ABSTRACT

Leveraging Available Data for Contaminants of Emerging Concern
to Develop an Understanding of Environmental Hazard

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Contaminants of emerging concern (CEC) are classes of compounds with relatively limited information available on environmental exposure, fate, and effects. The purpose of this research was to develop and test approaches that leverage available data using probabilistic models to advance an understanding of aquatic hazards of CECs. Pharmaceuticals are one such group of CECs. Though extrapolation approaches with fish models can provide important bridges between the biomedical and environmental sciences, little data is available addressing the sublethal effects of therapeutics in aquatic organisms. Seldom is the drug's Mode of Action (MOA) considered in selection of chronic endpoints for an assessment, though mammalian pharmacological information is available for most drugs. A statistically significant relationship (r^2=0.846, $p<0.001$) between mammalian pharmacology and toxicology data (margin of safety) and available fish acute to chronic data was identified, when therapeutic MOA was considered in selecting a chronic response variable. Based on this relationship, metrics to assess potency and internal effective dose were developed. These metrics were then evaluated

using probabilistic distributions in an effort to prioritize drugs based on potential hazard. These probabilistic assessments identified specific drugs and drug classes as potentially presenting greater hazard to fish. To test these models, toxicity experiments with diphenhydramine, an antihistamine drug, were conducted to characterize standardized endpoints and novel, MOA-related ecotoxicological endpoints. The results confirmed that sublethal endpoints (e.g., behavior) related to therapeutic may be more appropriate for fish and that leveraging mammalian pharmacology and toxicology data may be predictive for MOA related responses when evolutionary conservation of targets are considered. It further highlighted the importance of carefully selecting model organisms for study of pharmaceuticals with multiple MOAs, because reproduction of the invertebrate *Daphnia magna* was sensitive to diphenydramine, potentially resulting from its histaminergic and cholinergic activities. A similar probabilistic approach was applied to oil dispersants, another CEC class, to assess potential impacts to aquatic systems. Leveraging the limited acute toxicity data available for an invertebrate and a fish model, probabilistic distributions were employed to predict the likelihood of oil dispersants exerting acute toxicity in the presence or absence of oil. This approach can be utilized in prospective and retrospective assessments to support emergency response decisions to oil spills and prioritize substances for further study. Lastly, probabilistic methods were used to develop uncertainty factors for acute to chronic rations for select biological active chemicals. For many chemical classes chronic effects data is lacking. Typically, default uncertainty factors are utilized to bridge this data gap. By leveraging the available chronic data using probabilistic methods, novel data-driven uncertainty factors were developed, potentially providing more protective extrapolation models.

Leveraging Available Data for Contaminants of Emerging Concern to Develop an Understanding of Environmental Hazard Potential

by

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A Dissertation

Approved by the Institute of Biomedical Studies

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Robert R. Kane, Ph.D., Chairperson

Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of
Doctor of Philosophy

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August 2011

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ACKNOWLEDGMENTS

This research was supported by a Glasscock Fund for Excellence in Environmental Science grant to J.P. Berninger and the Baylor University Department of Environmental Science. The Center for Reservoir and Aquatic Systems Research and Institute of Biomedical Studies at Baylor University provided general support. Travel to conferences was supported by the Baylor University Graduate School, the Society of Environmental Toxicology and Chemistry (SETAC) travel award, Baylor University Department of Environmental Science, and South-Central regional SETAC chapter. Additional travel funds were provided through travel awards from the Society of Toxicology, PRIMO 16 Conference, the American Chemical Society, and SETAC/Health Canada

I thank my committee: Dr. Bryan Brooks, Dr. Erica Bruce, Dr. Kevin Chambliss, Dr. Ryan King, Dr. Sascha Usenko; and peers at Baylor University: Bowen Du, Kristin Connors, Stephanie Etycheson, Chris Gaskill, Mark Kolkmeier, Krista Prosser, ,Dr. Ted Valenti, and Dr. Spencer Williams. I would also like to thank Dr. Cindy Howard, Mark Smith, and Dr. Greg Kelly for the guidance and mentoring that brought me to this point as a scientist. I would also like to thank my family for the unconditional love and support throughout my life and these last few years. Barb and Roger, for being great parents and for encouraging me in science, and in life, to go beyond my limitations. Thomas and Gloria Banks and Louise Berninger for being everything grandparents should be. For my siblings, Mark, Brian, and Bethany putting up with it all.

DEDICATION

To my family for loving support

To my dog, Rio, for watching me write this dissertation

CHAPTER ONE

Introduction

General Overview

It is the role of an environmental risk / hazardassessor to reduce the sources of uncertainty in support of the decision making process (Suter 2007). A sound risk or hazard assessment acknowledges that uncertainty exists, identifies its sources, and takes appropriate steps to reduce it. Reduce is a key word in this statement, as it is not possible to remove all uncertainty. Reduction of uncertainty comes through defining its sources, sound scientific practice, and experimentation with appropriate quality management. It is the overarching theme of this dissertation to explore ways to reduce uncertainty in environmental assessments, through sound science.

There are a number of inherent challenges that underlie the uncertainty in environmental risk and hazard assessments. The first and foremost challenge is how to deal with data limitations. To assess the potential impact of a chemical in the environment, a number of different parameters need to be critically assessed, including the physical-chemical characteristics and toxicity to aquatic life. Yet reviews conducted before for REACH (Registration, Evaluation, and Authorization of Chemicals; a European Union effort requiring baseline toxicological data for chemical) implementation suggested that for a large number of chemicals, the required data is just not available (Williams et al. 2009; Williams et al. 2011). A review by Judson et al. (2009) evaluated over 9000 different chemicals and found about 1/3 had no available

toxicity data and the remaining 2/3 often only had limited data available. The type of data also presents a challenge. Having short-term acute laboratory studies are better than no data at all, but this type of data generally does not necessarily transfer to understanding the risks posed by a chemical in the environment. Thus, scaling and extrapolation, between short and long term studies, between lab and field, between different species, creates layers of uncertainty. This is not to say that such activities are inappropriate, but rather it is important to acknowledge and understand how data extrapolations ultimately influence the outcome of a risk or hazard assessment (Calabrese and Baldwin 1993).

Reducing uncertainties caused by inherent data gaps can be accomplished in a number of ways. Defensible empirical data, derived from experimentation, is without a doubt the most appropriate way to fill data gaps, but it is not always the most practical or effective method, particularly when gaps are large and screening level decisions are required. Often it is possible to bridge data gaps by leveraging available data. This data leveraging can take a number of different forms.

Leveraging available data can take the form of interspecies extrapolations. It is common place across all fields of toxicology for a lab species (e.g., rodent) to serve as a surrogate for a species of interest (e.g., human). For example, "read-across" approaches with fish models can provide important bridges between the biomedical and environmental sciences. In aquatic toxicology, it is common to use the responses of model species (e.g., fathead minnow, *Pimephales promelas,* or the cladoceran *Daphnia magna*) to represent components of an aquatic community in assessing effects from specific contaminants or effluents (US EPA 2002a). These types of interspecies

extrapolations are typically limited within specific taxa (fathead minnow – other fish species; *D. magna* – aquatic invertebrate community). As more and more interspecies data becomes available programs like the U.S. Environmental Protection Agency's (US EPA) WEB-ICE (Web-based Interspecies Correlation Estimation; Raimondo et al. 2010) can provide data-derived interspecies extrapolations, at least for acute toxicity threshold values. The advancements in computational toxicology also serve to reduce uncertainties, further supporting interspecies extrapolations (Kavlock et al. 2008).

Data leveraging can also take the form of acute to chronic extrapolations, where acute toxicity data is generally utilized with a default application factor to estimate a potential threshold of chronic toxicity. This is considered the most commonly employed data extrapolation (Calabrese and Baldwin 1993), which has a long history in environmental risk and hazard assessment, going back to some of the pioneering pollution effects assessments of the 1950s (Chapman et al. 1998). This type of extrapolation is so well accepted within risk and hazard assessment, it is embedded within many federal regulations (US EPA 1995). Although these extrapolations for aquatic organisms often take the simple form of default order of magnitude uncertainty factors (e.g., 10, 100, 1000), largely borrowed directly from mammalian toxicology and risk assessment, extensive work has been done over the years to develop more specific data-derived factors that general reduces the uncertainty within these extrapolations (Raimondo et al. 2007).

Data may also be leveraged within chemical or mode of action (MOA) classes. While less historically established than interspecies or acute to chronic extrapolations, there are several different ways to estimate the potential toxicity of a chemical when only

limited or no actual toxicity data is available. Data can be derived from specific classes of chemicals or specific MOA groups to predict the toxic potential of other member of that group. This is often employed when the acute to chronic extrapolation of other chemicals of a group can be utilized to estimate chronic response of a similar chemical within the group where chronic data is not available (Raimondo et al. 2007). When no toxicity data is availablefor a chemical, often quantitative structure activity relationship (QSAR) models are used. QSAR models make predictions about toxicity based on the specific chemical structures (Esher and Hermans 2002; Russom et al. 2003). Another approach to leveraging toxicity data across chemical groups is the use of chemical toxicity distributions (CTD). CTDs use probabilistic methods to examine responses among available data to provide potentially predictive estimates for unknown chemicals within the class or possessing a common MOA (Solomon and Takacs 2002). These CTDs have been used evaluate antibiotic effects on aquatic plants (Brain et al. 2006), a class of antimicrobial compounds (Dobbins et al. 2009), estrogenicity (Dobbins et al. 2008), and as a method for data leveraging in a REACH context (Williams et al 2011).

Data leveraging is critically important when assessing contaminants of emerging concern (CECs). Contaminants of emerging concern are classes of chemicals with relatively limited information on environmental exposure, fate, and effects. Aquatic toxicity data for these CECs are generally limited, and when available are largely derived from short term, standardized toxicity tests that may inadequately characterize sublethal responses of aquatic organisms. The U.S. EPA in a recent white paper has identified a number of different chemical groups/classes that it considers CECs (http://www.epa.gov/waterscience/criteria/library/sab-emergingconcerns.pdf). These

compounds present several problems for risk assessment (Brooks et al 2009, Sanderson and Solomon 2009). One principle problem is that because there is generally no history of environmental regulation for these CECs, as such very little data on their fate, transport or effects have been produced. Additionally, while many of these CECs are present at very low levels, some of these compounds (e.g., pharmaceuticals) elicit very specific biological/physiological responses often at very low concentrations.

One of approach examined in this document w was the use of probabilistic distributions, specifically chemical toxicity distributions (CTD) or probabilistic pharmaceutical distributions. For much of this dissertation the analysis of individual datasets was conducted using methodology reviewed by Solomon and Takacs (2002), which relies on the observation that most biological and chemical data have a log-normal distributions (Munro 1990; Hattis and Burmaster 1994; Burmaster and Hull 1997). From that it is reasonable to assume that toxicological data will fall into the same type of distribution (Solomon and Takacs 2002). Figure 1 provides an example of a CTD.

Scope of the Dissertation

The purpose of this research was to develop and examine approaches to leverage available data using probabilistic models to advance an understanding of aquatic hazards of CECs. Chapter Two provided (1) a novel probabilistic hazard assessment for pharmaceutical acute toxicity to mammalian and fish models, (2) identified a statistically signification relationship between mammalian margin of safety data and fish ACR values for pharmaceuticals and (3) presented an approach for prioritize drugs for future environmental assessments based on potential hazard. These probabilistic assessments identified specific drugs and drug classes as potentially presenting greater hazard to fish.

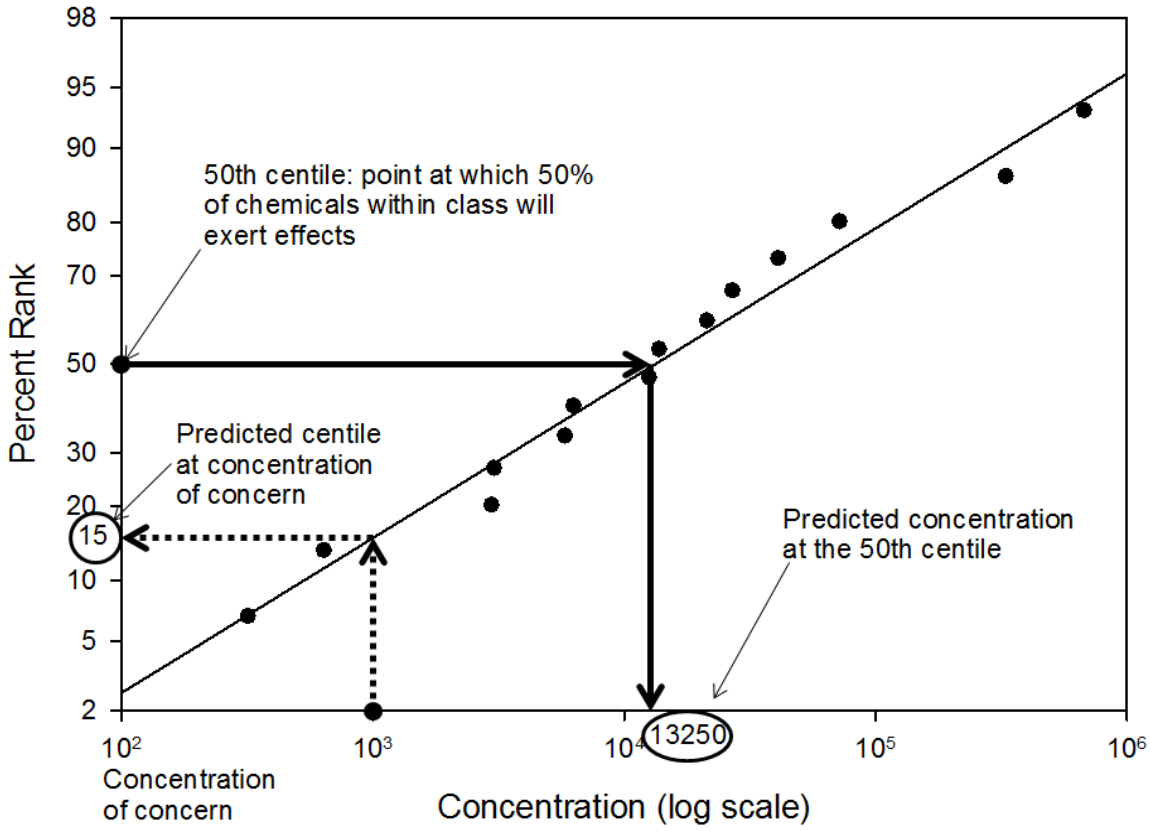


Figure 1. A theoretical Chemical Toxicity Distribution plotted on the standard probability versus log-transformed scale. Percent rank (or centile) can be used to determine at what concentration a certain percentage of drug distribution would have its effect. For example the 50th centile corresponds to a concentration of 13250 meaning that 50% of drugs within this distribution exert their effect at that concentration (solid arrows). By following a concentration through the distribution its corresponding centile can be predicted. For example a concentration of 1000 would correspond to the 15th centile meaning at a concentration of 1000 only 15% of drugs in the distribution will exert their effect (dashed arrows).

