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EFFECTS OF THE ANTIHISTAMINE DIPHENHYDRAMINE ON SELECTED AQUATIC ORGANISMS

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Abstract—In recent years pharmaceuticals have been detected in aquatic systems receiving discharges of municipal and industrial effluents. Although diphenhydramine (DPH) has been reported in water, sediment, and fish tissue, an understanding of its impacts on aquatic organisms is lacking. Diphenhydramine has multiple modes of action (MOA) targeting the histamine H1, acetylcholine (ACh), and 5-HT reuptake transporter receptors, and as such is used in hundreds of pharmaceutical formulations. The primary objective of this study was to develop a baseline aquatic toxicological understanding of DPH using standard acute and subchronic methodologies with common aquatic plant, invertebrate, and fish models. A secondary objective was to test the utility of leveraging mammalian pharmacology information to predict aquatic toxicity thresholds. The plant model, *Lemna gibba*, was not adversely affected at exposures as high as 10 mg/L. In the fish model, *Pimephales promelas*, pH affected acute toxicity thresholds and feeding behavior was more sensitive (no-observed-effect concentration = 2.8 μg/L) than standardized survival or growth endpoints. This response threshold was slightly underpredicted using a novel plasma partitioning approach and a mammalian pharmacological potency model. Interestingly, results from both acute mortality and subchronic reproduction studies indicated that the model aquatic invertebrate, *Daphnia magna*, was more sensitive to DPH than the fish model. These responses suggest that DPH may exert toxicity in *Daphnia* through ACh and histamine MOAs. The *D. magna* reproduction no-observed-effect concentration of 0.8 μg/L is environmentally relevant and suggests that additional studies of more potent antihistamines and antihistamine mixtures are warranted. Environ. Toxicol. Chem. 2011;30:2065–2072. © 2011 SETAC

Keywords—Diphenhydramine Pharmaceutical effects Comparative pharmacology Environmental risk assessment Prioritization

INTRODUCTION

Pharmaceuticals and personal care products (PPCPs) are found in most aquatic systems that receive large amounts of municipal effluent discharges, especially in areas where effluent makes up the majority of water entering the receiving system [1]. Although PPCPs have likely been present in the environment at low concentrations for some time, it is only over the last 20 years that advances in analytical techniques have allowed scientists to detect them [2]. Pharmaceuticals and personal care products are typically present at low levels (<1 μg/L), which historically represent concentrations of minimal concern for most environmental contaminants. However, pharmaceuticals are biologically active molecules developed to have specific effects at low concentrations. Although substantial work has examined potential PPCP exposure, comparatively less work has been done on understanding the adverse effects to aquatic life. Assessing the ecotoxicological impacts of these PPCPs is one of the primary needs identified by several authors [3,4] in addition to the U.S. Environmental Protection Agency (U.S. EPA) white paper on PPCPs (http://www.epa.gov/waterscience/criteria/library/sab-emergingconcerns.pdf). In fact, the scientific literature has few examples of well-characterized ecotoxicological effects of drugs, and of the available information most is limited to acute toxicity data [3,5]. Only a handful of drug classes are fairly well characterized, such as hormones, analgesics, antidepressants, beta blockers, and antibiotics [5]. The problem now becomes identifying which of the hundreds of active pharmaceutical ingredients (APIs) should be the focus of ecotoxicological study.

Beyond the need for a harmonized hazard prioritization approach that incorporates both effects and exposure elements [3,4], the most obvious need for analysis are those drugs that have been identified in field studies. One drug in particular, the antihistamine diphenhydramine (DPH), has been specifically identified in several major environmental compartments (water, sediment, tissue). In streams receiving significant discharges of treated municipal effluent, DPH has been detected in the water at concentrations ranging from 0.01 to 0.10 μg/L [6,7]. In the sediment, DPH concentrations were much higher (20–50 μg/kg) [7], two and three orders of magnitude higher than associated water concentrations. Perhaps most important, DPH has been found in the tissues of fish. Ramirez et al. [8] found DPH in the muscle tissue of fish living downstream of a North Texas municipal effluent outflow at a mean concentration of approximately 1 μg/kg. Furthermore, a U.S. EPA pilot study, conducted by the same group, found DPH in the muscle and liver (1–10 μg/kg) of fish residing near multiple large metropolitan areas in the USA [2]. Another study found 0.03 to 0.08 μg/kg of free DPH, which are those molecules unbound to protein, in fish tissue just downstream of an effluent outflow [9]. Actual DPH muscle concentrations might be as high as 0.2 to 8.0 μg/kg if the percent DPH bound to protein in fish is similar to the 86 and 99% protein binding reported in humans [10,11].

