pharmaceuticals with concentrations ranging from 0.0013 µg/L to 0.1757 µg/L (Table 1). Tennessee ranks 4th (of 50) in the United States for prescription drug use (Kaiser, 2011), making the Tennessee River watershed ideal to study

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Table 1: Summary of literature values for EC_{50}, LC_{50}, NOEC and LOEC on *Daphnia* species for individual pharmaceuticals found in the Tennessee River. Also presented are the 13 pharmaceuticals measured in the Tennessee River at 12 sample sites during four seasons, listed by frequency of detection (modified from Conley et al. (2008). These concentrations were the basis for the concentrations used in the present study.

Pharmaceutical Test Organism	Pharmaceutical Classification	Endpoint/duration	Conc. (mg/L)	Reference	TN River Range (mg/L) (Conley et al. 2008)	TN River Median (mg/L)	TN River Frequency (%)
Caffeine	Stimulant				8.10×10^{-5} 1.76×10^{-4}	2.88×10^{-4}	
D. magna		Reproduction/EC_{50}/17-19-d	>1	Olmstead and LeBlanc (2005)			
Sulfamethoxazole	Antibiotic				3.00×10^{-6} 3.30×10^{-5}	7.9×10^{-6}	
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				2.00×10^{-6} 2.30×10^{-5}	5.00×10^{-6}	
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	Ternes et al. (2002)			
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				2.00×10^{-6} – 6.00×10^{-6}	5.60×10^{-6}	
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)			
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				2.00×10^{-6} –1.2×10^{-5}	2.9×10^{-6}	13.3
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				1.00×10^{-6} – 1.00×10^{-5}	1.90×10^{-5}	10.2
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				4.00×10^{-6} – 5.40×10^{-5}	6.90×10^{-6}	10.2
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				6.00×10^{-6} – 5.90×10^{-5}	1.19×10^{-5}	6.3
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				2.00×10^{-6} – 1.20×10^{-5}	1.83×10^{-5}	2.3
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)			
D. magna		Immobilization/EC_{50}/48-h	0.92	Christensen et al. (2007)			
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				3.00×10^{-6} – 1.01×10^{-5}	7.00×10^{-6}	1.6
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	SSRI				N/A	N/A	N/A
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	SSRI				N/A	N/A	N/A
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. N/A = Not Applicable – these compounds were not searched for in the Tennessee Rive

environmental concentrations and relative hazard of these compounds. Ecological impacts of pharmaceuticals on non-target aquatic organisms have been investigated in the laboratory and to a lesser degree in the field. Current data on effective concentration (EC_{50}), lethal concentration (LC_{50}), no observed effect concentration (NOEC), and lowest observed effect concentration (LOEC) for the individual pharmaceuticals found in the Tennessee River on aquatic invertebrates are summarized in Table 1. One pharmaceutical that has received extensive scrutiny 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 *et a*l., 2006). Kolpin *et a*l., (2002) reported that fluoxetine concentrations averaged 0.012 µg/L in US streams. The highest detected level of fluoxetine in the Tennessee River was 0.0101 µg/L (Conley *et al*., 2008). 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). 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 5th 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 exposure than the amount that 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. However, when *D. magna* were exposed to fluoxetine and clofibric acid *in combination,* lower concentrations of fluoxetine and clofibric acid, 36 µg/L and 100 µg/L (respectively) resulted in mortality of 62.5%.

The aforementioned studies highlight 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, additional endpoints and chronic studies are essential to fully comprehend the ecological risks and impacts of compounds on aquatic organisms and their communities (Brooks *et al*., 2003; Dussault *et al*., 2008; Sanderson *et al*., 2004). Specifically, chronic exposures of organisms to the potential synergistic or antagonistic effects of pharmaceutical mixtures have been under-investigated.

