PERGAMON

Chemosphere 52 (2003) 135–142

CHEMOSPHERE

www.elsevier.com/locate/chemosphere

Waterborne and sediment toxicity of fluoxetine to select organisms

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Received 25 July 2002; received in revised form 3 January 2003; accepted 21 January 2003

Abstract

Ecological risk assessments of pharmaceuticals are currently difficult because little-to-no aquatic hazard and exposure information exists in the peer-reviewed literature for most therapeutics. Recently several studies have identified fluoxetine, a widely prescribed antidepressant, in municipal effluents. To evaluate the potential aquatic toxicity of fluoxetine, single species laboratory toxicity tests were performed to assess hazard to aquatic biota. Average LC_{50} values for *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas* were 0.756 (234 μg/l), 2.65 (820 μg/l), and 2.28 μM (705 μg/l), respectively. *Pseudokirchneriella subcapitata* growth and *C. dubia* fecundity were decreased by 0.044 (14 μg/l) and 0.72 μM (223 μg/l) fluoxetine treatments, respectively. *Oryias latipes* survival was not affected by fluoxteine exposure up to a concentration of 28.9 μM (8.9 mg/l). An LC_{50} of 15.2 mg/kg was estimated for *Chironomus tentans*. *Hyalella azteca* survival was not affected up to 43 mg/kg fluoxetine sediment exposure. Growth lowest observed effect concentrations for *C. tentans* and *H. azteca* were 1.3 and 5.6 mg/kg, respectively. Our findings indicate that lowest measured fluoxetine effect levels are an order of magnitude higher than highest reported municipal effluent concentrations.

Keywords: Environmental pharmaceuticals; Fluoxetine; Ecological risk assessment; Sediment toxicity; SSRI

1. Introduction

A goal of the US Clean Water Act is to protect aquatic life from deleterious anthropogenic activities. For example, Section 303 provides statutory support for water quality standards and the National Pollutant Discharge Elimination System (NPDES) regulates point source pollutant discharges to surface waters (Grothe et al., 1995). Prior to developing water quality criteria, minimum toxicity data are necessary for pollutants (USEPA, 1985). Whereas criteria exist for many contaminants and NPDES permits regulate discharges, recent research indicates that multiple classes of pharmaceutical chemicals are present in municipal wastewater effluents (Ternes, 1998; Kolpin et al., 2002; Huggett et al., 2003) and in sediments downstream from

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0045-6535/03/$ - see front matter
doi:10.1016/S0045-6535(03)00103-6

municipal discharges (Furlong etal., 2002). Currently, US EPA water quality criteria do not exist for steroid or non-steroid pharmaceuticals.

Webb (2001) recently identified several pharmaceuticals that have the potential to adversely affect aquatic ecosystems. Included among these compounds was fluoxetine, a selective serotonin reuptake inhibitor (SSRI). One of the most prescribed antidepressants (RxList, 2000), fluoxetine blocks serotonin reuptake transporters (Ranganathan et al., 2001). Fluoxetine and other SSRIs also act at a number of sites including norepinephrine uptake and Sigma receptors (Sanchez and Meier, 1997) and neuronal and muscle nicotinic acetylcholine receptors (Garcia-Colunga et al., 1997). Many SSRIs including fluoxetine bind with high affinity to other receptors like Sigma receptors, with unknown consequences. Fluoxetine exposure has also been shown to increase extracellular norepinephrine and dopamine in rats (Bymaster et al., 2002) and to potentially influence neuroendocrine function in Japanese medaka (Brooks et al., 2003). Fluoxetine, a racemic mixture of two lipophilic enantiomers, is metabolized by cytochrome P450 isoenzymes to norfluoxetine, its active metabolite. Fluoxetine is primarily excreted in the urine with less than 10% as unchanged parent compound (Hiemke and Härtter, 2000).

Whereas municipal effluents laden with steroids and other estrogenic compounds have received considerable attention, few peer-reviewed environmental hazard and exposure data are available for non-steroidal pharmaceuticals (Huggett et al., 2002, 2003; Brooks et al., 2003). Fewer studies have evaluated environmental effects of antidepressants (Fong, 2001). *Daphnia magna* and *Daphnia pulex* immobilization EC_{50} values for amitriptyline, a tricyclic antidepressant, were reported at 4.15 and 3.73 nM, respectively (Lilius et al., 1995), while Flaherty et al. (2001) found nominal treatment of 116 nM fluoxetine to significantly affect *D. magna* reproduction. Nominal fluoxetine treatment also affected embryonic *Physa elliptica* rotational behavior at 0.1–1 μM (Uhler et al., 2000) and potentiated male mussel spawning at 50 nM (Fong et al., 1998). However, responses of benthic organisms to sediment fluoxetine exposure have not been reported.