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The quantification of DPH in surface waters may be partially explained because it is fairly stable in the environment [12], although, like many drugs, it is subject to photodegradation [13]. In general, antihistamines, and likely DPH, are removed poorly through most wastewater processes [14]. With 2 to 15% of DPH excreted as unmetabolized by humans, it is likely continually discharged to receiving systems, resulting in potential life-cycle exposures, particularly in effluent-dominated streams [1]. An additional influx of DPH may come from the sewage treatment process where polar metabolites (e.g., diphenhydramine *N*-glucuronide [11]) are cleaved back to the parent compound, although this has not been studied directly [15]. Although studies have seldom examined seasonal differences in environmental exposures, it is possible that DPH usage, and consequently regional environmental loading, increases seasonally to coincide with seasonal allergy responses in human populations. Based on the relatively high log K_{OW} (Log *P*) of 3.27 (Table 1) and the empirical information summarized above, it appears likely that DPH will partition to the sediment and tissue matrices. Although DPH is present in multiple matrices in field samples, little work has been done to characterize its potential ecological effects [16].

As with many pharmaceuticals, it is possible that chronic aquatic risks of DPH exposure are related to the potential for therapeutic mechanism or mode of action (MOA) specific outcomes [3,5], rather than nonspecific narcosis responses typically seen with industrial chemicals [17]. Understanding mammalian pharmacological properties may help predict potential effects in nontarget species based on the conservation of critical drug receptors [18]. Diphenhydramine is a first-generation antihistamine drug found in many common over-the-counter formulations (Table 1) and crosses the blood–brain barrier [15]. In humans it has both antihistamine and sedative MOAs, which are reflected in the over-the-counter formulations that function either to reduce allergic reactions and motion sickness or serve as sleep aids. Table 1 summarizes the general physical, pharmacokinetic, and pharmacodynamics properties of DPH. Mechanistically, DPH targets a number of different receptors, although its primary target is the H1 histamine receptor [19]. Histamine, released from mast cells (a component of mammalian innate immune system) in response to an allergic trigger, targets the H1 receptors in the smooth muscles in the vasculature causing them to then dilate. This reaction allows blood and other immune cells to move into the affected area, causing the swelling and redness associated with an allergic reaction. This same mechanism is responsible for small localized reaction and larger systemic responses (e.g., anaphylactic shock). Diphenhydramine competitively binds the H1 receptors and reduces the allergic response by preventing histamine binding and allowing smooth muscle contraction. Diphenhydramine also targets the 5-HT reuptake transporter (SERT), preventing the reuptake of serotonin at the presynaptic nerve cleft [20]. In general, this MOA adds to the sedation response associated with DPH. Interestingly, discovery of this MOA led directly to the development of fluoxetine, the first selective serotonin reuptake inhibitor (SSRI) antidepressant, which exerts its therapeutic effect through the same mechanism, albeit with much greater specificity [20]. Furthermore, DPH acts as an anticholinergic agent by competitively antagonizing the acetylcholine receptor [19]. This reaction reduces the signal sent by the acetylcholine neurotransmitter, and as such has been suggested as a remedy for organophosphate poisoning [21] and in alleviating the symptoms of Parkinson's disease [19].

Table 1. Information on the antihistamine diphenhydramine (DPH), including physical, pharmacokinetics, and pharmacodynamics properties

Diphenhydramine

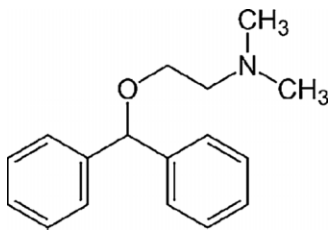
Common brands: Benadryl® McNeil-PPC, Unisom® Chattem, Sominex ® GlaxoSmithKline
Drugs commonly in mixture with DPH: Ibuprofen, acetaminophen, dextromethorphan, pseudoephedrine, benzocaine, ammonium chloride, codeine
Usage categories: Hypnotics and sedatives, antiemetics, antiparkinson agents, antidyskinetics, antipruritics, anti-allergic agents, histamine H1 antagonists, anesthetics – local, antitussives, anticholinergic

Physical properties	
CAS DPH-HCl	147-24-0
DPH	58-73-1
Formula	$C_{17}H_{21}N\,O$
Molecular weight	255.36 g/mol
IUPAC name	[2-(diphenylmethoxy) ethyl] dimethylamine
Solubility	3.06 mg/ml
Log *P*	3.27
Log *D* – pH 6.5 [31]	0.78
Log *D* – pH 8.5 [31]	2.66
pK_a	8.9

Pharmacodynamics	
Mammalian acute toxicity (Rat oral median lethal dose [LD50])	390 mg/kg
Human therapeutic dose – peak plasma concentration (C_{max})	0.05 μg/ml
Mammalian ATR[a] [5]	7,800
ATR predicted ACR in fish [5]	2,091

Pharmacokinetics	
Common adult dosage	25-50 mg – 400 mg/day
Bioavailability	43 – 72%
Protein binding	86 – 99%
Peak plasma concentration (T_{max})	<1.5 – 4 h
Plasma half life	3 – 9 h
Metabolism	Extensive hepatic metabolism; CYP2D6
Excretion	2 -15% parent compound unchanged
Volume of distribution	3.3 – 14.6 L/kg

DPH structure



[a] ACR = acute to chronic ratio; ATR = acute to therapeutic ratio.