Aquatic organisms are routinely exposed to complex mixtures of pharmaceuticals at low concentrations (Castiglioni *et al*., 2006; Conley *et al*., 2008). However, as indicated above, most literature reports bioassays using single pharmaceutical exposures. Currently, the Food and Drug Administration (FDA ,1998) examines a single 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 (EEC). 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 of the guideline. At this time, the US Environmental Protection Agency does not have any environmental testing requirements for human-use pharmaceuticals. Sulfamethoxazole, carbamazepine, trimethoprim, and acetaminophen have been tested individually on daphnids (Cleuvers, 2003; Dussault *et al*., 2008; Grung *et al*., 2007; Kim *et al*., 2007). In reality, these compounds occur in combination in surface waters (Castiglioni *et al*., 2006; Conely *et al*., 2008; Kolpin *et a*l., 2002), and thus mixtures should be analyzed to determine the potential effects of these pharmaceutical mixtures as they occur in the environment on aquatic organisms.

For the present study we utilized *D. magna*, a freshwater zooplankton with global distributions that plays a significant role in aquatic food webs and screening of toxicity in a regulatory context (Ternes *et al*., 2002). Moreover, they are sensitive to xenobiotics and undergo rapid reproduction (Flaherty and Dodson, 2005). Indeed, they have been shown to be more susceptible to effects from pharmaceutical exposure than other aquatic organisms, including fish (Kim *et al*., 2007). To address the data gap in pharmaceutical mixture studies for hazard assessment, *D. magna* 21-d chronic

reproduction tests were performed on pharmaceuticals as individuals, and as a complex mixtures modeled from those detected in the Tennessee River, USA.

MATERIALS AND METHODS

To evaluate the mixture toxicity of eleven pharmaceuticals detected in the Tennessee River by Conley *et al*., (2008) (Table 1), 21-d chronic laboratory toxicity test with *Daphnia magna* were conducted following standard methods of the American Society for Testing and Materials (ASTM) guidelines (ATSM 2002). After the NOEC and LOEC of the mixture were determined, concentration-response assays were then conducted with the individual pharmaceutical at the concentration in which they occurred in the mixture LOEC. The pharmaceuticals were tested individually in order to determine if any one pharmaceuticals was contributing significantly to toxicity in the mixture. Sub-lethal endpoints of toxicity were chosen to evaluate effects on the life cycle of *D. magna*. Hazard quotients (HQs) were then calculated based on the NOEC for the mixture and single pharmaceutical. Safety factors were applied to HQs values to account for uncertainty in both exposure and effects data. 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). Detailed methods are provided below.

D. magna Cultures

D. magna individuals were obtained from Aquatic BioSystems (Fort Collins, CO, USA). A laboratory culture was initiated from those individuals and maintained in an incubator set at 23 ^{o}C with a 16h:8h light to dark cycle. Mother cultures were housed in 1L beakers with approximately 40 adults per beaker. All culture water was tested to meet physiochemical requirements of ASTM guidelines (ATSM, 2002). Twice a week the culture media was renewed and neonates were removed from the mother culture and placed in individual chambers. Reproduction of these individuals was monitored daily. Neonates (<24 h old) from the 3rd through 7th brood of those individuals were then used in chronic toxicity assays.

Chronic Life Cycle Assay

As mentioned above, *D. magna* life-cycle toxicity assays were performed according to standard guidelines (ATSM, 2002) using an 11-pharmaceutical mixture. Toxicity was assessed through the endpoints of: 1) length of *D. magna* at the conclusion of the assay (growth), 2) survival of the first generation (mortality), 3) total number of neonates produced (reproduction), 4) time to the 1st brood (reproduction), and (5) number of young produced per adult female reproduction day (reproduction). The LOEC and NOEC were determined for each endpoint.