Recent findings indicate that fluoxetine is discharged in municipal effluents (Weston et al., 2001). Therefore, the primary objective of this study was to evaluate the environmental hazard of fluoxetine to select benthic and pelagic toxicity test organisms. This study provides novel waterborne fluoxetine toxicity data for *Pseudokirchneriella subcapitata*, *Ceriodaphnia dubia*, *Daphnia magna*, *Pimephales promelas* and *Oryias latipes*, and sediment toxicity information for *Hyalella azteca* and *Chironomus tentans*.

2. Materials and methods

2.1. Aqueous toxicity test methods

2.1.1. Algae

P. subcapitata stock cultures were maintained in 250-ml Erlenmeyer flasks containing 125 ml Gorham's media (ATCC, 1984). New stock cultures were inoculated from existing cultures every 7–10 days. Algal tests followed general procedures recommended by the USEPA (1989). Briefly, fluoxetine was added to three replicate 125-ml flasks at 0, 43.6, 87.3, and 174.4 nM. Twenty-five milliliter sterile AAP media (ATCC, 1984) was inoculated with 4 to 7-day-old stock culture algae such that flasks contained approximately 1×10^4 cells/ml at test initiation. Flasks were placed under cool white fluorescent lighting (2.15×10^3 lm m^{-2}) with a light cycle of 16 h light, 8 h dark. During a 120 h exposure period, temperature was maintained at 25 ± 1 °C and flasks were hand-swirled twice daily.

Algal growth was evaluated by enumeration and turbidity measurements (USEPA, 1989). Cell counts were made using a hemocytometer and a Nikon® compound microscope. Turbidity was determined as absorbance with a Beckman DU 64 spectrophotometer at 750 nm.

2.1.2. Cladocera

C. dubia and *D. magna* were mass cultured as previously described (Turner et al., 2001; Hemming et al., 2002). Static *C. dubia* and *D. magna* 48-h acute toxicity tests were performed with less than 24-h old neonates following standard methods (USEPA, 1991). A 7-day study was conducted to evaluate *C. dubia* fecundity responses to fluoxetine treatments at nominal levels of 0, 45.5, 91, 181, 362 and 724 nM (USEPA, 1989). This toxicity test received daily static renewals of test solutions that were stored in dark, 4 °C environmental chambers between renewals. Risley and Bopp (1990) previously demonstrated the persistence of fluoxetine for six weeks in water stored at temperatures up to 65 °C or exposed to ultraviolet light. Organisms were fed a 0.5 ml algae-Cerophyll® suspension following daily renewals of test solutions (Knight and Waller, 1992; Hemming et al., 2002). All studies were performed at 25 ± 1 °C with 16 h light, 8 h dark cycles.

2.1.3. Fish

P. promelas eggs were acquired from breeding cultures maintained at the Institute of Applied Sciences, Denton, TX. Prior to toxicity testing, juvenile fish were fed 24–48-h old *Artemia* nauplii twice daily. Two acute 48 h toxicity tests, hereafter tests 1 and 2, were conducted with 11- and 14-day old fathead minnows, respectively (USEPA, 1991). Organisms were not fed

during 48-h tests. A light cycle of 16 h light, 8 h dark was maintained at 25 ± 1 °C. *Oryias latipes* were cultured according to methods of Yamamoto (1975). Ten juvenile organisms each were placed in three replicate beakers at nominal concentrations of 0, 1.8, 3.6, 7.2, 14.5, and 28.9 μM for a 48 h acute study. Temperature was maintained at 25 ± 1 °C with a 16 h light, 8 h dark cycle.