Unfortunately, the consequences of DPH exposure are poorly understood in nontarget organisms. This data gap is especially disconcerting for aquatic species, as many may be exposed to DPH by way of multiple routes. Thus, the objective of this study was to develop a baseline aquatic ecotoxicological understanding of diphenhydramine by using a number of standardized toxicity test protocols with several species. In addition, we also explored the utility of leveraging mammalian pharmacological information to understand thresholds of adverse aquatic responses [3–5,22].

MATERIALS AND METHODS

Experimental conditions

The following experimental conditions described apply to all studies except where noted within individual methods. Reconstituted hard water (RHW), formulated according to U.S. EPA methods [23], was used as control and dilution water for invertebrate and fish studies. All experiments were performed in controlled environmental chambers at $25 \pm 1^{\circ}C$ under a 16:8 h light:dark regime. Water quality was monitored according to standard methods [24]. Water quality parameters were measured daily and mean (±standard deviation [SD]) values were well within acceptability criteria [23,25,26]: dissolved oxygen, 8.3 (±0.2) mg/L (YSI Model 55); conductivity, 580 (±4.6) μS /cm (YSI Model 30); alkalinity, 116 (±4) mg/L as $CaCO_3$; and hardness, 172.5 (±3.4) mg/L as $CaCO_3$.

The pH of each study solution was measured (Thermo Orion 720A pH/ISE meter) and recorded separately for each test conducted. A potential for shifts exists in the ionization state of DPH (pK_a 8.9; Table 1) resulting from slight differences in pH, which could influence toxicological responses [27]. All tests were generally conducted at higher pH (8.4–8.7) to approximate worst-case scenarios and realistic pH values for many effluent dominated streams in semiarid regions [1].

Diphenhydramine hydrochloride (CAS 147-24-0) was obtained from Sigma-Aldrich. Concentrations used in preliminary range finding testing were developed from U.S. EPA EPI Suite software [28] (96-h *P. promelas* median lethal concentration [LC50] = 13.7 mg/L; 48-h Daphnid LC50 = 1.2 mg/L), then adjusted based on preliminary results (not reported). All DPH concentrations were analytically verified following methods described below.

Pimephales promelas

Standardized acute studies. Standardized fathead minnow (*Pimephales promelas*) acute studies were conducted according to U.S. EPA acute toxicity protocols [23] with slight modifications [27,29]. Tests were run three times each at two different nominal pH levels, 6.5 and 8.5. To ensure test concentrations were the same across both pH treatments, a large volume (8 L) of each test solution at higher pH (8.5) was prepared, then subdivided into two 4-L aliquots, of which one was adjusted to the target pH 6.5 using 1.5 to 2.1 ml of 1N HCl. The higher pH study utilized five concentrations, while the lower pH required three additional (eight total) higher concentrations to establish the LC50. At each treatment level and control, four replicates of 600-ml glass beakers were loaded with 10 larval *P. promelas* (<24 h old). Prior to initiating the study, fish were fed brine shrimp nauplii but were not fed during the test. To reduce the likelihood of pH drift each replicate was covered tightly with parafilm for the entire 48-h test period. Survival was assessed at 24 and 48 h. Samples for analytical verification were taken at each concentration for each of the three replicate studies prior to pH adjustment (pH 8.5).

Standardized chronic study. A 7-d subchronic study was conducted following slightly modified U.S. EPA protocols [26,27,29]. Four replicates of eight concentrations and a control were prepared. Treatment levels for the fish subchronic study were selected based on acute response thresholds, a prediction of acute to chronic ratio (ACR) response using slope and intercept (0.254 and 0.788, respectively) of the regression between a mammalian margin of safety parameter (the acute to therapeutic ratio [ATR]; Table 1) and known ACR values (Eqn. 1) [5]

$$\mathrm{ACR} = (10^{\mathrm{intercept}}) \cdot (\mathrm{ATR}^{\mathrm{slope}}) \quad (1)$$

and predictions of plasma concentrations in fish [22,30]. Specifically, Fitzsimmons et al. [30] provided an empirical relationship for nonionic chemical bioaccumulation and partitioning to fish plasma (blood:water partition coefficients; P_{BW}), which was previously recommended for pharmaceutical prioritization [22]. Here we modified another Fitzsimmons et al. [30] equation (Eqn. 2), which is more appropriate for drugs with apparent log P values less than 3 [16], and substituted log D [31] at the study pH (8.5) for log P (Eqn. 3).