To evaluate contributions from Chapter Two and other researchers, toxicity experiments with diphenhydramine, an antihistamine with multiple MOA, were conducted to characterize standardized response thresholds and those from novel, MOA-related ecotoxicological endpoints. The results confirmed that sublethal endpoints (e.g., behavior) related to therapeutic may be more appropriate for fish and that leveraging mammalian pharmacology and toxicology data may be predictive for MOA related responses when evolutionary conservation of targets are considered. It further highlighted the importance of carefully selecting model organisms for study of pharmaceuticals with multiple MOAs, because reproduction of the invertebrate *Daphnia magna* was sensitive to diphenydramine, potentially resulting from its histaminergic and cholinergic activities.

In Chapter Four, a similar probabilistic hazard assessment approach with CTDs was applied to oil dispersants, another CEC, to assess potential impacts to aquatic invertebrates and fish in the presence and absence of different types of oil. These CTDs suggested that dispersants alone are generally less toxic than oil. In contrast, most dispersant:oil mixtures are more toxic than oil alone. For the datasets examined, CTDs predicted 95% of dispersant:oil mixtures to have acute toxicity values above 0.32 and 0.76 mg/L for *Mysidopsis* and 0.33 mg/L and 1.06 mg/L for *Menidia* (for Louisiana sweet crude and #2 fuel oil, respectively). These findings demonstrated the utility of CTDs as a means to evaluate the comparative ecotoxicity of dispersants alone and in mixture with different oil types. The approaches presented here also provided valuable tools for prioritizing prospective and retrospective environmental assessments of oil dispersants.

In Chapter Five, probabilistic methods were used to develop uncertainty factors for ACRs for biologically active compounds. For many chemical classes chronic effects data is lacking, making acute to chronic extrapolations a necessity. Typically, default uncertainty factors are utilized to bridge this data gap (Chapman et al. 1998). These default values are generally based on industrial chemical responses, where ACRs tend to be low. Biologically active compounds, by contrast tend to have higher ACR values, beyond what may be accounted for by default factors. By leveraging available chronic datausing probabilistic methods, novel data-driven uncertainty factors were developed, potentially providing more protective extrapolation models. Probabilistic models provide a way to leverage limited datasets to fill data gaps providing a robust assessment tool to address hazards of CECs.

CHAPTER TWO

Leveraging Mammalian Pharmaceutical Toxicology and Pharmacology Data to Predict Chronic Fish Responses to Pharmaceuticals

This chapter published as: Berninger JP and Brooks BW. 2010. Leveraging mammalian pharmaceutical toxicology and pharmacology data to predict chronic fish responses to pharmaceuticals. Toxicol. Lett. 193: 69 – 78.

Introduction

Extrapolating biological responses to chemicals among species is a key element in modern scientific research. Prior to initiating human clinical trials for a drug candidate, numerous in vitro and animal studies (e.g., rat model) are performed, from which data may be extrapolated to humans based on known differences in development, scaling, physiology, molecular genetics, and biochemistry (Calabrese 1987). Such cross species extrapolations has been well documented (Rhomberg and Lewanowski 2006; Mattes 2006), and is often known as read across. Although this approach has typically been applied from rodent models to humans, with increasing societal mandates and regulatory efforts for the reduction of mammalian testing, fish models are become more prevalent in biomedical research. In fact, fish to mammal read across approaches to cross species extrapolation is increasingly examined, particularly focusing on employing fish models in chemical carcinogenicity and other toxicological applications (Hinton et al. 2009). For example, studies of fish kidney functions have lead to a greater understanding of the mammalian kidney and the development of several human therapeutics (Bayenbach 2004). Further, fish models are increasingly used in early phases of pharmaceutical

development and safety testing, primarily because of the relatively lower costs of care, husbandry and testing, and the rapid rates of reproduction present important advantages for pharmaceutical development (Powers 1989).

Read across approaches with fish models can provide important bridges between the biomedical and environmental sciences (Owen et al. 2007; Brooks et al. 2008, 2009, 2010). Human pharmaceuticals are increasingly identified in various environmental matrices (e.g., water, sediment, soil, edible fish tissues), resulting in a need to identify potential environmental consequences of exposure. Traditional environmental approaches for assessing chemical impacts on fish have critical limitations for many pharmaceuticals, requiring development of alternative endpoints and testing approaches that *a priori* consider therapeutic mode/mechanism of action (MOA) to appropriately assess environmental risk (Brooks et al. 2003; Fent et al. 2006; Ankley et al. 2007; Brooks et al. 2009). Herein, leveraging mammalian pharmaceutical safety information using read across approaches (Brooks et al. 2009, 2010) presents important opportunities for predicting and determining environmental impacts, particularly for fish, where pharmaceutical targets may be more evolutionarily conserved (Huggett et al. 2003; Gunnarrson et al. 2008). Thus, an understanding of pharmacology and toxicology in fish models can advance the use of alternative models in drug development and support risk assessment and management efforts of pharmaceuticals in the environment. Because examining numerous drugs is impractical from a resource perspective, development of screening approaches is necessary to target pharmaceuticals for future environmental and biomedical studies.

A number of fundamental considerations are important in pharmaceutical read across approaches with fish models, but of particular importance is the presence of a pharmacological target (e.g., receptor, enzyme), functional activity of chemical-target interaction, and therapeutic potency. Ankley et al. (2007) proposed an approach for linking pharmaceutical MOA-related responses to physiological effects. Although Huggett et al. (2003) identified 50-60% homology of many therapeutic targets between fish and mammals, Gunnarsson et al. (2008) more recently reported that fishes share on average 65-75% genetic homology with humans at over a thousand different drug receptors. Owen et al. (2007) used a read across method in describing the effects of beta-adrenergic blockers in mammals and fish on physiology and biochemistry of target receptors. After the presence of a target has been identified, biochemical and physiological responses should be examined relative to pharmaceutical potency (Ankley et al. 2007). For mammals, therapeutic index and margin of safety, generally defined as a ratio of the lethal (or toxic) dosage and the therapeutic dosage of a drug, are key parameters in drug development to characterize the relationship between mammalian toxicity and physiological efficacy (Thummel and Shen 2001). Whereas larger therapeutic indices or margins of safety may result in greater drug safety for humans, such values related to potency may suggest increased potential for environmental risk.

A potential corollary for mammalian therapeutic index or margin of safety in aquatic toxicology models is the acute-to-chronic (ACR) ratio, defined as the ratio between an acute mortality benchmark concentration (LC_{50}) and a chronic threshold of effect (e.g., No Observed Effect Concentration; Kenaga 1982). In a recent review of common environmental contaminants Raimondo et al. (2007) found the median ACR to

be 8.3 and the 90th centile to be 79.5, a range that encompasses default ACRs (e.g., 10 - 100) used in regulatory environmental toxicology. Sanderson and Thomsen (2009) specifically examined aquatic ACRs for pharmaceuticals, reporting that for 23 drugs an ACR of 100 would be protective; however, this analysis relied primarily on invertebrates and algae studies, species which have lower therapeutic target similarities to mammals than fish (Gunnarsson et al. 2008). Selection of a chronic endpoint for calculation of a fish ACR that reflects the MOA of the pharmaceutical of interest and is ecologically relevant is critical. Because pharmaceuticals are designed to have specific biological activities, large ACRs are not unusual when examining MOA related responses; for example, an ACR of 48,000 was reported for the beta-adrenergic receptor blocker propranolol (Huggett et al. 2002) and an ACR of >1,000,000 was identified for 17-α ethinyl estradiol, a potent estrogen agonist (Schweinfurth et al 1996, Lange et al 2001). Thus, it is possible that ACRs may be used as a diagnostic for pharmaceutical studies with aquatic models, with larger ACRs potentially identifying if a therapeutic is eliciting chronic responses associated with therapeutic target interaction (Ankley et al. 2005; Clubbs and Brooks 2007).

In this study we hypothesized that using mammalian toxicological and pharmacological data can provide read across approaches for understanding the interactions between fish and pharmaceuticals. We specifically employed chemical toxicity distributions, an approach used in probabilistic hazard assessment for large and complex datasets. Chemical toxicity distribution approaches have been employed for identifying Thresholds of Toxicological Concern for many industrial chemicals (Kroes et al. 2005; de Wolf et al. 2005; Munro et al. 2008), comparing the sensitivities of in vitro

and fish models for estrogenicity (Dobbins et al. 2008), and predicting aquatic concentrations of ecotoxicological concern for chemical with common MOAs in plant models (Brain et al. 2006) and invertebrates and fish (Dobbins et al.. 2008, 2009). In fact, the Threshold of Toxicological Concern concept was identified to present a potentially valuable approach for supporting prioritization approaches for examining pharmaceutical hazards in the environment (Brooks et al. 2009). Subsequently, the primary objectives of this study included: (1) evaluating the relationship between fish and mammalian acute mortality data, (2) applying probabilistic methodologies to mammalian pharmacological data, (3) examining the probabilistic distributions of various drug classes (in mammals) as a way to inform future pharmaceutical studies in fish models, (4) comparing fish ACR values for available therapeutics with corresponding mammalian therapeutic indices or margin of safety data. We further propose a screening methodology for identifying environmental hazards to fish from pharmaceuticals, and relating sublethal fish physiological responses to mammalian therapeutic information.

Materials and Methods

Database Development

An initial list of active pharmaceuticals ingredients was developed. This list included the top 200 prescribed drugs (Verispan 2008; www.drugs.com), common therapeutics from important classes (e.g., antibiotics, benzodiazepines, beta blockers, antidepressants, contraceptive hormones, NSAIDs, narcotics), and pharmaceuticals for which acute aquatic toxicology data was available (Sanderson and Thomsen 2009). For each drug mammalian acute toxicity data (e.g., LD_{50}), therapeutic dose (e.g., C_{max}), therapeutic indices or margins of safety, physical-chemical properties (e.g., pK_a, log

K_{OW}), and information on acute and chronic toxicity to fish was collected. Acute mammalian dosage (LD_{50}) information was collected for 361 active pharmaceutical ingredients. Of those 361 pharmaceuticals, data for fish acute toxicity (LC_{50}) were found for 220. A total of 274 drugs were identified with mammalian therapeutic dose data. For fish chronic effects that could be plausible linked to therapeutic MOA (Ankley et al. 2007, Brooks et al. 2009, 2010), data for 15 pharmaceuticals were found that conformed to this MOA related response criteria and thus were used to calculate fish ACR values.

For mammalian acute toxicity data, only rat oral LD_{50} values (mg/kg) were selected to maintain consistency among drugs. Acute toxicity values were specifically collected from available refereed databases (Wishart et al. 2008; HSDB 2008; ChemIDplus 2008; Merck Index 2008), from FDA documents, or manufacturer supplied material safety datasheets. Because the majority of this information was gleaned from databases or secondary reference books, 25% of these values were checked against primary literature reported value as a measure of quality assurance; this QA/QC effort resulted in no discrepancies. Peak plasma concentrations (C_{max}; µg/ml) were collected for each drug to provide a measure of internal therapeutic dose. Human C_{max} data were more readily available, potentially because values from animal studies are often proprietary. These values were obtained from FDA, the Physicians' Desk Reference (2008) or the primary literature (Schulz and Schmoldt 1996). Unfortunately, information was not readily available to calculate therapeutic indices or margins of safety for these compounds and what was available was inconsistent in terms of calculations and uncertainty factors utilized. To provide a more consistent metric we subsequently developed an Acute to Therapeutic Ratio (ATR), which was derived as the ratio of rat

acute toxicity data (LD_{50}) and human therapeutic dose (C_{max} value) for each drug. We hypothesized that this metric is similar to the ACR often used in aquatic toxicology; similar to a fish ACR value, ATR is unitless, with an assumption that blood plasma density is equivalent to water.

Comparative Analysis of Fish and Mammalian Data

The potential relationship between available fish and mammalian data was examined. The first analysis compared the available acute toxicity data: fish LC_{50} (mg/L) and rat LD_{50} (mg/kg). The second analysis compared the mammalian ATR and fish ACR values for pharmaceuticals. An extensive review of chronic response of fish to pharmaceuticals was conducted, focusing on chronic fish responses plausibly related to the therapeutic MOA (Ankley et al. 2007). As noted above, only chronic responses matching those criteria, and not responses likely resulting from narcosis, were selected. Linear regression analyses were performed to examine potential data relationships (SigmaPlot Version 11.0, Systat Software, Inc., San Jose, CA, USA).

Probabilistic Analysis of Fish and Mammalian Data

We employed chemical toxicity distributions to further examine fish (LC_{50}) and rat (LD_{50}) acute data, therapeutic dose (C_{max}), and mammalian ATRs and fish ACRs. Because pharmaceuticals are not a single chemical class and have many MOAs, hereafter we identified these distributions of ATRs and C_{max} values as Probabilistic Pharmaceutical Distributions (PPD) instead of chemical toxicity distributions. Each PPD included a regression analysis of the distribution, which was used to identify the probability of finding a value at a certain centile or to determine the centile associated with a specific

concentration. For example, in a theoretical PPD the point at which the distribution is equal to a value of 1000 might corresponds to the 15th centile, predicting that there is a 15% probability of pharmaceuticals having an value of 1000 or less below this point in the distribution. PPDs for various data sets were calculated based on equations developed by Solomon and Takacs (2002) and modified by Brain et al. (2006). For each analysis, data were numerically ranked in descending order and ranks converted to a probability percentage calculated from the Weibull formula:

$$j = 100 * i / (n + 1) \quad (1)$$

where j is the plotting position, i is the numerical rank, and n is the total number of data points in the data set. Values and ranks were then plotted on a log-probability scale and a regression line fitted to these transformed distributions (SigmaPlot Version 10.0 Systat Software, Inc., San Jose, CA, USA). From each regression, slope, intercept, and r^2 values were determined. Using intercept and slope values it was possible to calculate centile values (Microsoft Excel 2007 Microsoft Corp, Redmond, WA, USA) using the equation:

$$\text{Centile Value} = \text{NORMSDIST}\,(m * \log_{10}(x) + b) \quad (2)$$

where NORMSDIST returns the standard normal cumulative distribution function, x is a selected value (C_{max} – μg/mg, LD_{50} – mg/kg, ATR – unitless), and m and b are the slope and intercept, respectively, of the regression line. PPD values were determined at 1, 5, 25, 50, 75, 95, and 99 centiles.