Over the course of the two-year study by Conley *et al*., (2008), 11 pharmaceuticals were consistently detected. Each pharmaceutical was detected in varying amounts over the course of the two years. In order to create the most conservative mixture (i.e., worst-case scenario mixture) of pharmaceuticals, the maximum concentration of each pharmaceutical detected by Conley *et al*., (2008), (Table 1) was prepared and combined into a single mixture. This maximal, worst-case scenario mixture was considered the 1x mixture in the present study. In order to determine the potential threshold of toxicity, solutions were created containing 10, 25, 50, 75, 100, and 1000 times as much as the 1x solution (Table 2). These concentrations (10x - 1000x) ranged from a single pharmaceutical concentration of 0.01 µg/L (diltiazem/fluoxetine) to 176 µg/L (caffeine) and a total pharmaceutical concentration ranging from 4.96 µg/L (10x) to 496 µg/L (100x) (Table 2). For example, the maximum concentration of caffeine detected by Conley *et al*., (2008) was 0.176 µg/L. Therefore, the 10x mixture concentration contained 1.76 µg/L of caffeine and the 10x total maximal values of all the pharmaceuticals quantified in the Tennessee River resulted in 4.96 µg/L (Table 2). After the completion of the tests on the 11 pharmaceutical mixture, and the 21-d LOEC and NOEC was determined, 21-d life cycle tests were conducted with each individual pharmaceutical at those LOEC and NOEC concentrations using the same testing approach as described above.

All 21-d tests exposed individual *D. magna* (n = 11 chambers, each containing one individual) for 21 days to a mixture of pharmaceuticals or single pharmaceuticals (total volume test volume 160 mL), with pharmaceutical exposures renewed every 3 days. (Table 2). The controls were treated identically (n = 5, housed individually), but exposed to only reconstituted hard water (RHW). A methanol solvent control (ACS grade) was conducted simultaneously. Methanol was used as a solvent for the pharmaceuticals that were not water soluble with a maximum concentration of 0.03 µl methanol / L of RHW. Test

Table 2: 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. An asterisk indicates pharmaceuticals that were solubilized with methanol. The 10x mixture contained 10 times the maximum concentration of each pharmaceutical detected. Accordingly, the 25x mixture contained 25 times the maximum concentration of each pharmaceutical detected. The LOEC (reproduction) was found at the 100x concentration.

	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

and control chambers were 80 × 65 (w × h) mm glass wide-mouth jars (Jarden Home Brands; Daleville, IN). 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 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 *et al*., 2010). Reproduction (number of neonates) was monitored every day but neonates were only removed when solution was renewed to avoid added stress to adult *D. magna*. Neonates were removed via a large-mouth pipette 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. Dead organisms were removed upon each renewal.

Statistics

Effects of various pharmaceutical exposures (single compound and mixtures) on each endpoint was compared to control performance using one-way repeated-measures analysis of variance (ANOVA) followed by a post-hoc Tukey test (α=0.05). Assumptions of homogeneity of variances and normality were satisfied by converting reproduction data raw values 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 SAS (Statistical Analysis System) (SAS, 2008).

Hazard Assessment

Environmental hazard posed to the organisms in the Tennessee River by the 11 pharmaceutical mixture and the individual pharmaceuticals was assessed by the calculation of hazard quotients (HQs) (FDA, 1998). The HQ was calculated by using the following equation:

$$HQ = \frac{MEC}{NOEC} \quad (1)$$

Where MEC represents the maximum measured environmental concentration detected in the Tennessee River and NOEC represents the no observed effect concentration for the most sensitive endpoint for this study. 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 whereas an HQ value >1 indicates an apparent hazard to *D. magna*

(Han, 2006). FDA and European standards recommended safety factors of 10 and 100 (EMEA, 2003; FDA, 1998), respectively. Safety factors account for interspecies variability and interaction that may cause stress that cannot be duplicated or accounted for in the laboratory (Robinson *et al*., 2005) – especially in lower-tier hazard assessments such as the present study (Solomon *et al*., 2008). While safety factors (also referred to in the literature as uncertainty factors) are somewhat arbitrary in their numerical value, they have utility being a conservative estimate, and hence highly protective.