2.2. Sediment toxicity test methods

Sediment toxicity tests were performed using a Zumwalt testing system (USEPA, 2000). Dechlorinated, activated carbon treated tap water served as overlying water in sediment tests (USEPA, 2000). Two volume additions per day were performed on overlying water. Reference sediments were obtained from pond mesocosms at the University of North Texas Water Research Field Station. Physical sediment parameters were evaluated including organic carbon, particle characterization, and percent water. Sediments were spiked with fluoxetine according to methods of Suedel and Rodgers (1996). Ten-day *C. tentans* and *H. azteca*, and 42-day *H. azteca* toxicity tests followed standard methods (USEPA, 2000). Following preliminary range finding toxicity tests, 10-day *C. tentans* and *H. azteca* treatment levels were selected at 0, 1.4, 2.8, 5.6, 11.2 and 22.4 mg/kg, and 0, 5.4, 10.8, 21.6 and 43.2 mg/kg, respectively. Treatment levels for the 42-day *H. azteca* reproduction study were selected at 1.4, 2.8, 5.6, 11.2, and 22.4 mg/kg.

2.3. Analytical measures

Fluoxetine ([±]-*N*-methyl-γ-[4- trifluoromethyl)phenoxy]benzenepropanamine) hydrochloride was obtained from Sigma Chemical (St. Louis, MO). Waterborne fluoxetine treatment concentrations were verified according to methods of Weston et al. (2001). Briefly, 100 ml subsamples of lowest, mid, and highest treatment level solutions were adjusted to pH = 9, pre-filtered with 1.2 μm GFF, and extracted with hexane and methanol conditioned Empore® C18 solid phase extraction disks. Disks were eluted with methanol, brought to dryness, resolublized in 100 μl methanol, and analyzed by a Waters Alliance model 2690 liquid chromatograph with Waters 996 photo diode array and Waters Micromass ZQ mass selective detectors. Method detection limits were 0.2 ng/l. Sediment fluoxetine concentrations were not verified.

C. dubia, *D. magna*, and *P. promelas* bioassays were performed in reconstituted hard water (RHW) (APHA et al., 1995). *O. latipes* tests were performed in a balanced saline solution (BSS) (Yamamoto, 1975). For each study, temperature, pH, dissolved oxygen, conductivity or salinity, alkalinity and hardness measures followed standard methods (APHA et al., 1995).

2.4. Statistical analyses

P. subcapitata EC_{50} levels were estimated using nonlinear regression with the SAS system (Version 8.0 for Windows; Bruce and Versteeg, 1992). LC_{50} values for *C. dubia*, *D. magna*, *P. promelas* and *C. tentans* tests were estimated using Trimmed Spearman Karber (Hamilton et al., 1977). *P. subcapitata*, *H. azteca* and *C. tentans* growth, and *C. dubia* and *H. azteca* fecundity responses were evaluated using a one-way ANOVA with Dunnett's test for multiple comparisons (Zar, 1984).

3. Results and discussion

3.1. Analytical measures

Reconstituted hard water quality parameters were within guidelines recommended by USEPA (1991) and are listed in Table 1. Percent fluoxetine recoveries from aquatic treatment levels ranged from 95% to 113%; therefore, nominal concentrations are presented in Figs. 1 and 2, Table 2, and throughout this manuscript. Sediment total organic carbon was 22340 mg/kg; percent moisture was 60%. Sediment grain size was distributed as 41.2% sand, 39.2% silt and 19.6% clay.

3.2. Acute fluoxetine toxicity

O. latipes and *H. azteca* survival was unaffected across treatment levels. An LC_{50} of 15.2 mg/kg was estimated for *C. tentans*. Average LC_{50} values for *C. dubia*, *D. magna*, and *P. promelas* toxicity tests were 0.756, 2.65, and 2.28 μM, respectively. This average *D. magna* LC_{50} is similar to a nominal value of 2.72 μM previously reported for a *Daphnia* spp. (FDA-CDER, 1996). *P. promelas* LC_{50}'s for tests 1 and 2 were 2.22 and 2.88 μM, respectively. Such a toxicity decrease from test 1 to test 2 may be related to age of test organisms; test 1 organisms were 11 d old, whereas organisms were 14 d old in test 2. Fluoxetine, similar to other xenobiotics, is metabolized from the parent compound by cytochrome P-450 isoenzymes to an active metabolite, norfluoxetine.