Analysis of ATR Values for Drug Classes

PPDs of ATR values were subsequently developed for selected pharmaceuticals based on broadly defined drug classes. Each PPD of a drug class contained a minimum of seven values for individual pharmaceuticals to meet assumption of a chemical toxicity distribution (Brain et al. 2006, Dobbins et al. 2008, 2009), resulting in 15 different PPDs of common drug classes. These included reproductive hormones, corticosteroids, antihistamines, acetyl choline inhibitors, lipid lowering agents, beta blockers, calcium channel blockers, angiotensins, benzodiazepines, antidepressants, narcotics, NSAIDs, antibiotics,anti-neoplastics, and anti-seizure medications. PPDs of ATR values for each of these drug class ATR values were then compared to the ATR PPD for all drugs, using a Kruskal-Wallis ANOVA to determine if a significant difference existed among the drug classes. To determine if a drug class was different from the all drug distribution, each class was independently compared to the all drug ATR data using a Mann Whitney rank sum test (SigmaPlot Version 11.0, Systat Software, Inc., San Jose, CA, USA). Threshold values for specified centiles were determined for each PPD using the methods described previously.

Results

Acute Toxicity Data

An examination of fish LC_{50} and rat LD_{50} values for common pharmaceuticals resulted in a poorly correlated relationship ($r^2 = 0.033$; $p < 0.004$; Figure 2). Figure 3 presents chemical toxicity distributions for both fish (LC_{50}, n=220) and rat (LD_{50}, n=361) acute toxicity data. The probabilities of finding a pharmaceutical that elicits acute

mortality to fish or rodent models were derived using the equations in Table 1. Fish acute 5th, 50th and 95th centile values were 0.84, 52.8 and 3,310 mg/L, indicating a 5%, 50% and 95% probability, respectively, of encountering a pharmaceutical in the environment that causes acute mortality to fish at or below those values (Table 1). Similarly, rat acute 5th, 50th, and 95th centile values were 33.5, 942.1, and 26,488 mg/kg, respectively (Table 1). To explore how chemical toxicity distributions may be used to examine pharmaceutical toxicity relative to other xenobiotics, mammalian and fish acute toxicity data were compared to existing toxicity scales. Specifically, when comparing information from the chemical toxicity distribution approach to the Hodge and Sterner (1949) scale for mammalian toxicity (Table 2), the majority of drugs (62%) corresponded to the slightly toxic to relatively harmless categories (>500 mg/kg), while few (8%) were highly to extremely toxic (LD_{50} <50 mg/kg). The fish acute chemical toxicity distribution for pharmaceuticals generally compared to a similar threshold of ecotoxicological concern distribution for other environmental contaminants (Table 3), where it was predicted that very few pharmaceuticals are acutely toxic at concentrations below 100 µg/L (0.75%) and the majority (74%) of drugs are acutely toxic to fish at concentrations above 10 mg/L.

Mammalian Therapeutic and ATR Data

The range of values observed in the C_{max} and ATR PPDs exemplifies the differences in potencies among pharmaceuticals (Figure 4). The 5th, 50th and 95th centile values from the human C_{max} PPD were 0.0011, 0.226 and 47.7 µg/mL, respectively, identifying that there was a 5%, 50% and 95% probability, respectively, of observing a therapeutic dose for a pharmaceutical at or below these concentrations (Table 1). From

the ATR distribution 5th, 50th and 95th centile values were identified as 12.19, 3771 and 1.16×10^6, respectively (Table 1). Not surprisingly, for the majority of drugs both acute toxicity and therapeutic dose values influenced the specific locations on the ATR PPD. Those pharmaceuticals plotted on the extreme ends of the distributions (e.g., warfarin LD_{50} in Figure 3, 17-α ethinyl estradiol C_{max} in Figure 4A) generally had corresponding positions in the ATR PPD (Figure 4B).

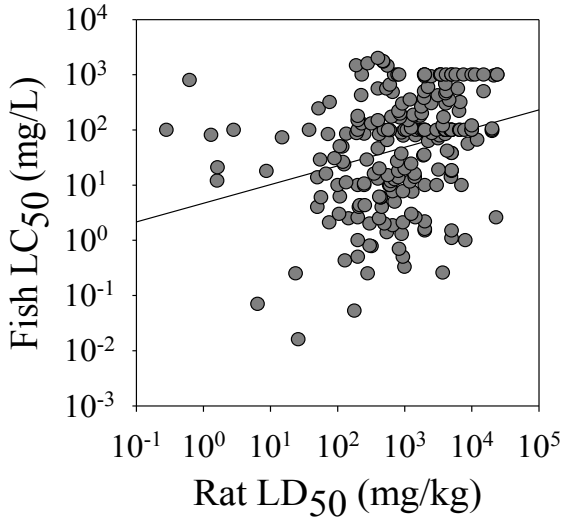


Figure 2. Relationship between fish acute toxicity (LC_{50}) and rat acute toxicity (LD_{50}, oral) values for a common group of 220 pharmaceuticals ($p < 0.004$, $r^2 = 0.033$).

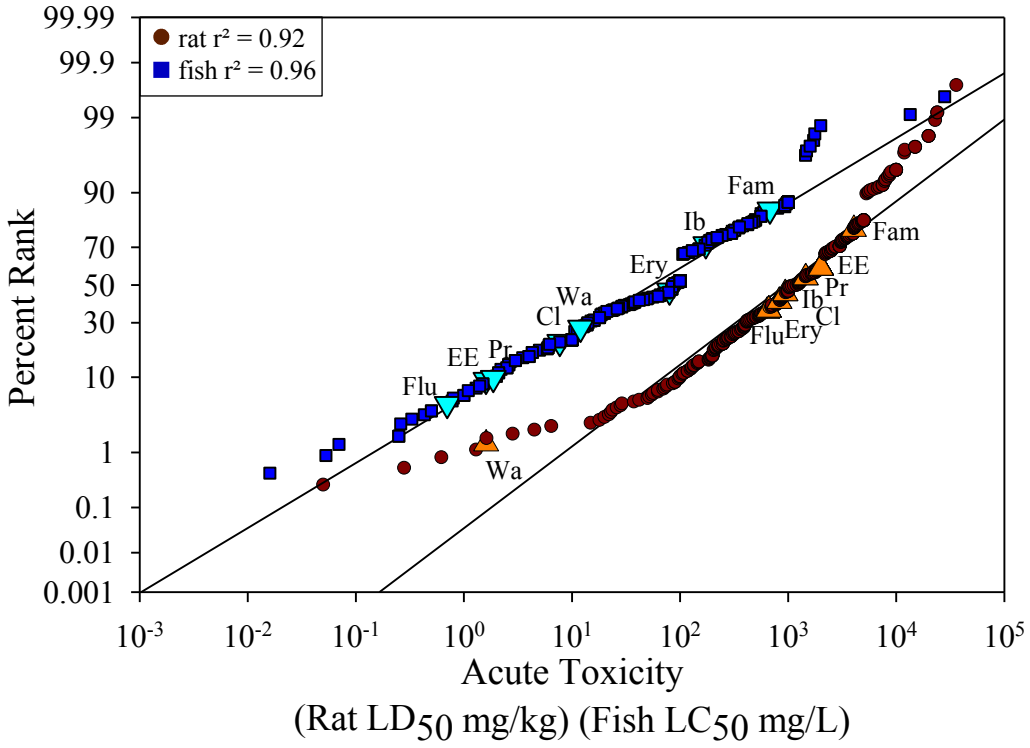


Figure 3. Chemical toxicity distributions of fish acute LC50 (n = 220) and rat acute LD50 (n = 361). For reference purposes triangles denote several common drugs: lipid lowering agent - clofibrate (Cl), antibiotic - erythromycin (Ery), reproductive hormone - 17α-ethinyl estradiol (EE), antihistamine - famotadine (Fam), antidepressant - fluoxetine (Flu), NSAID - ibuprofen (Ib), beta blocker - propranolol (Pr), and anticoagulant - warfarin (Wa).

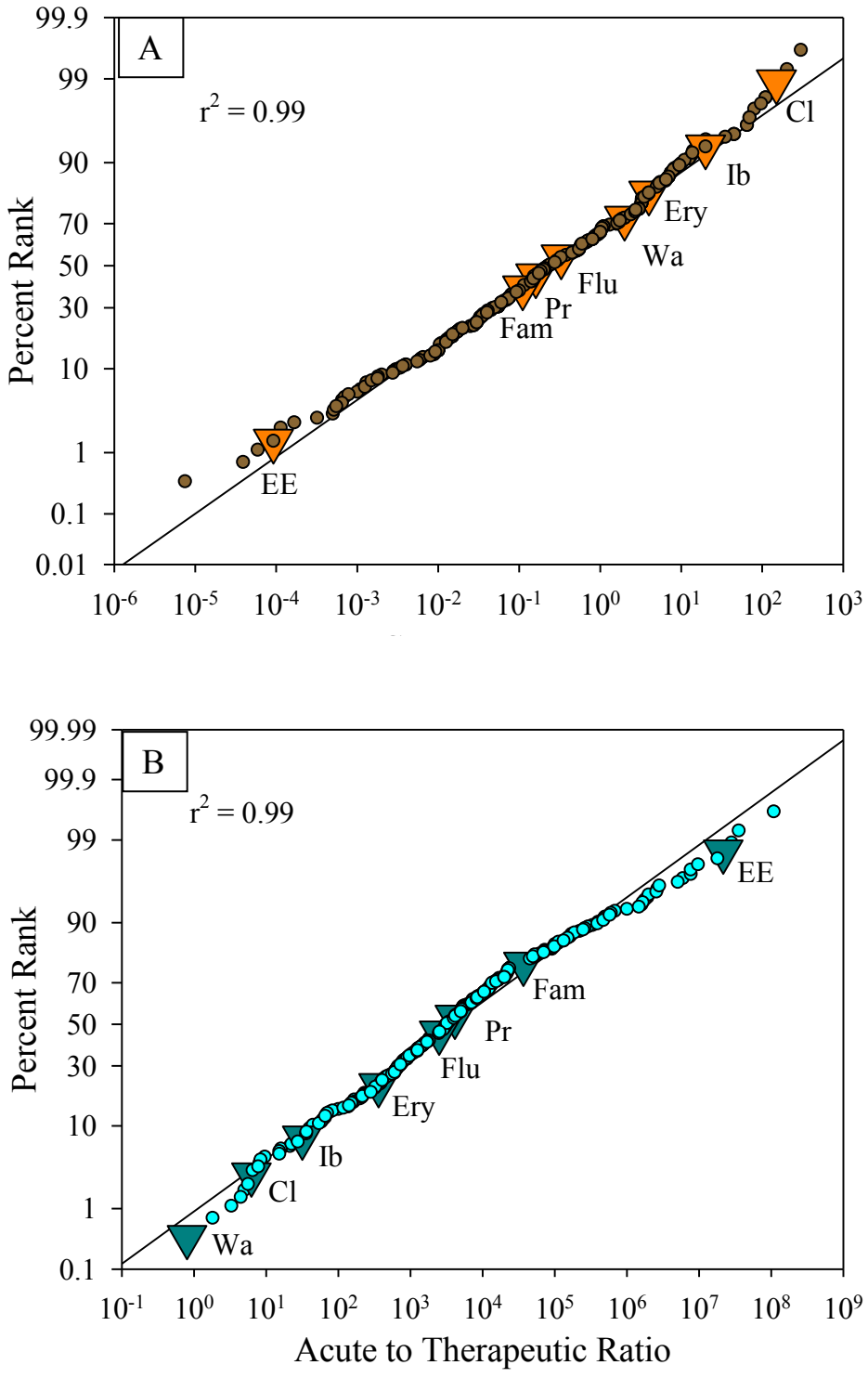


Figure 4. Probabilistic Pharmaceutical Distributions for human therapeutic dose (A; Cmax, mg/mL), and Acute to Therapeutic Ratio (B; LD50/Cmax). For reference purposes triangles denote several common drugs: clofibrate (Cl), erythromycin (Ery),17α-ethinyl estradiol (EE), famotadine (Fam), fluoxetine (Flu), ibuprofen (Ib), propranolol (Pr), and warfarin (Wa).

Table 1. Equations for regression lines and values corresponding to the 1st, 5th, 25th,50th, 75th, 95th, and 99th centiles for probabilistic distributions of acute toxicity in fish (LC_{50}, mg/L) and rats (LD_{50} oral, mg/kg), human therapeutic dosage (C_{max}, µg/ml), and the Acute to Therapeutic Ratio (ATR, unitless).

Distribution	*n*	r^2	*a*	*b*	Centile value						
					1%	5%	25%	50%	75%	95%	99%
Fish LC_{50}	220	0.958	0.915	-1.577	0.152	0.84	9.68	52.8	288.1	3310	18383
Rat LD_{50}	361	0.920	1.135	-3.376	8.41	33.5	239.9	942.1	3701	26488	105532
Human C_{max}	274	0.991	0.708	0.457	$1.18x10^{-4}$	0.00108	0.0252	0.226	2.03	47.6	436
ATR	274	0.989	0.661	-2.364	1.14	12.2	359.7	3771	39537	$1.16x10^{6}$	$1.25x10^{7}$

n = number of compounds, a = slope of regression line, b = y-intercept of regression line.

Table 2. Hodge and Sterner (1949) toxicity classes for pharmaceuticals and the frequency of occurrence in each class based on a rodent LD_{50} chemical toxicity distribution for 361 common pharmaceuticals.