RESULTS AND DISCUSSION

Pharmaceutical Mixture Chronic Assay

Of the five endpoints evaluated in the mixture life cycle assay (i.e., length, survival of first generation, reproduction, time to the first brood, and number of young produced per adult female reproduction), only reproduction was statistically different from the control (Fig. 1 and 2). All criteria were met for an acceptable assay i.e., *D. magna* control survival (>70%) and reproduction (>60 neonates per female) (ATSM, 2002). The number of young produced per female reproduction day was statistically similar for control (mean = 9.8) and concentrations up to the 75x mixture. At the 100x and 1000x concentrations, mean number of young produced per female reproduction day dropped significantly, to 8.6 neonates (88% of control, $p < 0.05$) and to 2.1 (28% of control, $p < 0.0001$) neonates per female reproduction day (Fig. 1), respectively. Therefore, fecundity was significantly reduced at 100x (49.6 µg/L total concentration) and 1000x (496.0 µg/L total concentration). The NOEC for the 11 pharmaceutical mixture was the 75x exposure (37.2 µg/L total concentration of pharmaceuticals) (Fig. 2).

Individual Chronic Assay

Once the LOEC (100x) was calculated for the pharmaceutical mixture, life cycle tests were performed on individual pharmaceuticals (at their individual concentrations that were present in the LOEC for the mixture assays) to determine if one or more pharmaceuticals might be driving any observed decrease in reproduction. All single pharmaceutical exposures were not statistically different from their control for all endpoints ($p > 0.05$). However, ciprofloxacin and levofloxacin showed a non-significant decrease in reproduction at 87% and 88% of control, respectively. Caffeine, sulfamethoxazole, carbamazepine, trimethoprim, sertraline and fluoxetine showed a slight increase (non-significant) in reproduction when compared to control treatments with fluoxetine having the maximum percentage increase of 10% (Fig. 3).

Hazard Assessment

Hazard quotients (HQs) were calculated in order to estimate the hazard that individual pharmaceuticals and a mixture of pharmaceuticals may pose for invertebrates in the Tennessee River. When all pharmaceuticals were

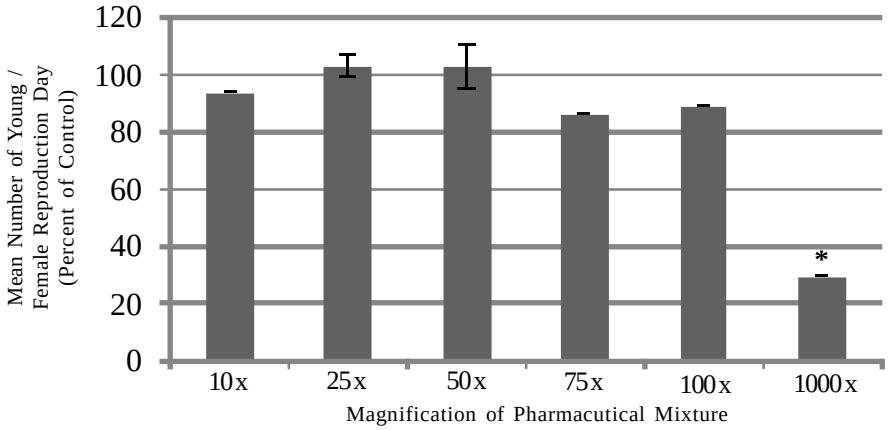

Fig. 1: Mean number of young produced per female reproduction day (expressed as the percent of the control value) following exposure to magnified concentrations of maximum concentrations found in the Tennessee River. An asterisk indicates that the mean response was significantly different from that of the control ($p \leq 0.05$). Error bars represent ±1 Standard Error.