Table 1
Water quality characteristics of aqueous toxicity tests

Parameter	Reconstituted hard water	Balanced saline salt solution
pH	8.0	6.637
Temperature (°C)	25 ± 1	25 ± 1
Dissolved oxygen (mg/l)	7.8	8.83
Hardness (mg/l $CaCO_3$)	170	82.5
Alkalinity (as mg/l $CaCO_3$)	110	34.3
Conductivity (μmhos)	500	NM
Salinity (g/l)	NM	1.64

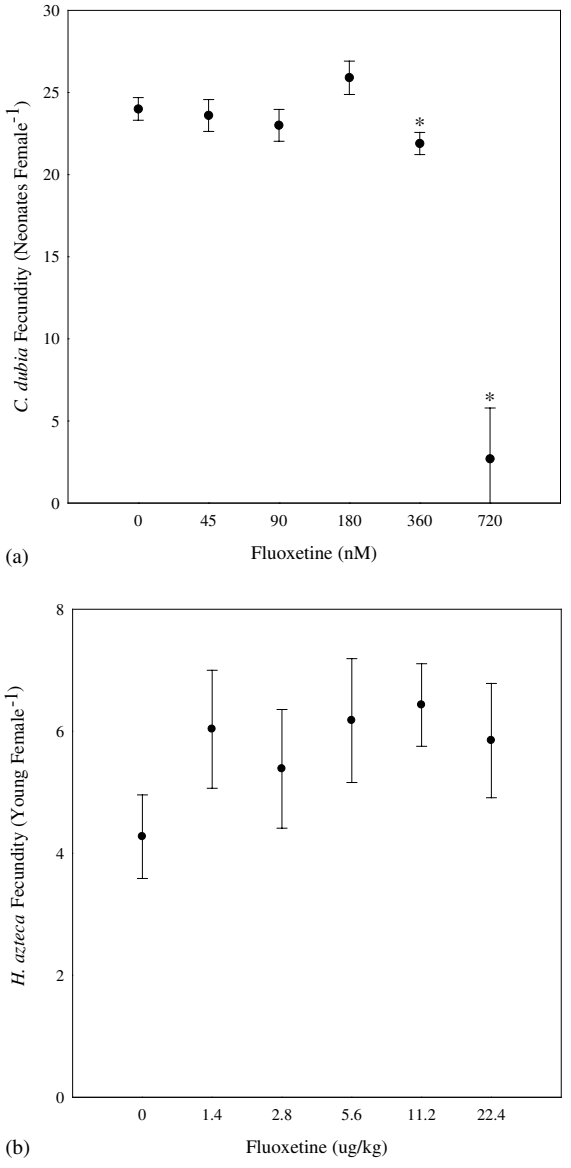

Fig. 1. Effects of fluoxetine on: (a) *C. dubia* reproduction (±SE; $N = 10$; $F = 5.093$; df $= 40, 4$; *: $P < 0.05$) and (b) *H. azteca* reproduction (± SE; $N = 8$, $P = 0.05$).

For example, Hamm et al. (2001) found that bioactivation of diazinon to its potent metabolite increased with development in juvenile Medaka. Observed differences in fluoxetine toxicity to fathead minnows may be associated with an age dependent developmental increase in P-450 metabolism. In this study, older *P. promelas* were less sensitive to fluoxetine treatment.

3.3. Sublethal fluoxetine toxicity

Lowest observed and no observed fluoxetine effect levels on *C. dubia* reproduction were 360 and 180 nM,

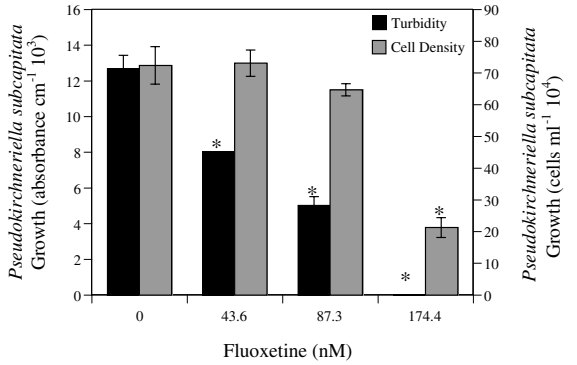

Fig. 2. *P. subcapitata* growth responses, measured as turbidity (±SD; $N = 3$; $F = 101.7$, df $= 8, 3$; *: $P < 0.05$) and enumeration (±SD; $N = 3$; $F = 28.9$, df $= 8, 3$; $P < 0.05$), to fluoxetine treatments.