Hodge and Sterner Classes			Rodent LD_{50} Distribution	
Class	Description	mg/kg	% drugs per class	Cumulative %
1	Extremely Toxic	1 or below	0.05%	0.05%
2	Highly Toxic	1 to 50	8%	8%
3	Moderately Toxic	50 to 500	30%	38%
4	Slightly Toxic	500 to 5000	42%	80%
5	Practically Non-Toxic	5000 to 15000	13%	93%
6	Relatively Harmless	15000 or above	7%	100%

Table 3. General aquatic chemical toxicity classes for fish based on distribution of LC50 values (Russom et al. 1997; de Wolf et al. (2005) in comparison to acute chemical toxicity distribution of pharmaceuticals.

Fathead Minnow Narcotic Toxicity				Fish LC_{50} Distribution	
Class	Concentration (mg/L)	% per class	Cumulative %	% drugs per class	Cumulative %
1	0.1	2%	2%	0.75%	0.75%
2	1	8%	10%	5%	6%
3	10	30%	40%	20%	26%
4	100	40%	70%	34%	60%
5	1000	20%	90%	27%	87%
6	10000	9%	99%	11%	98%

ATR Values for Drug Classes

PPDs were developed for fifteen pharmaceutical classes (Figure 5), and these distributions were compared to the PPD for available ATR values (Figure 4). A comparison at specific centiles (Table 4) shows considerable variability across the various drug classes. For example, at the 50th centile for all drugs the ATR value is 3,771, while the same centile for reproductive hormones is 2,090,000 and the NSAID 50th centile value is 35, a difference of 5 orders of magnitude. Many drug classes had differences in slopes and intercepts compared to original data set (Figure 5 A-H). Differences in slope are indicative of the variability within a class (e.g., antibiotic have a steeper slope indicating less variation, while lipid lowering agents have shallower slopes). Intercept differences indicate variation in potency (e.g., the NSAID intercept value of -1.14 indicates less potency compared to reproductive hormones at -5.13). Figure 6 summarizes centile ranges among these PPDs, highlighting the variation among ATRs among the fifteen drug classes. In particular, 25th centile ATR values for reproductive hormones, antihistamines, corticosteroids, benzodiazapines and calcium channel blockers were higher than the median ATR for all pharmaceuticals evaluated (Figure 6, Table 4). A Kruskal Wallis ANOVA was used to identify significant differences among drug classes (Figure 6). Further statistical comparisons between the all drug ATR distribution and the individual classes determined some classes to be significantly higher (reproductive hormones, $p < 0.001$; corticosteroids, $p < 0.001$; calcium channel blockers, $p = 0.036$; antihistamines, $p = 0.007$) and others to be significantly lower (NSAIDs, $p < 0.001$; and anti-seizures, $p = 0.003$; antibiotics, $p < 0.001$).

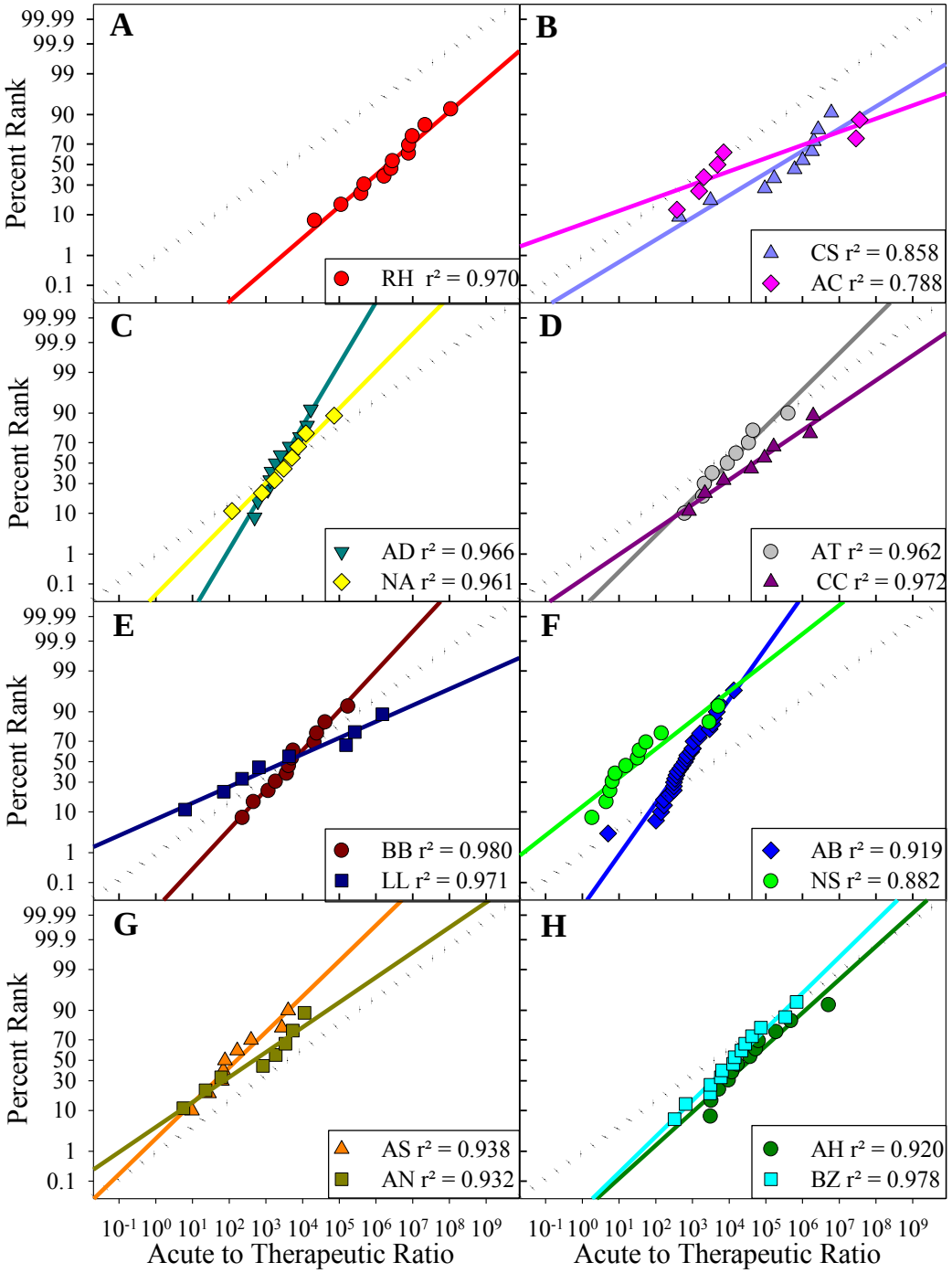


Figure 5. Probabilistic Pharmaceutical Distributions (PPD) of Acute to Therapeutic Ratio (LD_{50}/ C_{max}) for fifteen drug classes. A PPD for all drugs (Figure 4B) is plotted as a dotted black reference line. A) reproductive hormones; B) acetyl cholinesterase inhibitors and corticosteroids; C) narcotics and antidepressants; D) angiotensins and calcium channel blockers; E) beta blockers and lipid lowering agents; F) antibiotics and NSAIDs; G) anti-seizure and anti-neoplastics; H) benzodiazepines and antihistamines.

Table 4. Equations for regression lines and values corresponding to the 1st, 5th, 25th,50th, 75th, 95th, and 99th centiles for Probabilistic Pharmaceutical Distribution of mammalian Acute to Therapeutic Ratio in fifteen different drug classes.

					Centile value						
Distribution	*n*	r^2	*a*	*b*	1%	5%	25%	50%	75%	95%	99%
AC	7	0.79	0.338	-1.523	0.0042	0.44	326.1	32453	$3.23x10^6$	$2.42x10^9$	$2.53x10^{11}$
AT	9	0.96	0.932	-3.712	30.66	165.1	1816	9610	50869	559300	$3.01x10^6$
AB	29	0.92	1.325	-3.708	11.03	36.05	194.60	628.21	2028	10947	35771
AD	11	0.93	1.583	-5.376	63.97	193.1	931.4	2780	8299	40021	120823
AH	12	0.92	0.85	-3.874	66.32	420.6	5835	36310	225937	$3.13x10^6$	$1.99x10^7$
AN	8	0.93	0.639	-1.708	0.11	1.25	41.30	468.7	5319	175209	$2.04x10^6$
AS	9	0.94	0.911	-2.002	0.44	2.47	28.67	157.6	866.9	10070	56366
BZ	14	0.98	0.92	-3.791	39.12	215.5	2447	13246	71698	814079	$4.48 x10^6$
BB	12	0.98	1.014	-3.76	25.92	121.7	1102	5093	23546	213076	$1.00 x10^6$
CC	8	0.97	0.637	-2.973	10.35	121.6	4057	46466	532157	$1.78 x10^7$	$2.09 x10^8$
CS	10	0.86	0.569	-3.062	19.69	310.6	15778	241967	$3.71 x10^6$	$1.88 x10^8$	$2.97 x10^9$
LL	8	0.97	0.417	-1.469	0.0088	0.38	80.36	3333	138272	$2.94 x10^7$	$1.27 x10^9$
NA	8	0.97	0.954	-3.062	12.21	63.24	657.8	3350	17064	177502	919462
NS	12	0.88	0.737	-1.145	0.025	0.21	4.34	35.71	293.6	6082	51104
RH	12	0.97	0.812	-5.132	2854	19709	308722	$2.09x10^6$	$1.41x10^7$	$2.22x10^8$	$1.53x10^9$

n = number of compounds, *a* = slope of regression line, *b* = y-intercept of regression line. AC - acetyl cholinesterase inhibitor, AT - angiotensins, AB - antibiotics, AD – antidepressants, AH - antihistamines, AN – antineoplastics, AS - anti-seizure, BZ - benzodiazepines, BB - beta blockers, CC - calcium channel blockers, CS - corticosteroids, LL - lipid lowering agents, NA - narcotics NS - non steroidal anti-inflammatory, RH - reproductive hormones.

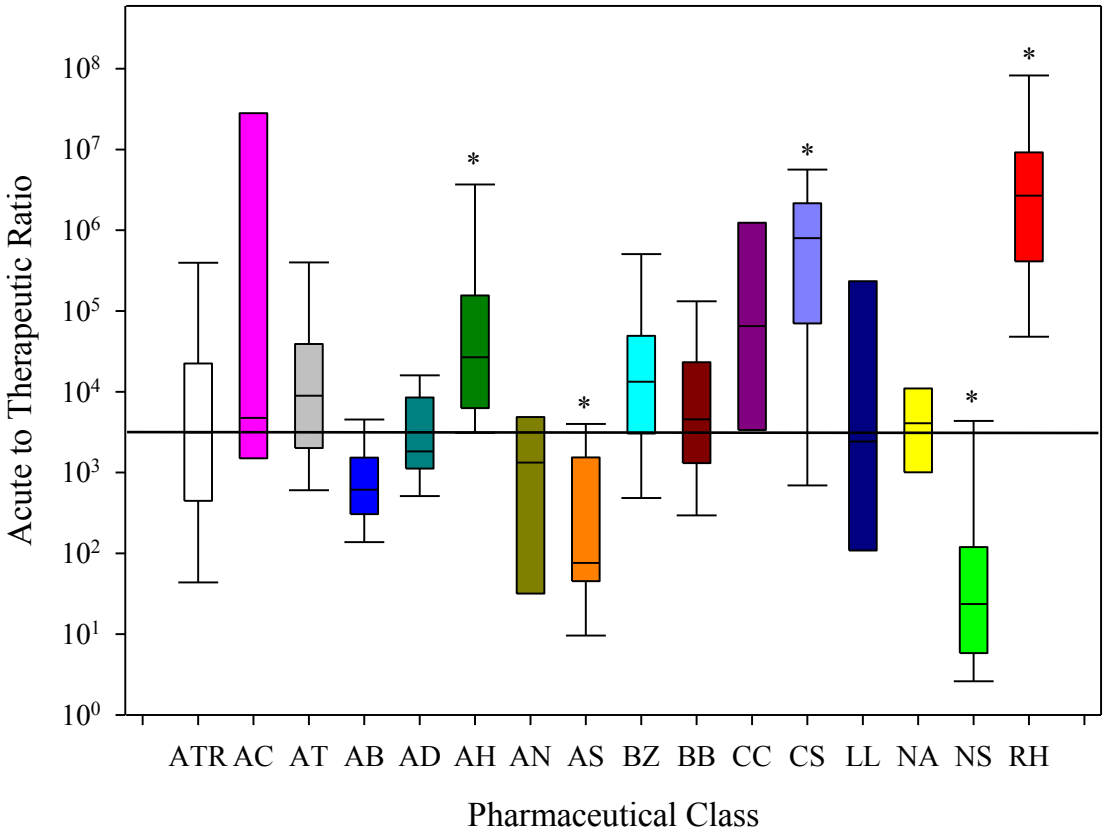


Figure 6. Centiles among Acute to Therapeutic Ratios (ATRs) for all drugs and various drug classes. Boxes represent values within the 25th and 75th centiles; error bars represent 10th and 90th centiles. The ATR value for all drugs at the 50th centile is plotted as a reference line. * Represents significantly different drug class (Kruskal–Wallis ANOVA; $p < 0.05$) from all drug ATR. Drug class abbreviations follow previous figures: AC- acetylcholinesterase inhibitors; AT- angiotensins; AB- antibiotics; AD- antidepressants; AH- antihistamines; AN- anti-neoplastics; AS- antiseizure; BZ- benzodiazepines; BB- beta blockers; CC- calcium channel blockers; CS- corticosteroids; LL- lipid lowering agents; NA- narcotics; NS- non-steroidal anti-inflammatory; RH- reproductive hormones.