$$P_{\mathrm{BW}} = (10^{0.73 \log P} \cdot 0.16) + 0.84 \quad (2)$$

$$P_{\mathrm{BW}} = (10^{0.73 \log D}\,(\mathrm{pH}\,8.5 \cdot 0.16) + 0.84 \quad (3)$$

We then conceptually applied the plasma model approach recommended by Huggett et al. [22], where the fish plasma concentration (FPC) is determined by multiplying the aqueous concentration (Aq) of a drug by its P_{BW} (Eqn. 4). The model considers an effect likely to occur any time the FPC is greater than the human plasma therapeutic dose (C_{max}) and the point at which C_{max} = FPC is considered an effect threshold (ET). Because C_{max} and P_{BW} are constants, it is then possible to solve for the aqueous concentration at the effect threshold (AqET) (Eqn. 5) [32], and to derive Equation 6, which predicts the concentration of DPH in water necessary to result in plasma accumulation equal to a human C_{max} value:

$$\mathrm{FPC} = P_{\mathrm{BW}} \cdot \mathrm{Aq} \quad (4)$$

$$C_{\max} = \mathrm{FPC} = (P_{\mathrm{BW}} \cdot \mathrm{AqET}) \text{ and } C_{\max}/(P_{\mathrm{BW}} \cdot \mathrm{AqET}) = 1 \quad (5)$$

$$\mathrm{AqET} = C_{\max}/(P_{\mathrm{BW}}) \quad (6)$$

Consistent with the acute studies, experimental units were 600-ml beakers filled with 500 ml of test solution and loaded with 10 <24-h-old *P. promelas*. This was a static renewal experiment with feeding of brine shrimp nauplii twice daily. The test solution was renewed daily 2 h after the morning feeding with 80 to 85% renewal [25]. Stock solutions for each exposure concentration were made fresh daily and analytically verified. Tests were monitored daily for survival. At the completion of the 7-d study, three fish from each replicate were randomly selected for a feeding trial (see *Discussion*). The remaining seven fish were euthanized according to standard methods [25] and placed in aluminum weigh pans. Weigh pans with fish were then placed into an 80°C drying oven for 48 h. Pans and fish were allowed to come to room temperature in a desiccation chamber for 1 h. Fish were then weighed on a Mettler Toledo Model MX5 microbalance.

Feeding behavior. Three randomly selected fish from each replicate were placed in 100-ml glass beakers filled with fresh exposure media of the appropriate concentration and held for 24 h without food. Experiments were conducted according to the approach outlined in Stanley et al. [29] with the modifications suggested by Valenti et al. [27]. The trial started by adding 40 brine shrimp nauplii to the beaker containing a single fish. Fish were given 15 min to feed, after which time the fish was removed and the remaining nauplii counted.

Daphnia magna

Acute study. A 48-h static acute study for *D. magna* was conducted according to established U.S. EPA protocols [23]. It was conducted at a single pH, 8.59 (±0.05). Four replicates were used for each of five concentrations and a control. Each replicate was loaded with five *D. magna*. All *D. magna* used were <24 h old and hatched within a single 4-h window. This acute test design was performed three times. Water samples for analytical verification were taken from each concentration prior to the initiation of testing.

Subchronic study. A 10-d *D. magna* subchronic toxicity test was performed following standard protocols [33] with slight modifications [34,35]. The endpoints assessed were immobilization (mortality) and reproduction (young per female). *Daphnia magna* used to initiate the study were <24 h old and hatched within a 4-h period. Eight concentrations and a control were used in this study, with 10 replicates per treatment level. The experiment was static renewal with daily renewal. To ensure consistency in renewal concentrations a 4-L stock solution of each concentration was made at test initiation. Stock solutions were analytically verified three times: day 0, day 5, and day 8. Experimental units were 30-ml disposable plastic cups with a test volume of 30 ml. Each replicate was fed 0.6 ml per day of a mixture of *Pseudokirchneriella subcapitata* and cereal grass media [23,36]. Neonates were counted and removed daily during renewals.

Lemna gibba

Diphenhydramine toxicity to a model aquatic plant was assessed by exposing *L. gibba* (a duckweed) to five concentrations (10, 5, 2.5, 1.25, 0.63 mg/L DPH, nominal) and a control and measuring effects on frond number, wet weight, and growth rate after 7 d. *Lemna gibba* G-3 culture was obtained from the Canadian Phycological Culture Center and maintained in Hunter's media, as described by Brain and Solomon [37]. Prior to experimentation, plants were acclimatized to test media (Hunter's media) for one week before the study was initiated. The 7-d static renewal experiments were conducted according to the standardized protocol outlined in Brain and Solomon [37]. After the acclimatization period, two *Lemna* plants, each with four fronds, were transferred from the acclimatized mass culture into a 250-ml Erlenmeyer flask containing 100 ml sterilized test solution. Test solutions were created through serial dilutions. Flasks were arranged in a randomized complete block design and maintained in a growth chamber (25°C) under constant cool white fluorescent light (6800 lux). Frond number and fresh weight were measured on day 7. The number of doubling events (n) (Eqn. 7)

$$n = \log(F_t/F_0)/\log(2) \quad (7)$$

where F_t is the number of fronds at time, t; F_0 is the number of fronds at time zero, is divided by the total exposure time (t) to calculate growth rate [37].