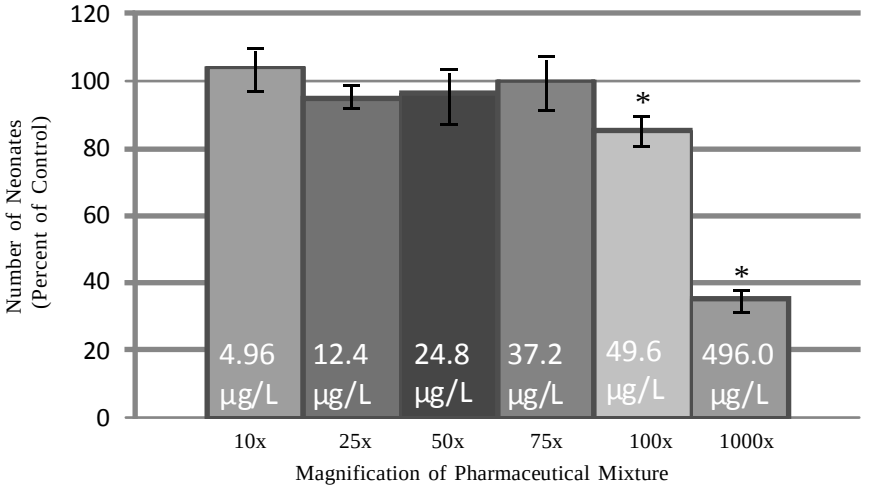

Fig. 2: Effects of varying concentrations of the 11 pharmaceutical mixtures on the reproduction of *D. magna*. Mean numbers of *D. magna* neonates produced are expressed as the percent of the control during a 21-day life cycle assay. The mixtures are magnified concentrations of maximum concentrations found in the Tennessee River. An asterisk indicates that the mean response was significantly different from that of the control ($p \leq 0.05$). Total concentrations are inset in the corresponding bars. Error bars represent ±1 Standard Error.

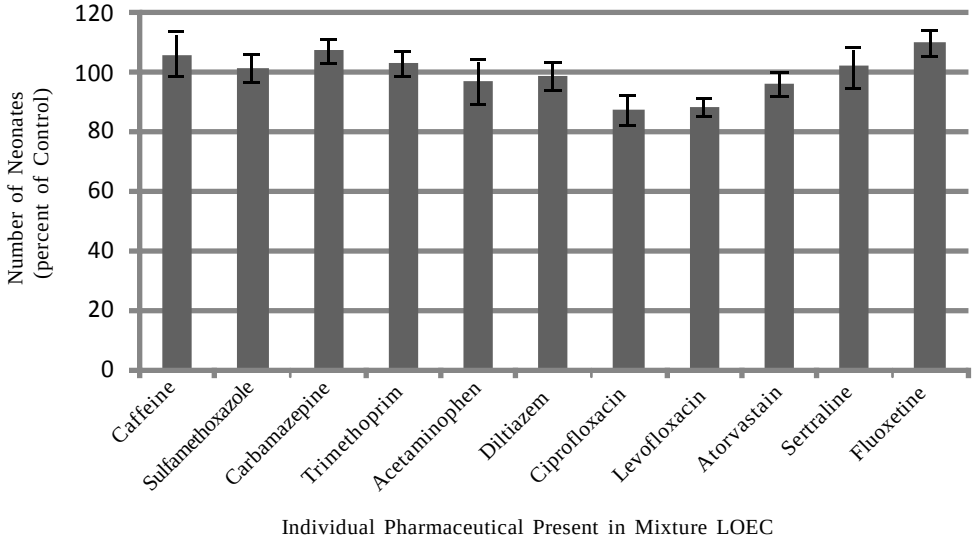

Fig. 3: Effects on *D. magna* reproduction from 21-day exposure to single pharmaceutical concentrations. *D. magna* neonates produced are expressed as percent of control values during a 21-day life cycle assay. There was not a statistically significance difference for any of the single pharmaceuticals at their concentration in the LOEC for the mixture (see Table 2 for those specific concentrations). Error bars represent ±1 Standard Error.