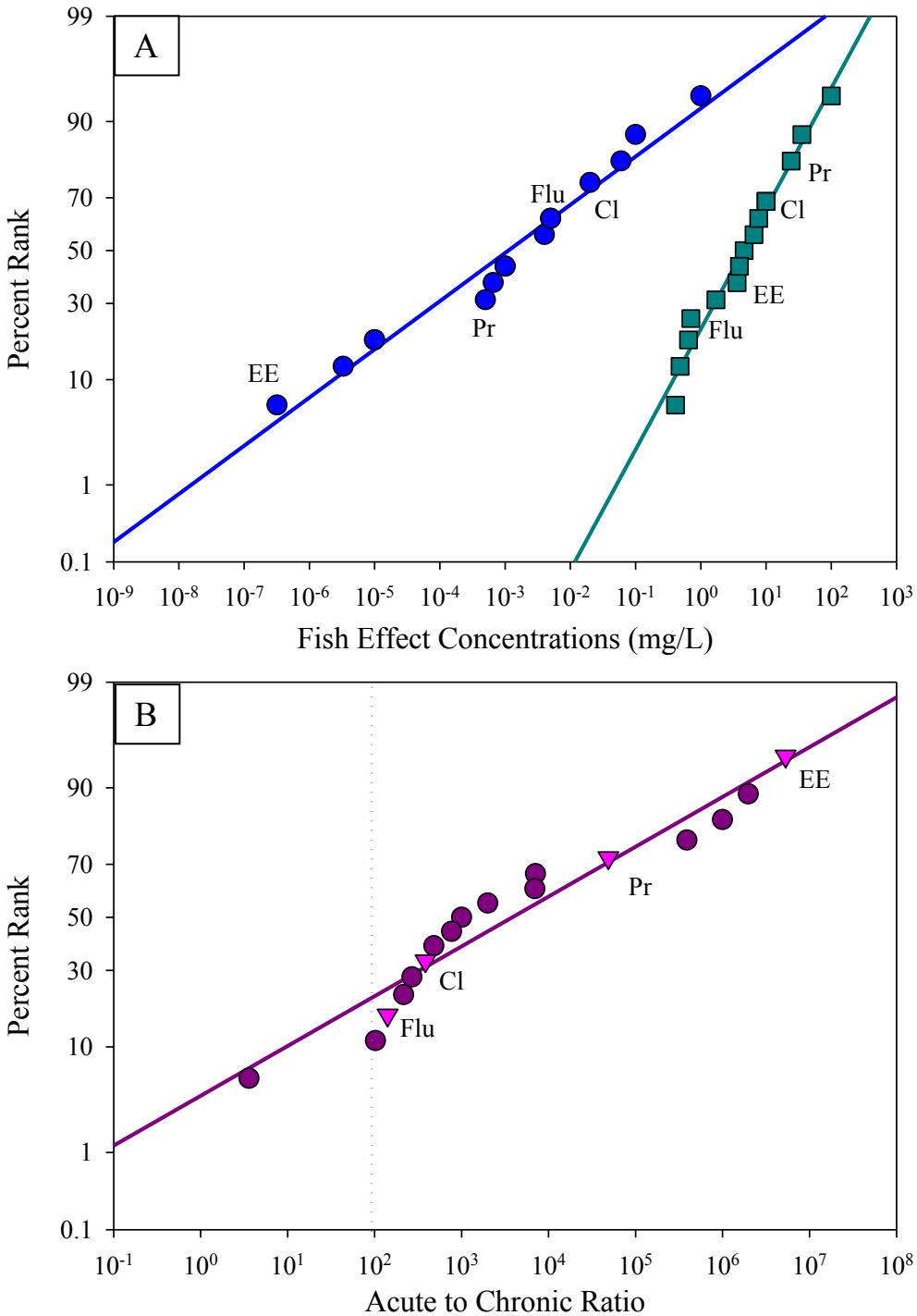


Figure 7. (A) Probabilistic distributions of toxicological benchmarks for fish acute (LC_{50}) and chronic (NOEC) effects concentrations. (B) Probabilistic distributions of fish Acute to Chronic Ratios for studies with endpoints plausibly linked to therapeutic MOA. Dotted line represents 90th centile ACR value of 79.5 for aquatic toxicants (Raimondo et al., 2007). For reference positions of several drugs are highlighted on distributions: clofibrate (Cl), ethinyl estradiol (EE), fluoxetine (Flu), and propranolol (Pr).

Table 5. Pharmaceutical data used to (A) calculate fish Acute to Chronic Ratios and to (B) calculate mammalian Acute to Therapeutic Ratios.

	(A) Fish Toxicological Responses				(B) Mammalian Pharmacological Data (Rat-Human Model)		
Compound	Species	Acute Response (mg/L)	Sub-Lethal/Chonic Response (µg/L)	ACR	Acute [1] (mg/kg)	Cmax (µg/mL)	ATR
Flutamide	MED	3.6 96h LC_{50} [2]	1000 NOEC sex reversal [2]	3.6	787	0.95 [3]	828
Sertraline	FHM	0.647 48h LC_{50} [4]	60 NOEC feeding [4]	10.8	840	0.19 [5]	4421
Atenolol	RT/FHM	100 96h LC_{50} [6]	100 NOEC 21day condition index [6]	100	2000	0.55 [3]	3636
Tamoxifen	RT/FHM	0.41 96h LC_{50} [7]	4.01 NOEC fecundity [8]	101	1190	0.04 [5]	29750
Fluoxetine	FHM/MED	0.705 96h LC_{50} [9]	5 NOEC 28day fecundity [9]	141	825	0.33 [3]	2500
Clofibrate	MF/RT	7.7 96h LC_{50} [10]	20 NOEC 28day gill function [11]	385	940	150 [3]	6.26
Diclofenac	ZF/RT	0.48 96h LC_{50} [12]	1 NOEC 28day liver function [11]	480	62.7	1.75 [3]	35.7
Drospirenone	ES/FHM	4.6 96h LC_{50} [13]	0.66 NOEC 21day fecundity [14]	6970	1250	0.0595 [5]	21008
Carbamazepine	MED/CC	35.4 96h LC_{50} [15]	5 NOEC 28day kidney function [11]	7080	1957	1.9 [3]	1030
Metoprolol	MED/RT	100 96h LC_{50} [16]	5 NOEC 28day liver function [11]	20000	5500	0.268 [3]	20560
Propranolol	MED	24.3 48h LC_{50} [16]	0.5 NOEC 28day fecundity [16]	48600	660	0.079 [5]	8354
Estradiol	MED	3.9 96h LC_{50} [2]	0.01 NOEC sex reversal [2]	390000	100	0.0000438 [3]	2283105
Testosterone	MED	10 96h LC_{50} [2]	0.01 NOEC sex reversal [2]	1000000	1000	0.00901 [5]	110988
Levonorgestrel	ES/FHM	6.53 96h LC_{50} [13]	0.0033 NOEC 21day fecundity† [14]	1978788	5000	0.0128 [5]	390625
Ethinyl Estradiol	ZF	1.7 96h LC_{50} [17]	0.00032 NOEC 150day fecundity [18]	5312500	2000	0.0000922 [5]	21691974

MOA – Mode of Action; ES - (ECO)SAR derived (US EPA, 2009b), FHM- Fathead Minnow (*Pimephales promelas*), RT- Rainbow Trout (*Oncoryncus gardeneri*), MED- Japanese Medaka (*Oryzias latipes*), CC – Common Carp (*Cyprinus carpio),* MF – Mosquito Fish (*Gambusia holbrooki*), ZF- Zebrafish (*Danio rerio)*. † actual NOEC is lower but could not be calculated based on results. [1] ChemIDplus, 2008; [2] Hutchinson et al. 2003; [3] Schulz and Schmoldt, 1997; [4] Valenti et al. 2009; [5] Physician Desk Reference, 2008; [6] Winter et al. 2008; [7] Astra Zeneca, Brixham Environmental Laboratory, Devon, UK, unpublished data; [8] Williams et al. 2007; [9] Foran et al. 2004; [10] Nunnes et al. 2005; [11] Triesbskorn et al. 2007; [12] Dietrich et al. 1999; [13] US EPA 2009b; [14] Zeillinger et al. 2009; [15] Kim et al. 2007; [16] Huggett et al. 2002; [17] Versonnen et al. 2003; [18] Parrott and Blunt 2005

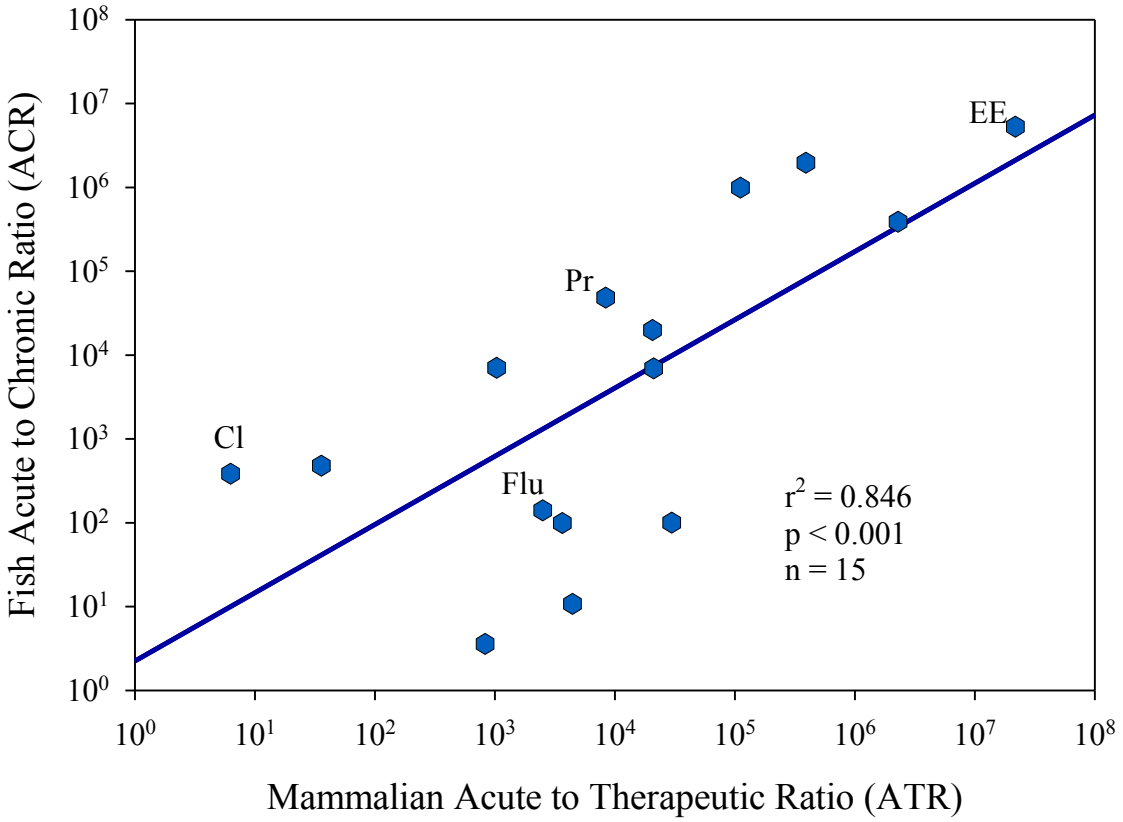


Figure 8**.** A comparison between fish Acute to Chronic Ratios and mammalian Acute to Therapeutic Ratios for human pharmaceuticals (Table 5; n = 15; $p < 0.001$, $r^2 = 0.846$). Select compounds (ethinyl estradiol – EE; fluoxetine – Flu, propranolol – Pr, clofibrate – Cl) are denoted.

Relationship between Fish ACR and Mammalian ATR

A review of available research on the chronic effects of pharmaceuticals in fish resulted in a limited dataset meeting our established criteria with which to develop fish ACRs (Table 5A). The majority of fish studies conducted with pharmaceuticals have focused on acute mortality or sub-chronic responses likely resulting from narcosis MOAs (Sanderson and Thomsen 2009), instead of evaluating endpoints plausibly linked to a therapeutic MOA in mammals (Ankley et al. 2007). For comparative purposes, for those drugs selected for fish ACR development, the corresponding mammalian values for

acute, therapeutic, and ATR are shown in Table 5. For these drugs chemical toxicity distributions were developed for acute and chronic threshold values (Figure 7A) and for the fish ACR values (Figure 7B). Despite the paucity of fish ACR data, a statistically significant relationship was observed between fish ACR and mammalian ATR values ($p < 0.001$, $R^2 = 0.841$; Figure 8), indicating that mammalian ATRs appear predictive of fish ATR values for pharmaceuticals.

Discussion

One of the primary objectives of this study was to evaluate the acute toxicological and pharmacological relationships between fish and mammals exposed to pharmaceuticals. We observed a poorly correlated ($r^2 = 0.042$) relationship between fish and rat acute toxicity for the drugs examined in this study. Such acute toxicity data is important to both pharmaceutical safety assessments and environmental risk assessments. For fish model responses to human pharmaceuticals it has been suggested that the MOA associated with pharmaceutical acute toxicity is likely narcosis, a different MOA than therapeutic MOAs of drugs (Ankley et al. 2005; Sanderson and Thomsen 2007, 2009). Whereas the practice of using rodent models to predict the toxicity of pharmaceuticals to humans and employing model fish responses to predict chemical toxicity to other fish (US EPA 2009a) is relatively well developed, extrapolation between rodent models and fish is less understood. Previous researchers have used similar approaches to those employed in the present study to examine acute toxicity relationships between fish and mammalian models. Janardan et al. (1984) observed a statistically significant relationship between fish and rat acute toxicity for 47 priority pollutants. The low correlation in the present study may have resulted from a number of factors.

Pharmaceuticals include numerous MOAs and even therapeutics within the same class may exhibit different physical-chemical properties, which can influence ionization, partitioning and toxicity to fish (Valenti et al. 2009). Further, different routes of exposure (e.g., gill, dietary) lead to dispositional differences, and genetic, biochemical and physiological variability among fish models influence toxicokinetics, toxicodynamics and organismal sensitivities to toxicants (Rand 1995). Delistraty et al. (1998) examined acute toxicity relationships between rat and fish models for 217 chemicals, noting significant relationships when route of exposure was considered. Quantitative structural activity relationship modeling is likely a predictor of acute fish toxicity (Sanderson and Thomsen 2007, 2009) for most pharmaceuticals, but potential relationships between rat LD_{50} and fish LC_{50} within specific drug classes and MOAs require future study.