Analytical methodology

Exposure concentrations of DPH were verified in each stock solution and all experiments by way of liquid chromatography-tandem mass spectrometry. Instrumentation consisted of a Varian model 410 autosampler, ProStar model 212 binary pumping system, and model 1200L triple quadrupole mass analyzer. Fifty μl of a 10-ppm solution of the isotopically labeled internal standard (DPH-d3) was added to all samples and calibration standards. To ensure that analyte concentrations fell within the calibrated range of the instrument, sample aliquots were diluted with 95:5 0.1% (v/v) aqueous formic acid-methanol prior to analysis.

Analyses were carried out using a 15 cm × 2.1 mm (5 μm, 80 Å) Extend-C18 analytical column (Agilent Technologies) and 12.5 × 2.1 mm (5 μm, 80 Å) guard cartridge connected in series. A binary gradient consisting of 0.1% (v/v) formic acid in water and 100% methanol was employed to promote elution of target analytes within 6 min. Additional chromatographic parameters were as follows: injection volume, 10 μl; column temperature, 30°C; flow rate, 350 μl/min. Analytes were ionized using positive electrospray ionization and monitored using the following optimized MS/MS transitions: m/z 256 > 167 and 259 > 167 for DPH and DPH-d3, respectively. Internal standard calibration curves were constructed using linear or quadratic regression, as appropriate ($R^2 \geq 0.998$) used to determine DPH concentrations in all analyzed samples. During analysis, one continuing calibration verification sample was analyzed every 6th injection with an acceptability criterion of ±20%.

Statistical analysis

An $\alpha = 0.05$ was used in evaluating response variables for all experiments. The LC50 values were calculated using U.S. EPA Toxstat. The probit method was used if data met assumptions; otherwise, the trimmed Spearman–Karber method was applied [23]. The LC50 values were calculated based on analytically verified concentrations for individual test. No-observable-effect concentration (NOECs) and lowest-observable-effect concentrations (LOECs) were calculated using analysis of variance with Dunnett's post-hoc test, as suggested by U.S. EPA protocols [25,33].

RESULTS

Analytical confirmation of DPH concentrations

Table 2 provides analytical verified concentrations of DPH for each treatment level of the acute and subchronic experiments with the various model organisms. For acute studies (Table 2) concentration reported are mean ($n = 3$; ±SD) values from triplicate studies.

Pimephales promelas

Control survival was >95% for all *P. promelas* tests (acute and chronic). Mean (±SD) pH treatment levels for the acute studies were 6.45 (±0.03) and 8.52 (±0.02). Acute studies showed clear dose-dependent responses to DPH exposure, although mortality occurred at a much higher concentrations in acute studies at lower pH (6.5; Table 3). The mean LC50 for *P. promelas* acute toxicity studies was 2.09 (±0.41) mg/L at pH 8.5 and 59.28 (±6.64) mg/L at pH 6.5. The responses for *P. promelas* growth and feeding trials were similarly dose-dependent (Fig. 1). Subchronic exposure survival was 100% except at the highest concentration tested in this study. The LOEC for growth and behavioral (feeding) responses were

Table 2. Analytically verified mean (± standard deviation) diphenhydramine concentrations for acute and subchronic studies (μg/L)

D. magna		*P. promelas*	
Acute[a]	Subchronic[b]	Acute[a]	Subchronic[b]
38 (±10)	0.10 (±0.01)	570 (±11)	0.09 (±0.02)
63 (±13)	0.46 (±0.07)	1162 (±117)	0.63 (±0.14)
170 (±28)	0.83 (±0.21)	2136 (±2)	2.82 (±0.32)
368 (±30)	3.44 (±0.96)	4930 (±60)	5.62 (±1.10)
1087 (±66)	6.93 (±0.42)	9330 (±1430)	24.49 (±2.01)
1606 (±89)	27.80 (±0.61)	19115 (±940)	49.08 (±5.90)
	46.08 (±1.53)	33370 (±3012)	388.26 (±63.1)
	273.40 (±4.65)	72190 (±2340)	836.70 (±103)

[a] Acute studies samples were taken from each replicate ($n = 3$).
[b] Subchronic studies multiple samples were taken for *Daphnia magna* ($n = 3$) and *Pimephales promelas* ($n = 7$).

measured at much lower concentrations: 49.1 and 5.6 μg/L for growth and behavioral endpoints, respectively (Table 3). Acute to chronic ratios for growth and behavior endpoints were calculated at 85 and 746, respectively (Table 3).