considered as a mixture, the HQ, with a safety factor of 100, was >1 (Table 3). When MECs were combined with the NOEC values observed herein, an HQ >1 for the 11 pharmaceutical mixture was produced (1.9) – *without* the addition of a safety factor (Table 3). The individual pharmaceuticals caffeine, sulfamethoxazole, and diltiazem also had HQ values > 1 when a safety factor of 100 was applied to the pharmaceutical maximum MEC found in the Tennessee River (Table 3). Four of the 11 pharmaceuticals in the present study have been studied previously for effects on *D. magna* 21-day reproduction (Lurling *et al*., 2006; Minagh *et al*., 2009; Park and Choi, 2008; Yamashita, 2006). In those studies, carbamazepine, trimethoprim, levofloxacin, and sertraline had NOECs at 200 µg/L, 6,000 µg/L, 310 µg/L, and 32 µg/L, respectively. Those values are orders of magnitude greater than the current study's NOECs. If the NOEC values reported from those studies are used to calculate HQ for these four pharmaceuticals the result is < 1 even after a safety factor of a 100 was applied.

Pharmaceutical Mixture Toxicity

The present experiment and HQ calculations were designed to reflect a possible worst-case scenario for a pharmaceutical mixture found in the Tennessee River; however, we recognize that many other factors were not captured or considered that would reflect the full potential for toxicity in the Tennessee River. Realistically, factors in the Tennessee River could increase or decrease the potential for pharmaceuticals to adversely impact aquatic invertebrates. Foremost, not all pharmaceuticals tested in the present mixture were detected in every surface water sample, nor was every sample detected at the maximum concentration (Conley *et al*., 2008). By assuming that all components are occurring together, at their maximum concentrations, we are adding conservatism to our assessment. Our results indicate that the mixture of pharmaceuticals has a lower LOEC for reproduction (Fig. 2), than any of the individual pharmaceuticals separately. This suggests that there are interactions of the mixture components, though whether additive, synergistic, or even a multiple types, including antagonistic is not known. 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 was not measured in the present study.

The fact that reproduction was the only endpoint significantly reduced is not unexpected; reproduction has

Table 3: Hazard quotients (HQs) for *D. magna*. HQs were calculated using the highest measured concentration in the Tennessee River (TN. MEC) and the no observed effect concentration observed for reproduction in the present 21-day study (TN. NOEC). Values >1 indicate a potential hazard to *D. magna*. Literature MEC (Lit. MEC) are maximum concentrations detected globally. The Lit. NOEC is the NOEC value found in the literature (Table 2).

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

been shown to be one of the most sensitive endpoints for *D. magna* (Minagh *et al*., 2009). In the 100x concentration, reproduction was reduced to just 85% of control. The 1000x concentration caused reproduction to be reduced to 35% of control indicating reproduction was affected in a concentration-dependent manner by the mixture. This decline 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 are supported by previous studies. For example, Park and Choi, (2008) found that *D. magna* reproduction is concentration-dependent for eleven antibiotics. Cleuvers, (2008) conducted a study investigating the chronic effects of a mixture of naproxen, ibuprofen, and diclofenac mixture on *D. magna* and found that reproduction was also reduced in a concentration-dependent manner by the mixture, where 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 as the mortality endpoint was not observed at these concentrations. Data such as these and ours reaffirm the need to use sub-lethal parameters as endpoints to address the pharmaceuticals' potential environmental risk.

The HQ for the 11 pharmaceutical mixture of this study (calculated using Tennessee River MECs) was 0.013. When an uncertainty factor of 10 was applied the mixture HQ was still below 1. This indicates that the mixture of 11 pharmaceuticals currently detected in the Tennessee River poses little hazard to *D. magna* at concentrations 10x greater than what is currently maximally detected – according to FDA guidelines (FDA, 2008). 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 uncertainty factor of 100 to HQs (EMEA, 2003). Thus, the mixture of 11 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, 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.