We further examined fish and mammalian acute toxicity data with chemical toxicity distributions for rat LD_{50} and fish LC_{50} values and used these distributions to classify acute pharmaceutical toxicity to mammals and fish. The rat acute chemical toxicity distribution (Figure 3) indicated that the majority of pharmaceuticals (62%) are predicted to be slightly toxic to harmless, while very few (8%) are predicted to be highly toxic and none were predicted to be extremely toxic (Table 2). Such predictions are not surprising because drugs are intentionally designed for human consumption. The fish acute toxicity distribution (Figure 3) specifically predicts that very few human pharmaceuticals will be acutely toxic to fish below 10 µg/L, and generally was similar to a distribution for a wide range of environmental contaminants (de Wolf et al. 2005). Probabilistic distributions have also been used in aquatic toxicology to identify threshold values for select pharmaceutical and personal care product classes (Brain et al. 2006;

Dobbins et al. 2009) and endocrine active substances (Dobbins et al. 2008; Gross et al. in press). Dobbins et al. (2008) used a similar probabilistic approach to compare the sensitivities of common in vitro and in vivo models for estrogenic activity. Applying this methodology to the acute toxicity distributions (Figure 3), the 5th centile value (0.84 mg/L, or parts per million) from the fish LC_{50} PPD is markedly lower than the 5th centile value (33.5 mg/kg, or parts per million) from the rat LD_{50} PPD (Table 1). Subsequently, for acute mortality benchmark concentration estimation, fish models are predicted to be 40 fold more sensitive than rat models for 95% of human pharmaceuticals. Similar to such applications of chemical toxicity distributions, the Thresholds of Toxicological Concern concept presents an approach to identify exposure concentrations of chemicals not resulting in significant risk to humans (Kroes et al. 2005, Munro et al. 2008). Although Threshold of Toxicological Concern approaches have primarily been used in human health risk assessment, it can provide an approach to focus inherently limited resources on chemicals exhibiting responses above concentrations determined to represent environmental thresholds or trigger values. Thus, Threshold of Toxicological Concern approaches, such as those presented in the present study, may be useful in regulatory toxicology efforts such as the European Union's Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH; Williams et al. 2009), and may support prioritization efforts for pharmaceutical hazards in the environment (Brooks et al. 2009).

PPDs were employed to evaluate human C_{max} values and mammalian ATRs, a value that may provide a reasonable surrogate for margin of safety or therapeutic index data, which were not as readily available and are not consistently developed for toxicity

thresholds. The C_{max} PPD predicted that only 5% of pharmaceuticals would have C_{max} values lower than or equal to 0.0011 μg/mL, whereas the ATR PPD identified that 5.5% of all pharmaceuticals would have ATR values greater than or equal to 1,000,000 (Figure 4, Table 1). For both the C_{max} and ATR PPDs, values spanned more than 8 orders of magnitude, suggesting that these distributions may be useful for modeling and predicting similar values for pharmaceuticals not examined in the present study. This is informative because an assumption of this probabilistic method is that the distribution of a toxicological or pharmacological property of compounds is representative and predictive of the toxicological or pharmacological properties of all possible pharmaceuticals, including those therapeutics presently on the market and chemicals yet to be developed or distributed (Burmaster and Hull 1997; Kroes et al. 2005; Munro et al. 2008; Dobbins et al. 2008, 2009; Gross et al. in press).

Probabilistic approaches like the PPDs used in this study have also been used to predict thresholds of sublethal responses to chemicals with shared MOAs (Dobbins et al. 2008, Dobbins et al. 2009, Gross et al. in press). In the present study we also developed PPDs to predict potential differences in ATR distributions among 15 classes of pharmaceuticals (Table 4). These PPDs allowed for identification of drug classes that have lesser or greater ATRs (Figures 4 and 5) than the distribution of ATRs for all pharmaceuticals. There was a significant difference in the ATR response among classes; for example, 50^{th} centile (Table 4) values spanned 5 orders of magnitude. As might be expected from previous fish toxicity studies and an understanding reproductive hormone potencies (e.g., C_{max}), these ATR values exhibited the greatest statistically significant difference ($p<0.001$; Figure 6) from the ATR values for all drugs. Other classes,

including the corticosteroids ($p<0.05$), calcium channel blockers ($p=0.037$), and antihistamines ($p=0.007$) ATRs were also significantly greater than ATR values for all drugs included in the present study. Future examination of values at specific centiles can allow for predictions of the magnitude of ATR and other pharmacological and toxicological responses expected among compounds with specific MOAs.

Although a number of researchers (Kenaga 1982; Roex et al. 2000; Ahlers et al. 2006; Raimondo et al. 2007) identified that for >90% of chemicals and aquatic organisms an ACR of 100 is protective of adverse chronic responses, compounds outside of this range generally elicit chronic responses through chemical specific MOAs rather than narcosis (Rand 1995; Ahlers et al. 2006). Sanderson and Thomsen (2009) agreed that an ACR of 100 may be adequate for estimating the chronic aquatic responses to pharmaceuticals because the majority (~70%) of drugs apparently exert acute toxicity through narcosis. However, the largest portion of chronic responses used by Sanderson and Thomsen (2009) were algal and *Daphnia* models, where a non specific, narcosis MOA could reasonably be expected based on the relatively higher evolutionary conservation of drug targets among mammals and aquatic vertebrates (Huggett et al. 2003, Ankley et al. 2007, Brooks et al. 2008, Gunnarrson et al. 2008) than plants, algae and invertebrates, with antibiotic effects to plants and algae as an important exception (Brain et al. 2008). Subsequently, Sanderson and Thomsen (2009) only included one fish chronic response to an antibiotic when examining ACRs for 23 pharmaceuticals. Based on the probabilistic distribution of fish ACRs examined in this study (Figure 7), an ACR threshold value of 100 corresponds to the 20^{th} centile, highlighting that default ACR

values derived for other industrial chemicals may not be protective when therapeutic MOA related responses in fish are used to calculate an ACR.

Ahlers et al. (2006) noted that the MOA of a chemical may change with exposure concentration, where at acutely toxic exposure levels aquatic mortality may result from a narcosis MOA, while lower exposure levels may result in responses mediated by interaction with a specific target (e.g., receptor, enzyme). Such an observation is particularly relevant for pharmaceutical effects in fish models. For example, very high fish ACRs have been identified for reproductive hormones such as 17α-ethinyl estradiol (Caldwell et al. 2008) and synthetic gestins (Zellinger et al. 2009) when the chronic reproduction responses evaluated were mechanistically linked to pharmaceutical MOAs (Ankley et al. 2007). In standardized aquatic testing approaches, short term (e.g., 7 d) sub-chronic growth responses in larval fish is a common endpoint, though this response may not result from a therapeutic MOA in fish models (Brooks et al. 2003; Ankley et al. 2007). For example, if 7 d growth responses in fathead minnow models were used to derive ACRs for a reproductive hormone instead of adverse reproduction thresholds, the magnitude of difference between ACRs calculated for the same compound can vary by 5 orders of magnitude (Brooks et al. 2008). Stanley et al. (2007) and Valenti et al. (2009) reported lower EC_{10} values for feeding behavior of fish, a response related to the mammalian therapeutic MOA of the antidepressants fluoxetine and sertraline, respectively, than EC_{10} values for growth. Further, enantiomer specific differences were also previously identified with the fluoxetine enantiomer known to be more potent at the therapeutic target (serotonin reuptake transporter) exerting greater toxicity (e.g., lower EC_{10} values) on juvenile fish behavior than growth (Stanley et al. 2007).

To examine the potential utility of using the mammalian ATRs to predict pharmaceuticals that may result in relatively high ACRs in fish models, we identified a statistically significant relationship between mammalian ATRs and fish ACRs ($p < 0.001$, $R^2 = 0.846$; Figure 8). It is important to note that in the present study we only included chronic responses of fish to pharmaceuticals that appear to have been elicited through a therapeutic MOA for calculating ACRs and for statistical analysis of the relationship with mammalian ATRs (Figure 8, Table 5). Using this approach mammalian ATR values appear useful for predicting pharmaceuticals with higher fish ACRs if the chronic response used in ACR calculation is plausibly linked to the therapeutic MOA of a pharmaceutical (Ankley et al. 2007). Environmental hazards of pharmaceutical classes with larger ATR values warrant further examination. Huggett et al. (2003) proposed a pharmacokinetic-based screening approach for pharmaceutical effects in fish, which used physiologically based pharmacokinetic (PBPK) equations for non-polar organics to predict plasma concentrations in fish then related these predictions of mammalian therapeutic dose (e.g., C_{max}). Unfortunately, appropriate PBPK models have not been developed for fish models exposed aqueously to pharmaceuticals, which are often ionizable chemicals. Future studies are specifically needed to determine whether the screening approach presented in the present study, when coupled with pharmacokinetic (Huggett et al. 2003) and bioinformatic (Gunnarsson et al. 2008) approaches, is useful in identifying whether pharmaceuticals with larger mammalian ATR values exert potential environmental hazards to fish, particularly if therapeutic targets are present, and their functional interactions with a drug and associated physiological outcomes are understood.

CHAPTER THREE

Effects of the Antihistamine Diphenhydramine on Selected Aquatic Organisms

This chapter published as: Berninger JP, Du B, Connors KA, Eytcheson SA, Prosser KN, Valenti TW, Chambliss CK, and Brooks BW. 2011. Effects of the Antihistamine Diphenhydramine to Selected Aquatic Organisms. Environ. Toxicol.Chem.
Published Online: 6 June 2011

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 (Brooks et al. 2006). 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 (Ramirez et al. 2009). 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. Though 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 (Ankley et al. 2007; Brooks et al. 2009) 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 (Ankley et al. 2007; Brooks et al. 2009). Only a handful of drug classes are fairly well characterized, such as hormones, analgesics, antidepressants, beta blockers, and antibiotics (Berninger and Brooks 2010). 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 (Ankley et al. 2007; Berninger and Brooks 2010), 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 (Stackelberg et al. 2004; Ferrer et al. 2004). In the sediment, DPH concentrations were much higher (20-50 μg/kg) (Ferrer et al. 2004); two and three orders of magnitude higher than associated water concentrations. Perhaps most importantly, DPH has been found in the tissues of fish. Ramirez et al. (2007) 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, an 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 (Ramirez et al. 2009). 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 (Zhou et al. 2008). 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 (Au-Yeung et al. 2006; Knox et al. 2011).

The quantification of DPH in surface waters may be partially explained because it is fairly stable in the environment (Beijersbergen van Henegouwen et al. 1987), although like many drugs, it is subject to photodegradation (Boreen et al. 2003). In general, antihistamines, and likely DPH, are removed poorly through most wastewater processes (Kosonen and Kronberg 2009). 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 (Brooks et al. 2006). An additional influx of DPH may come from the sewage treatment process where polar metabolites (e.g., diphenhydramine N-glucuronide (Knox et al. 2011)) are cleaved back to the parent compound, although this has not been directly studied (Heberer 2002). 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 6) 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 (Daughton and Brooks 2011).

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 (Ankley et al. 2007; Berninger and Brooks 2010), rather than nonspecific narcosis responses typically seen with industrial chemicals (van Wezel and Opperhuizen 1995). Understanding mammalian pharmacological properties may help predict potential effects in non-target species based on the conservation of critical drug receptors (Gunnarsson et al. 2008). Diphenhydramine is a first generation antihistamine drug found in many common over-the-counter formulations (Table 6), and crosses the blood-brain barrier (Au-Yeung et al. 2006). 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 6 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 (Brown et al. 2001). 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. DPH also targets the 5-HT reuptake transporter (SERT), preventing the re-uptake of serotonin at the presynaptic nerve cleft (Wong et al. 2005). 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 re-uptake inhibitor (SSRI) antidepressant, which exerts its therapeutic effect through the same mechanism, albeit with much greater specificity (Wong et al. 2005). Interestingly, DPH acts as an anticholinergic agent by competitively antagonizing the acetylcholine receptor (Brown et al. 2001). This reaction reduces the signal sent by the acetylcholine neurotransmitter, and as such has been suggested as remedy for organophosphate poisoning (Bird et al. 2002) and in alleviating the symptoms of Parkinson's disease (Brown et al. 2001).

Unfortunately, the consequences of DPH exposure are poorly understood in non-target organisms. This data gap is especially disconcerting for aquatic species as many may be exposed to DPH via multiple routes. Thus, the objective of this study was to develop a baseline aquatic ecotoxicological understanding of DPH 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 (Ankley et al. 2007; Brooks et al. 2009; Berninger and Brooks 2010; Huggett et al. 2003).

Materials and Methods

Experimental Conditions

The following experimental conditions described apply to all studies except where noted within individual methods. Reconstituted hard water, formulated according to U.S. EPA methods (US EPA 2002a), was used as control and dilution water for invertebrate and fish studies. All experiments were carried out in controlled environmental chambers

at 25± 1°C under a 16:8 h light-dark regime. Water quality was monitored according to standard methods (APHA 1998). Water quality parameters were measured daily and mean (± standard deviation; SD) values were well within acceptability criteria (US EPA 2002a; US EPA 2002b; OECD 2008): dissolved oxygen, 8.3 (±0.2) mg/L (YSI Model 55, Yellow Springs, OH, USA); conductivity, 580 (±4.6) µS /cm (YSI Model 30, Yellow Springs); 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, MA, USA) and recorded separately for each test conducted. There is potential for shifts in the ionization state of DPH (pK_a 8.9; Table 6) resulting from slight differences in pH, which could influence toxicological responses (Valenti et al. 2009). 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 semi-arid regions (Brooks et al. 2006).

Diphenhydramine hydrochloride (Chemical Abstracts Service No.147-24-0) was obtained from Sigma-Aldrich (MO, USA). Concentrations used in preliminary range finding testing were developed from U.S. EPA EPISuite software (US EPA 2009) (96h *P. promelas* median lethal concentration (LC_{50}) = 13.7 mg/L; 48h Daphnid LC_{50}= 1.2 mg/L), then adjusted based on preliminary results (not reported). All DPH concentrations were analytically verified following methods later described.

Pimephales promelas

Standardized acute studies. Standardized fathead minnow (*Pimephales promelas*) acute studies were conducted according to U. S. EPA acute toxicity protocols (US EPA

2002a) with slight modifications (Valenti et al. 2009; Stanley et al. 2007). 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 (8L) 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 (8 total) higher concentrations to establish the LC_{50}. At each treatment level and control, four replicates of 600 ml glass beakers were loaded with 10 larval *P. promelas* (<24h 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 48h test period. Survival was assessed at 24 and 48h. 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 7d sub-chronic study was conducted following slightly modified U.S. EPA protocols (OECD 2008; Valenti et al. 2009; Stanley et al. 2007). 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 6) and known ACR values (Equation 1) Berninger and Brooks 2010),

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

and predictions of plasma concentrations in fish (Huggett et al. 2003; Fitzsimmons et al. 2001). Specifically, Fitzsimmons et al. (2001) 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 (Huggett et al. 2003). Here we modified another Fitzsimmons et al. (2001) equation (Equation 2), which is more appropriate for drugs with apparent log *P* values less than 3 (Daughton and Brooks 2011), and substituted log *D* (Scherrer and Howard 1977) at the study pH (8.5) for log *P* (Equation 3).