Daphnia magna

Control survival was >95% for both acute and chronic experiments. Acute tests showed dose-dependent responses with a mean ($n = 3$) LC50 of 0.37 (±0.14) mg/L. The 10-d studies also exhibited a dose-dependent pattern. Survival in the control and lower concentrations was 100% through the 10-d exposure, while 100% mortality occurred at concentrations 27.8, 46.1, and 273.4 μg/L by days 7, 5, and 4, respectively. Reproduction LOEC and NOEC values were determined at 3.4 and 0.8 μg/l, respectively (Fig. 2, Table 3). The corresponding ACR value for *D. magna* was 467.5 (Table 3).

Lemna gibba

No statistically significant ($p > 0.05$) effects of DPH on *L. gibba* responses were observed (Table 3). For example, mean (±SD) growth rate for all plants was 0.358 (±0.014), compared to a mean growth rate in the highest concentration of 0.357 (±0.014) and 0.345 (±0.015) in control. No significant differences were observed among any of the various parameters measured (e.g., frond number, wet wt, growth rate). Because no treatment level adversely affected this plant model, only the highest concentration was confirmed analytically at 10.75 mg/L (±0.13).

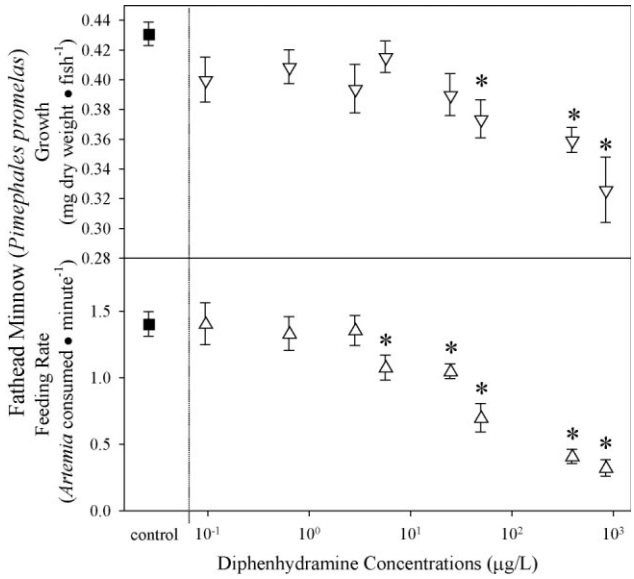


Fig. 1. Mean (±standard error) growth (mg dry wt per fish; $n = 7$ per replicate) and behavioral responses (*Artemia* consumed per min; $n = 3$ per replicate) of larval fathead minnows (*Pimephales promelas*) following 7-d diphenhydramine study. *Significantly different from control ($p \leq 0.05$).

DISCUSSION

The primary objective of this study was to establish a baseline understanding of aquatic toxicological effects of a drug commonly reported in various environmental compartments (tissue, sediment, water) [7,8]. Here we observed that an aquatic plant model was insensitive to DPH, even at very high exposure levels (>10 mg/L). Such an observation is consistent with previous reports for several other classes of pharmaceuticals (e.g., nonsteroidal antiinflammatory drugs, SSRIs, lipid lowering agents, beta-blockers) [38], likely because the histamine-H1, SERT, and muscarinic ACh receptors targeted by DPH were not present in either plant or algae models analyzed for homologs [18]. However, significant acute and subchronic effects of DPH were observed to a model fish and an invertebrate (Table 3).

A second objective of this study was to employ approaches previously proposed [3,5,22] to leverage mammalian pharmacological information to understand aquatic hazards of pharmaceuticals. Fish are known to possess some degree of genetic homology for the three critical DPH targets (histamine-H1, SERT, muscarinic ACh receptor), although the percent sim-

Table 3. Toxicological thresholds of mean acute ($n = 3$; ± standard deviation) and subchronic endpoints of select organisms exposed to diphenhydramine and associated acute to chronic ratios (ACR)

		Subchronic endpoints			
Species	Mean 48 h LC50 (mg/L)	Type	LOEC (μg/L)	NOEC (μg/L)	ACR
Pimephales promelas	pH 6.5: 59.28 (±6.6)	Survival	836.7	388.3	5.4
	pH 8.5: 2.09 (±0.405)	Growth	49.1	24.5	85.3
		Behavior (feeding rate)	5.6	2.8	746.4
Daphnia magna	0.374 (±0.142)	Survival	46.1	27.8	13.5
		Reproduction	3.4	0.8	467.5
Lemna gibba		Growth (frond#)	—	>10,750	—
		Growth (wet weight)	—	>10,750	—
		Growth (growth rate)	—	>10,750	—

LC50 = median lethal concentration; LOEC = lowest observed effect concentration; NOEC = no observed effect concentration.