Pharmaceutical use occurs worldwide, so we can apply this HQ approach to surface waters elsewhere. If the greatest reported MEC is applied to the NOEC found in the current study for the mixture, the HQ would be greater than 1 with no uncertainty factor applied (Table 3). Thus, in surface waters that approach the worldwide MEC for all pharmaceutical in the present mixture, there is a potential hazard to *D. magna*. 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 individual pharmaceuticals was to determine if one (or more) of the single pharmaceuticals were solely or contributing significantly to reduced reproduction. No single pharmaceutical was the driving factor (Fig. 3) (i.e., no singular pharmaceutical was as toxic as the mixture) (Table 3). All HQs values were <1 for MECs - even when a safety factor of 10 was applied.

Two individual pharmaceutical tests did result in a decline (though statistically insignificant) in *D. magna* reproduction. The fluoroquinolone antibiotics ciprofloxacin and levofloxacin showed a decrease in reproduction 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 *et al*., 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 *et al*., 1999).

A previous study by Robinson *et al*., (2005) found that neither levofloxacin nor ciprofloxacin posed a significant hazard when tested in an acute 48-hour survival test of *D. magna* with a 24 hr NOEC at 10 mg/L. However that study's lone endpoint was acute survival. 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 48-h NOEC mortality of 60 mg/L when *D. magna* were exposed to ciprofloxacin. In general, 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 assay were available. Yamashita *et al*., (2006) found reproduction NOEC and LOEC for levofloxacin in *D. magna* to be much higher than that reported in the present study: 310 µg/L and 630 µg/L, respectively (Table 1). In the present study, both ciprofloxacin and levofloxacin were found to have NOEC at 5.4 µg/L and 5.9 µg/L, respectively.

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 and 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 *D. magna* reproduction is 1000x greater than what was tested in the current study.

The longest assay that could be found in the literature for sulfamethoxazole was a 96hr 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 *et al*., 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 present study, the NOEC for carbamazepine was 2.3 µg/L; although, there was a statistically insignificant increase in reproduction that resulted in 100% percent of control (p=0.11) (Table 2). A significant increase in reproduction would strongly 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 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, 2006; Richards and Cole, 2006; Schloss and 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 and 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 *D. magna* 21-day reproduction LOEC of 100 µg/L and a NOEC of 32 µg/L (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 and Choi 2008). Sertraline had the lowest NOEC at 32 µg/L (Minagh *et al*., 2009). The lack of data available for these individual pharmaceuticals needs to be addressed in order to better

characterize 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 present study. As a result, our NOEC values for single pharmaceuticals may be orders of magnitude lower than the actual NOECs. 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 0.6 µg/L whereas the NOEC reported in the literature is 6 mg/L (Park and Choi, 2008). Our NOEC is 1,000x lower than what is reported in the literature; i.e., our HQ for trimethoprim would be equivalent to the addition of an uncertainty factor of 1,000-fold relative to other reported values. The NOECs 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.

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 previously 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, simple comparisons of literature NOEC and present-study NOEC cannot be made.

CONCLUSION

The occurrence of pharmaceuticals as complex mixtures and at small concentrations is well documented globally. Most studies that address the ecological hazard of pharmaceuticals only account for the toxicity of single pharmaceutical exposure, usually under acute conditions, and do not take into account chronic additive or synergistic affects that can occur in mixtures. This is a concern 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*. Our 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 deemed low to non-existent.

Our data suggest that the current hazard of this 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 added to the system increases, the potential for adverse responses is likely increase as well. With over 3,000 active ingredients in use today, the possibility of many more pharmaceuticals in the environment cannot be ignored. The use of uncertainty factors will help compensate for some of this knowledge gap, but it is difficult to determine to what degree. 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* is exposed to 11 pharmaceuticals simultaneously at the maximum environmental concentration 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 margin could be maintained as human populations grow.

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