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

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

We then conceptually applied the plasma model approach recommended by Huggett et al (2003), where the fish plasma concentration (FPC) is determined by multiplying the aqueous concentration (Aq) of a drug by its P_{BW} (Equation 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 *Aq*ueous concentration at the *E*ffect *T*hreshold (AqET) (Equation 5) (Fick et al. 2010), and to derive Equation 6, which predicts the concentration of DPH in water that would be necessary to result in plasma accumulation equal to a human C_{max} value:

$$\text{FPC} = P_{BW} \bullet \text{Aq} \quad (4)$$

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

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

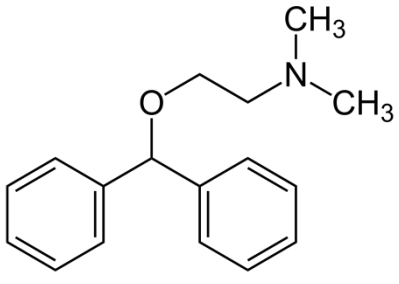
Consistent with the acute studies, experimental units were 600 ml beakers filled with 500 ml of test solution and loaded with 10 <24h old *P. promelas*. This was a static renewal experiment with feeding of brine shrimp nauplii twice daily. The test solution was renewed daily 2h after the morning feeding with 80 to 85% renewal (US EPA 2002b). Stock solutions for each exposure concentration were made fresh daily and analytically verified. Tests were monitored daily for survival. At the completion of the 7d study, 3 fish from each replicate were randomly selected for a feeding trial (see discussion below). The remaining seven fish were euthanized according to standard methods (US EPA 2002b) and placed in aluminum weigh pans. Weigh pans with fish were then placed into an 80°C drying oven for 48h. Pans and fish were allowed to come to room temperature in a desiccation chamber for 1h. Fish were then weighed on a Mettler Toledo Model MX5 microbalance (OH, USA).

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. (2007) with the modifications suggested by Valenti et al. (2009). 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.

Table 6. 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		Pharmacokinetics	
CAS # DPH-HCl DPH	147-24-0 58-73-1	Common adult dosage	25-50 mg – 400 mg/day
Formula:	$C_{17}H_{21}NO$	Bioavailability	43 – 72%
Molecular weight:	255.36 g/mol	Protein binding	86 – 99%
IUPAC Name:	[2-(diphenylmethoxy) ethyl] dimethylamine	Peak plasma concentration (T_{max})	<1.5 – 4h
Solubility	3.06 mg/ml	Plasma half life	3 – 9h
Log *P*	3.27	Metabolism	Extensive Hepatic Metabolism; CYP2D6
Log *D* – pH 6.5 [35] Log *D* – pH 8.5 [35]	0.78 2.66	Excretion	2 -15% parent compound unchanged
pK_a	8.9	Volume of distribution	3.3 – 14.6 L/kg

Pharmacodynamics		DPH Structure
Mammalian acute toxicity (Rat oral median lethal dose [LD_{50}])	390 mg/kg	
Human therapeutic dose – peak plasma concentration (C_{max})	0.05 µg/ml	
Mammalian acute to therapeutic ratio (ATR) [9]	7800	
ATR predicted acute to chronic ratio (ACR) in fish [9]	2091	

Daphnia magna

Acute study. A 48h static acute study for *Daphnia magna* was conducted according to established U.S. EPA protocols (2002a). It was conducted at a single pH, 8.59 (±0.05). Four replicates were used for each of six concentrations and a control. Each replicate was loaded with five *D. magna*. All *D. magna* used were <24h old and hatched within a single 4h 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 10d *D. magna* subchronic toxicity test was performed following standard protocols (US EPA 1994) with slight modifications (Dzialowski et al. 2006; Stanley et al. 2006). The endpoints assessed were immobilization (mortality) and reproduction (young per female). *Daphnia magna* used to initiate the study were <24h old and hatched within a 4h period. Eight concentrations and a control were used in the study. The experiment was static renewal with daily renewal. To ensure consistency in renewal concentrations a 4L 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 (US EPA 2002a; Hemming et al. 2002). Neonates were counted and removed daily during renewals.

Lemna gibba

Diphenhydramine toxicity to a model aquatic plant was assessed by exposing *Lemna gibba* (duckweed) to five concentrations (0.63, 1.25, 2.5, 5, 10 mg/L DPH,

nominal) and a control and measuring effects on frond number, wet weight, and growth rate after 7d. *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 (2007). Prior to experimentation, plants were acclimatized to test media (Hunter's media) for one week before the study was initiated. The seven day static renewal experiments were conducted according to the standardized protocol outlined in Brain and Solomon (2007). 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 seven. The number of doubling events (n) (Equation 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 (Brain and Solomon 2007).

Analytical Methodology

Exposure concentrations of DPH were verified in each stock solution and all experiments via 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 (5µm, 80 Å) Extend-C18 analytical column (Agilent Technologies, California, USA) and 12.5 mm x 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 ºC; flow rate, 350 µl/min. Analytes were ionized via 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 LC_{50} 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 (US EPA 2002a). The LC_{50} 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 (US EPA 2002b; US EPA 1994).

Results

Analytical Confirmation of DPH Concentrations

Table 7 provides analytical verified concentrations of DPH for each treatment level of the acute and sub-chronic experiments with the various model organisms. For acute studies (Table 7) concentration reported are mean (n = 3; ±SD) values from triplicate studies.

Table 7. Analytically verified mean (± standard deviation) diphenhydramine concentrations for acute and sub-chronic studies (µg/L). For acute studies samples were taken from each replicate (n = 3). For the sub-chronic studies multiple samples were taken for *D. magna* (n = 3) and *P. promelas* (n = 7). No DPH was detected in any control samples (not shown).

Daphnia magna		*Pimephales promelas*	
Acute	Sub-Chronic	Acute	Sub-Chronic
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)	836.7 (±103)

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 8). The mean LC_{50} 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 (Figure 9). Sub-chronic exposure survival

was 100% except at the highest concentration tested in this study. The LOEC for growth and behavioral (feeding) responses were measured at much lower concentrations: 49.1 and 5.6 µg/L for growth and behavioral endpoints, respectively (Table 8). Acute to chronic ratios for growth and behavior endpoints were calculated at 85 and 746, respectively (Table 8).

Daphnia magna

Control survival was >95% for both acute and chronic experiments. Acute tests showed dose-dependent responses with a mean (n = 3) LC_{50} of 0.37 (±0.14) mg/L. The 10d studies also exhibited a dose-dependent pattern. Survival in the control and lower concentrations was 100% through the 10d 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 (Figure 10, Table 8). The corresponding ACR value for *D. magna* was 467.5 (Table 8).

Lemna gibba

No statistically significant (p > 0.05) effects of DPH on *L. gibba* responses were observed (Table 8). 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).

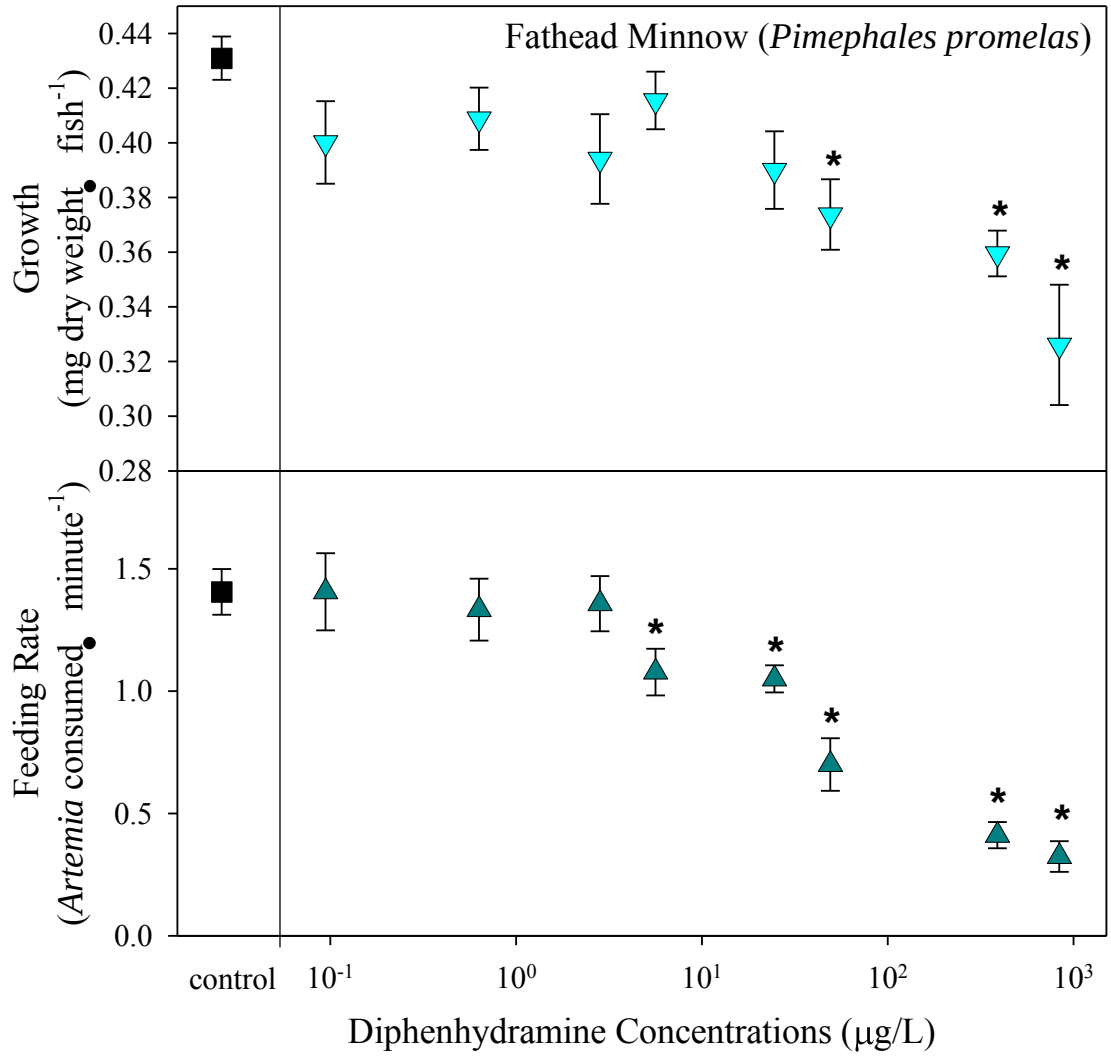


Figure 9. Mean (± standard error) growth (mg dry wt per fish) and behavioral responses (Artemia consumed per min) of larval fathead minnows (Pimephales promelas) following 7 d diphenhydramine study. * = significantly different from control (p=0.05).

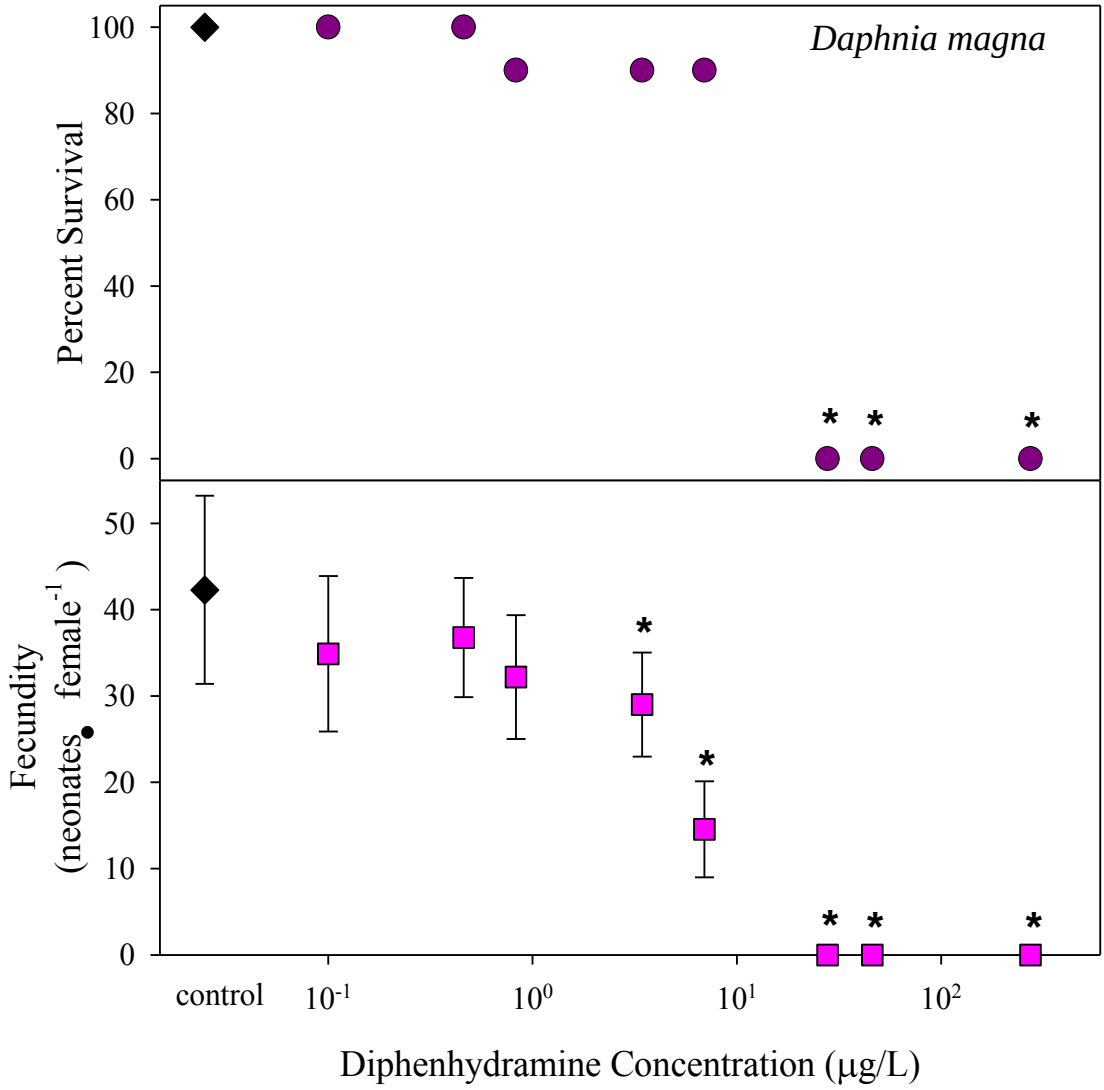


Figure 10. Percent survival and mean (± standard deviation) *Daphnia magna* fecundity (neonate per female) following 10 d diphenhydramine study. * = significantly different from control (p=0.05).