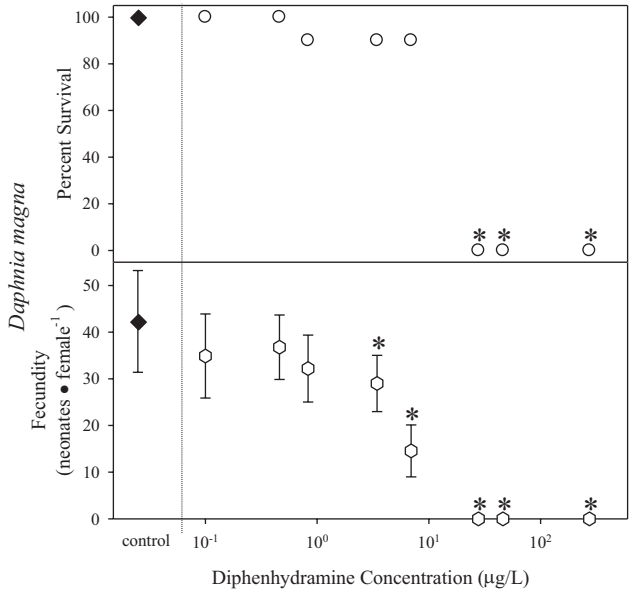


Fig. 2. Percent survival and mean (±standard deviation) *Daphnia magna* fecundity (neonate per female) following 10-d diphenhydramine study ($n = 10$). *Significantly different from control ($p \leq 0.05$).

ilarity is reported to vary between 40 to 70% [18]. When observations of the present study are compared to similar studies with the SSRIs fluoxetine [29] and sertraline [27], DPH potency was very similar to these SSRIs, exerting subchronic toxicity on growth and feeding behavior with comparable NOEC values (≈10 μg/L). However, DPH was found to be much less effective in producing mortality in the 48-h and 7-d studies (Table 3) than comparable mortality thresholds for sertraline [27] and fluoxetine [29]. Similar to observations previously reported for sertraline [27] and fluoxetine [39], this study demonstrated that pH is a critically important factor influencing aquatic toxicity of ionizable weak bases, because a 28-fold higher DPH LC50 value was observed for *P. promelas* at pH 6.5 than pH 8.5 (Table 3).

In the present study the standardized growth endpoint in the *P. promelas* model was not the most sensitive fish response to DPH (Fig. 1, Table 3); rather, a behavioral response was more sensitive than the standardized growth endpoint. For example, the 5.6 and 24.5 μg/L DPH treatment levels significantly suppressed feeding behavior but not growth (Fig. 1, Table 3). Feeding behavior was examined here and in previous studies with the SSRIs sertraline [27] and fluoxetine [29] because it represents an alternative sublethal endpoint that may be plausibly related to the drug MOA (e.g., targeting the SERT). For example, previous work by Gould et al. [40] demonstrated that SSRIs target the SERT in fish with similar binding kinetics as observed in mammals. Such MOA-related responses are recognized as critical for pharmaceutical effects on aquatic organisms because therapeutic-related responses are often observed at much lower levels than traditional standardized survival and growth endpoints in fish [3–5].

Although similarities were found between DPH and sertraline and fluoxetine potencies to the *P. promelas* model in the present study, DPH toxicity to cladocerans differed drastically from previous studies of SSRIs. The responses of *D. magna* to DPH exposure were two to three orders of magnitude lower than SSRI thresholds [29,41–43]. The only other study available on the aquatic toxicology of DPH found similar results in *D. magna* [44]. Meinertz et al. [44] recently evaluated effects of DPH on *D. magna* over 21 d, but only at three widely separated concentrations, resulting in an NOEC of 0.12 μg/L and LOEC of 70 μg/L. Subsequently, Meinertz et al. [44] were unable to report differences between concentrations affecting survival and reproduction, as all *D. magna* above reported NOEC died and did not reproduce. In the present study, a reproduction NOEC value of 0.8 μg DPH /L for *D. magna* is in general agreement with this previous research, although we detected reproductive effects at an order of magnitude lower concentration than a survival NOEC of 27.8 μg/L (Table 3). One interesting observation in the Meinertz et al. [44] study was that even at the highest concentration tested (620 μg/L, reported as diphenhydramine hydrochloride) *D. magna* generally survived for about 10 d, whereas in the present study *Daphnia* were only able to survive for up to 7 d at the lowest lethal concentration (28 μg/L). It is possible the observed differences in time to death resulted from the ionization of DPH, as we demonstrated here with *P. promelas* (Table 3) and was observed previously for sertraline [27]. Meinertz et al. [44] reported a pH range between 7.2 and 7.6, whereas pH was 8.63 (±0.05) in the present study. With a pK_a of 8.98 DPH and other weak bases would be expected to shift ionization states within environmental relevant pH ranges [27]. In this study, at a pH closer to the pK_a value, DPH was more un-ionized and more toxic to *D. magna* than in the Meinertz et al. study. Thus, based on the information from the present study and others [27,39], it appears important to consider pK_a during the environmental assessment of ionizable pharmaceuticals in the environment.