Table 2
P. subcapitata growth (EC_{50}) and *C. dubia*, *D. magna*, *P. promelas* and *C. tentans* survival (LC_{50}) responses to fluoxetine treatments

Organism	LC/EC_{50}
P. subcapitata	
Cell density	0.126 μM (39 μg/l)
Turbidity	0.077 μM (24 μg/l)
C. dubia	0.756 μM (234 μg/l)[a]
D. magna	2.65 μM (820 μg/l)[a]
P. promelas	2.28 μM (705 μg/l)[a]
C. tentans	15.2 mg/kg

[a] LC_{50} values are averaged for duplicate experiments.

respectively (Fig. 1a; $P = 0.05$, $F = 5.093$, df $= 40, 4$). However, such an observed statistically significant decrease in fecundity may not be of ecological relevance because the mean difference between 360 nM and 0 treatments was just 2.1 neonates. Each fluoxetine treatment level significantly reduced *C. tentans* growth such that a lowest observed effect level (LOEC) of 1.3 mg/kg ($P = 0.05$, $F = 10.632$, df $= 15, 2$) was observed. *H. azteca* growth was also significantly reduced by all treatment levels such that a LOEC was determined at 5.6 mg/kg ($P = 0.05$, $F = 6.566$, df $= 15, 4$). Therefore, *C. tentans* was more sensitive to fluoxetine than *H. azteca*. A plausible explanation for greater *C. tentans* sensitivity to fluoxetine is increased exposure via ingestion of sediments. Because *H. azteca* is epibenthic, it is likely that dietary exposure to fluoxetine is less than that *C. tentans* would experience during sediment burrowing.

Following a 42-day study period, *H. azteca* fecundity was not significantly affected by fluoxetine treatments (Fig. 1b). However, all treatment levels appeared to stimulate *H. azteca* reproduction (Fig. 1b). We also

observed an increase in *C. dubia* fecundity at the 180 nM treatment level (Fig. 1a). Flaherty et al. (2001) observed a similar reproductive stimulation when *D. magna* were nominally exposed for thirty days to 116 nM fluoxetine. In fish, serotonin stimulates release of gonadotropin in some species, which stimulates sex steroid synthesis and controls the development of oogenesis, including vitellogenesis (Arcand-Hoy and Benson, 2001). In invertebrates, serotonin has been shown to stimulate ecdysteroids, ecdysone, and juvenile hormone which control oogenesis and vitellogenesis (Nation, 2002). Whereas serotonergic effects on ecdysteroids, ecdysone, and juvenile hormone are less understood (LeBlanc et al., 1999), observed fecundity stimulation may result from increased synaptic serotonin levels. Such an increase in *C. dubia* and *H. azteca* reproduction need not be associated with maintenance of offspring fitness. Numerous studies identified that low-level contaminant exposure may result in higher fecundity; however, increased egg and neonate production is often associated with reduced egg and neonate body size (Bodar et al., 1988; Ebert, 1993). Future research is warranted to identify the potential mechanism(s) of crustacean reproductive stimulation by fluoxetine and potential changes in the timing of invertebrate reproduction (Hoonkoop et al., 1999) following fluoxetine exposure.

P. subcapitata growth responses to fluoxetine treatments were evaluated by two methods: cell density and turbidity, the later reported previously as a sensitive measure of algal growth (USEPA, 1989). *P. subcapitata* EC_{50} values were estimated at 126 nM by cell density and 77 nM by turbidity (Table 2). These values bracket a previously reported nominal value of 89 nM for an unknown green alga (FDA-CDER, 1996). *P. subcapitata* growth as turbidity was significantly reduced at 43.6 nM ($P = 0.05$, $F = 101.7$, $df = 8, 3$) and at 174.4 nM ($P = 0.05$, $F = 28.9$, $df = 8, 3$) as cell number (Fig. 2). Whereas cell density was not significantly affected by fluoxetine treatment at 87.3 and 174.4 nM, cell deformities were observed and cell sizes were reduced at both treatment levels. Cells appeared shriveled and frequently were not crescent shaped, a normal characteristic of *P. subcapitata*. The mechanism of toxicity for observed cell deformities is not known; however, fluoxetine is known to have bacteriostatic effects, perhaps exerted by efflux pump inhibition (Munoz-Bellido et al., 2000). Although cell deformities and biovolumes were not quantified in this study, such an effect of fluoxetine on algal cells warrants further investigation.