The differences in *D. magna* response thresholds for DPH (Table 3) compared to SSRIs are likely related to other MOAs of DPH and conservation of relevant targets in invertebrates. Although SSRIs were derived based on the SERT activity of DPH, SSRIs have been designed to more specifically target the SERT, while DPH also has histamine and cholinergic targets. Invertebrate physiology and neurochemistry is highly reliant on both histamine and acetylcholine as neurotransmitters. For example, organophosphate (OP) pesticides are much more effective in invertebrates. Whereas OPs target acetylcholineesterase, DPH and other antiacetylcholinergics (e.g., atropine) bind to the ACh receptor, preventing ACh neurotransmission [45]. This binding is generally reversible, and over the short term less toxic, but given continuous exposure and the likelihood for bioaccumulation, particularly in effluent-dominated streams [1], the probability of deleterious effects can increase. Thus, DPH may have exerted its toxicity to *D. magna* in the present study through an ACh MOA, which resulted in greater toxicity than previously reported for SSRIs. It may have also been that an antihistamine MOA played a role in the observed toxicity to cladocerans, because DPH also targets histamine ion channel transporters in invertebrates [46]. It is important to note that DPH is not even the most potent antihistamine. For example, Berninger and Brooks [5] recently ranked desloratadine and loratadine much higher than DPH. Both of these drugs are also known to be much more potent at histamine H1 and ACh receptors [47]. Clearly these findings deserve additional study.

When we selected treatment levels for the subchronic fish study, an ACR value of 2,100 was predicted for DPH, based on mammalian margin of safety information presented in Equation 1 [5]. Based on results from the *P. promelas* feeding behavior study an ACR value of 746 was calculated (Table 3); an order of magnitude higher than previously reported feeding

behavior ACR values for sertraline (ACR = ≈15) [27] and fluoxetine (ACR = 22) [29]. Although the observed ACR value was lower than predicted by Equation 1, a DPH ACR value of 746 is an order of magnitude higher than ACR values for 90% of all industrial chemicals [48]. Such an observation highlights the importance to pharmaceutical risk assessment of understanding a priori pharmacological potency and if pharmacological targets are present and maintain physiologically important functions in nontarget organisms [3–5,19,22]. Furthermore, we also employed a plasma model approach modified from that presented by Huggett et al. [22] and advanced by Fick et al. [32]. We employed a partitioning equation (Eqn. 3) more appropriate for chemicals with apparent log *P* values less than 3. Additionally, due to the appreciable effects of lowering pH on acute toxicity to fish (Table 3) log *D* was substituted at the study pH (8.5) for log *P* using Equation 3. Then, using Equation 6, at an aqueous exposure concentration it was predicted that an AqET of 2.53 μg/L would be required to potentially result in a fish plasma concentration equaling the human therapeutic dose for DPH (C_{max} = 50 ng/ml). As noted above, NOEC values for fish growth (24.5 μg/L) were not as sensitive as behavioral responses (2.8 μg/L).

Although plasma measurement of DPH was not possible due to the size of *P. promelas* employed, this plasma model approach, when the effects of log *D* were considered, appears useful for predicting thresholds related to the therapeutic MOA of DPH because the NOEC value of 2.8 μg/L approximated the predicted threshold of 2.53 μg/L. If log *D* was not considered in Equation 3, and instead Equation 2 was used, a slightly lower potential threshold value of 1.25 μg/L was predicted. Thus, the observations in the present study generally support use of a plasma model approach for fish in further definitive studies, particularly when sublethal responses are plausibly linked to therapeutic MOAs and plasma concentrations can be measured.

CONCLUSIONS

Observations in the present study highlight the importance of carefully selecting study organisms and endpoints for pharmaceuticals that possess multiple MOAs. Because standardized toxicity testing methodologies may not account for specific aquatic MOAs of pharmaceuticals, environmental risks may be underestimated by current testing approaches [3–5]. Here we demonstrated that an alternative behavioral endpoint was more sensitive in the *P. promelas* model than survival or growth responses, which is consistent with previous studies of the SSRIs fluoxetine [29] and sertraline [27], which possess a common MOA as DPH (e.g., the SERT). Such alternative endpoints that may be related to a specific therapeutic MOA (e.g., the SERT) and are relevant to organismal and population level consequences are necessary to appropriately characterize environmental risks [3,4]. It is also important to note that responses might be related to another DPH MOA, ACh activity, which appeared to be appropriately characterized by the *D. magna* model. Thus, employing a priori knowledge of comparative pharmacology among target and nontarget organisms remains critical during environmental hazard and risk assessments of pharmaceuticals in the environment [3–5].

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