3.4. Preliminary ecological risk characterization for fluoxetine

Presently, the US Food and Drug Administration requires environmental assessments of new pharmaceuticals only if predicted environmental introduction concentrations (EIC) are greater than 1 μg/l (3.32 nM for fluoxetine; FDA-CDER, 1998). Daughton and Ternes (1999) identified that this approach: does not consider additive effects of therapeutics with similar mechanisms of action, does not consider interactions of compounds with different mechanisms of action, and relies on traditional ecotoxicology bioassay response variables. In addition, a default dilution factor of 10 is applied to an EIC in an attempt to approximate realistic environmental exposures. Such an approach is generally appropriate because 77% of permitted effluent dischargers receive greater than 10 fold dilution at annual mean flow (Dorn, 1996). This exercise becomes problematic in areas where effluent discharges do not benefit from upstream dilution. For example, perennial municipal effluents often influence historically ephemeral streams in the southwestern US; 90% of the flow of the Trinity River south of Dallas/Fort Worth, TX is dominated by reclaimed waters from treatment plants (Dickson et al., 1989). Environmental pharmaceuticals in these regions may present the greatest risk to aquatic organisms.

Ecological risk is often simplistically characterized by evaluating the relationship between an ambient exposure concentration (AEC) and a toxicologically effective concentration (TEC; Suter et al., 2000). Conservative safety factors are also applied to overcome the uncertainties inherent in risk estimation procedures (Warren-Hicks and Moore, 1998). If a hazard quotient (HQ) derived from a ratio of AEC/TEC is >1, then risk to the environment may become a concern. Weston et al. (2001) found fluoxetine concentrations in municipal effluents to range from 0.3 to 1.6 nM. This range is consistent with an ambient exposure concentration of 1.2 nM predicted for United Kingdom effluents (Webb, 2001). The lowest fluoxetine effect level reported in this study was 43.6 nM for algal growth. Therefore, the lowest observed adverse effect level of fluoxetine on aquatic biota is an order of magnitude higher than the highest reported environmental fluoxetine concentrations. Based on data presented in this study an HQ is calculated at $\ll 1$.

Previous investigators discussed reliance on aquatic toxicity test responses for regulatory decisions (Cairns, 1983; Mount, 1994). Laboratory bioassays may not be representative of the most sensitive species (Cairns, 1986) and are not intended to predict structural or functional community responses to stressors (La Point and Waller, 2000). Others suggested that longer term studies with low concentrations are necessary to assess pharmaceuticals effects on higher levels of biological organization (Daughton and Ternes, 1999; Brooks et al., 2003). Further, Daughton and Ternes (1999) identified that bioassays focused on specific mechanisms of action are necessary to assess therapeutic effects on non-target biota. Identifying such mechanistic toxicity will be challenging because the responses of aquatic organisms to

fluoxetine are largely unknown. For example, the beta-adrenergic receptor blocking pharmaceuticals propranolol and metaprolol significantly reduce cladoceran heart rate and metabolic activity at concentrations an order of magnitude lower than those that affect reproduction (Dzialowski et al., 2002).

4. Conclusions

Sensitivities of organisms tested in this study to fluoxetine were *P. subcapitata* > *C. dubia* > *P. promelas* > *D. magna* > *C. tentans* > *H. azteca*. Our results indicate that fluoxetine adversely affects aquatic organisms at levels at least an order of magnitude higher than that reported in municipal effluent concentrations. When pesticide exposure and effect levels are within an order of magnitude, additional data are often necessary to adequately characterize risk. Unlike pesticides, environmental pharmaceutical concentrations that we measured are not acutely toxic to aquatic organisms. Chronic responses of non-target biota, many of which are unidentified, may result from nanomolar or potentially even picomolar exposure (Fong, 1998). Aquatic systems in regions where municipal effluents do not benefit from upstream dilution may present maximal risk to aquatic organisms.

Acknowledgements

This research was supported in part by a US Congressional Environmental Sensors and Signals grant to M. Slattery and C.M. Foran, a Texas Water Resources Institute/United States Geological Survey grant to B.W. Brooks and T.W. La Point, and the Environmental Toxicology Research Program at the University of Mississippi. The authors thank Erica March and Bethany Peterson for technical support.

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