Table 8. Toxicological thresholds of mean acute (n = 3; ± standard deviation) and sub-chronic endpoints of select organisms exposed to diphenhydramine and associated acute to chronic ratios (ACR).

Species	Mean 48 h LC_{50} (mg/L)	Sub-chronic endpoints			
		Type	LOEC (µg/L)	NOEC (µg/L)	ACR
Pimephales promelas	pH 6.5: 59.28 (±6.6)	Survival	836.7	388.3	5.4
		Growth	49.1	24.5	85.3
	pH 8.5: 2.09 (±0.405)	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#)	---	>10750	--
		Growth (wet weight)	---	>10750	--
		Growth (growth rate)	---	>10750	--

LC_{50} – median lethal concentration, LOEC – lowest observed effect concentration, NOEC – No observed effect concentration, ACR – acute to chronic ratio.

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) (Ferrer et al. 2004; Ramirez et al. 2007). 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 anti-inflamatory drugs, SSRIs lipid lowering agents, beta-blockers) (Brain et al. 2008), 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 (Gunnarsson et al. 2008). However, significant

acute and subchronic effects of DPH were observed to a model fish and an invertebrate (Table 8).

A second objective of this study was to employ approaches previously proposed (Ankley et al. 2007; Berninger and Brooks 2010; Huggett et al. 2003) 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 similarity is reported to vary between 40 to 70% (Gunnarsson et al. 2008). When observations of the present study are compared to similar studies with the SSRIs fluoxetine (Stanley et al. 2007) and sertraline (Valenti et al. 2009), 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 8) than comparable mortality thresholds for sertraline (Valenti et al. 2009) and fluoxetine (Stanley et al. 2007). Similar to observations previously reported for sertraline (Valenti et al. 2009) and fluoxetine (Nakamura et al. 2008), this study demonstrated that pH is a critically important factor influencing aquatic toxicity of ionizable weak bases, because a 28 fold higher DPH LC_{50} value was observed for *P. promelas* at pH 6.5 than pH 8.5 (Table 8).

In the present study, the standardized growth endpoint in the *P. promelas* model was not the most sensitive fish response to DPH (Figure 9, Table 8); 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 (Figure 1, Table 8). Feeding behavior was examined here and in previous studies

with the SSRIs sertraline (Valenti et al. 2009) and fluoxetine (Stanley et al. 2007) 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. (2007) 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 (Ankley et al. 2007; Brooks et al. 2009; Berninger and Brooks 2010; Huggett et al. 2003).

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 (Stanley et al. 2007; Minagh et al. 2009; Brooks et al. 2003; Oakes et al. 2010). The only other study available on the aquatic toxicology of DPH found similar results in *D. magna* (Meinertz et al. 2010). Meinertz et al. (2010) recently evaluated effects of DPH on *D. magna* over 21 d, but only at employed three widely separated concentrations, resulting in a NOEC of 0.12 µg/L and LOEC of 70 µg/L. Subsequently, Meinertz et al. (2010) 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, though we detected reproductive effects at an order of magnitude lower concentration than a survival NOEC of 27.8 µg/L (Table 8).

One interesting observation in the Meinertz et al. (2010) study was 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 8) and was observed previously for sertraline (Valenti et al. 2009). That study reported a pH range between 7.2 and 7.6 (Meinertz et al. 2010), 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 (Valenti et al. 2009). In this study, at a pH closer to the pK_a value, DPH was more unionized and more toxic to *D. magna* than in the Meinertz et al. study. Thus, based on the information from the present study and others (Valenti et al. 2009; Nakamura et al. 2008), 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 8) compared to SSRIs are likely related to other MOAs of DPH and conservation of relevant targets in invertebrates. Though 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, organo-phosphate (OP) pesticides are much more effective in invertebrates. Whereas OPs target acetylcholineesterase, DPH and other anti-acetylcholinergics (e.g., atropine) bind to the ACh receptor preventing ACh neurotransmission (Carvalho et al. 2003). 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 (Brooks et al. 2006), 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 (Haas et al. 2008). It is important to note that DPH is not even the most potent antihistamine. For example, Berninger and Brooks (2010) 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 (Orzechowski et al. 2005). Clearly these findings deserve additional study.

When we selected treatment levels for the subchronic fish study, an ACR value of 2100 was predicted for DPH, based on mammalian margin of safety information presented in Equation 1 (Berninger and Brooks 2010). Based on results from the *P. promelas* feeding behavior study an ACR value of 746 was calculated (Table 8); an order of magnitude higher than previously reported feeding behavior ACR values for sertraline (ACR = ~ 15) (Valenti et al. 2009) and fluoxetine (ACR = 22) (Stanley et al. 2007). Though 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 (Raimondo et al. 2007). 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

non-target organisms (Ankley et al. 2007; Brooks et al. 2009; Berninger and Brooks 2010; Gunnarsson et al. 2008; Huggett et al. 2003). Further, we also employed a plasma model approach modified from that presented by Huggett et al. (2003) and advanced by Fick et al. (2010). We employed a partitioning equation (Equation 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 8) log *D* was substituted at the study pH (8.5) for log *P* using Equation 3. Then, using Equation 6, 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 (Ankley et al. 2007; Brooks et al. 2009; Berninger and Brooks 2010). 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 (Stanley et al. 2007) and sertraline (Valenti et al. 2009), 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 (Ankley et al. 2007; Brooks et al. 2009). 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 (Ankley et al. 2007; Brooks et al. 2009; Berninger and Brooks 2010).

CHAPTER FOUR

An Initial Probabilistic Hazard Assessment of Oil Dispersants Approved by the United States National Contingency Plan

This chapter published as: Berninger JP, Williams ES and Brooks BW. 2011. An initial probabilistic hazard assessment of oil dispersants approved by the United States National Contingency Plan. Environ. Toxicol.Chem. 30: 1704 – 1708

Introduction

In response to oil spills, such as the recent event in the Gulf of Mexico, several classes of chemical agents are employed for environmental mitigation and cleanup (Chapman et al., 2007). These include dispersants, surface cleaning agents, bioremediation agents, and miscellaneous oil spill control agents. The National Oil and Hazardous Substances Pollution Contingency Plan (NCP) Subpart J (US EPA 2010) establishes a list of products that are authorized for use in oil spill cleanup in the United States (http://www.epa.gov/osweroe1/content/ncp/product_schedule.htm). Inclusion on the NCP Product Schedule requires the submission of aquatic toxicology information in accordance with requirements set forth in the Code of Federal Regulations (US EPA 2010; 1997). Specifically, acute toxicity estimates (LC_{50}s) must be determined for the dispersant alone and in a 1:10 mixture with #2 fuel oil (#2FO) in two marine species: *Menidia beryllina* (Inland Silverside) and *Mysidopsis bahia* (Opossum Shrimp). Additionally, NCP Subpart J requires LC_{50} values for #2 Fuel Oil, and reagent grade dodecyl sodium sulfate (DSS) as a reference toxicant (although other reference toxicants have been accepted). Whereas the testing methodologies are well established (US EPA

2002a), toxicity or variability parameters for addition to the NCP-PS are not explicitly stated in the regulations (US EPA 1997). Currently, the product schedule lists only 14 dispersants as having met the requirements of Subsection J of the NCP (US EPA 2010) (http://www.epa.gov/osweroe1/docs/oil/ncp/notebook.pdf).

Establishing the comparative toxicity of oil dispersants is challenging. Standardized toxicity information is generally only available for those compounds that are listed on the NCP-PS. For compounds failing to receive regulatory approval, or those that are later delisted, associated toxicity data are not made publicly available. Further, the chemical components of specific dispersants are largely proprietary with limited or no additional aquatic fate and acute or chronic effects information (Judson et al. 2010), which challenges any comparative understanding of ecological and environmental health implications. As the purpose of a dispersant is to facilitate the acceleration of natural attenuation and dilution of spilled oil (Swannell and Daniel 1999), the aquatic toxicity of the dispersant:oil mixture is also an important consideration. This further complicates a comparative toxicity evaluation, as the course of toxicity in mixture may be unknown and potentially different for each dispersant. For example, Ramachandran et al. (2004) suggested that the increased toxicity of a Corexit 9500 oil mixture may be due to an increase in the availability of polycyclic aromatic hydrocarbons (PAHs), rather than being directly toxic. Although the presence of PAHs does not represent the only factor in determining oil toxicity (Barron 1999), there is evidence linking the increased presence of PAHs in chemically dispersed oils to increased toxicity to aquatic organisms (Carls et al. 2008). The combination of these factors makes direct comparative toxicity, and therefore risk-based decision making, difficult. It may be that probabilistic hazard

assessment techniques, specifically chemical toxicity distributions (CTDs) provide a useful alternative approach to comparative toxicity of dispersants.

Chemical toxicity distributions provide a way to utilize existing data to estimate the probability of response (in this case, an LC_{50}). This probability may be associated with either fixed centiles (e.g., the 5^{th} centile, analogous the hazardous concentration (HC5) used in a species sensitivity distribution (Solomon et al. 2000)) or a designated concentration or benchmark. Chemical toxicity distributions are useful in comparative toxicity because they allow for comparison across different types of groups, such as a chemical class or a specific mode of action (MOA) category. The CTD approach has been previously employed to examine various chemical classes (antimicrobial agents (Dobbins et al. 2009), pharmaceuticals (Brain et al. 2006; Berninger and Brooks 2010), surfactants (Williams et al. 2011)) and common modes of action (estrogen agonists (Dobbins et al. 2008), Verhaar MOA categories (de Wolfe et al. 2004), acetylcholinesterase activity (Williams et al. 2011), and human therapeutic MOAs (Berninger and Brooks 2010)). In this case, dispersants, while differing in components and toxic potential, can be placed in a group because they all share a common environmental application: the dispersion of spilled oil. Dispersants are by nature amphiphatic and comprised of three principal components: surfactants, solvents, and additives (Clayton et al. 1992). The mixture of surfactants (ionic and nonionic) in currently approved dispersants exhibit a hydrophilic-lipophillic balance (HLB) between 9 to11 (NRC 2005). The similarity of components and dispersive mechanisms make dispersants a viable group for the application of CTDs.

The purpose of the present study was to perform a novel probabilistic hazard assessment with CTDs to examine the relative acute aquatic toxicity of oil dispersants, alone and in combination with oil. We further explored the utility of CTDs as a potential tool for decision-making in oil spills, evaluation of data for NCP listing, and prioritization of prospective and retrospective environmental management efforts.

Methods

The use of the single standardized endpoint for a common in vitro or in vivo model is one of the underlying assumptions of a CTD. For this initial probabilistic hazard assessment, datasets were sought based on two critical components: that the datasets use a standardized method for assessing a single endpoint (e.g., acute toxicity LC_{50}s); that the datasets are used in decision making. Toxicity data from the NCP-PS on the 14 currently listed dispersants provided one such dataset. As noted above, a series of acute toxicity tests on two marine species, *M. beryllina* and *M. bahia,* must be conducted as part of NCP-PS listing. Tests must include determination of acute LC_{50} for the dispersant, #2 fuel oil (#2FO), a 1:10 mixture of dispersant and #2FO, and dodecyl sodium sulfate (DSS) as a reference toxicant (summary information provided in Supplemental Data Table S1) following protocols listed in the NCP subsection J appendix C (US EPA 1997).

An additional dataset of eight dispersants (a subset of the 14 NCP-PS dispersants), reevaluated in the wake of the Deepwater Horizon oil spill, recently became available through the U.S. EPA. The dataset contains acute toxicity (LC_{50}) information for *Menidia beryllina* and *Mysidopsis bahia* on the dispersants alone (Hemmer et al. 2010a) and in mixture with Louisiana Sweet Crude Oil (LSC) (Hemmer et al. 2010b), in

addition to LSC alone and DSS. Whereas both datasets evaluate similar endpoints, testing methodologies were very different and as such the datasets will be treated separately here. For the purposes of the present study, hereafter the dataset provided as part of the National Contingency Plan Product Schedule will be referred to as the NCP-PS dataset and the 8 dispersants dataset retested after the Deepwater Horizon Oil Spill by Hemmer et al. (2010a, b) will be referred to as the DHOS dataset.

Chemical toxicity distributions for oil dispersants were developed based on the methodologies outlined by Solomon and Takacs (2002) and modified by Brain et al. (2006) and described further elsewhere (Solomon et al. 2000; Dobbins et al. 2009; Berninger and Brooks 2010). Specifically, CTDs were developed from acute LC_{50} values for dispersants alone and dispersant:oil mixtures (#2FO or LSC) for both datasets. The toxicity of #2FO and LSC were used as benchmarks in evaluating the CTDs.

As the individual test data in the NCP-PS was compiled by U.S. EPA from multiple sources, variability exists among LC_{50} values for the reference toxicants: #2FO and DSS. To characterize this variability, a number of different descriptive metrics were calculated, including the coefficient of variation (standard deviation / mean) and the range factor (maximum value / minimum value) (Supplemental Data, Table S2, Table S1). Some level of inter-laboratory variability is acceptable in toxicity testing. For example, the U.S. EPA whole effluent toxicity guidelines (US EPA 2001) for reference toxicants suggest that a coefficient of variation of 31.2% for *Mysidopsis* and 38.5% in *Menidia* and an earlier U.S. EPA document suggests a range factor of 3.5 for marine test species (US EPA 1981). Based on our analysis, the variability of both #2FO and DSS for both species fell well outside these ranges. Outlier analysis ($p<0.05$; Grubb's Outlier

Analysis, GraphPad Software, CA, USA) was performed, identifying three outlier values: *Menidia*: 201.8 and 100 mg/L; *Mysidopsis*: 72.7 mg/L. Removal of the outlier values greatly reduced the variability of #2FO (Supplemental Data, Table S2). Therefore, the benchmark value for #2FO will be based on values calculated after outlier removal.

For both datasets, the oil benchmark was the measure of central tendency of the LC_{50} of the oil specified in that dataset (LSC or #2FO). In the DHOS dataset, a single LC_{50} value (and 95% confidence interval) was reported (as total petroleum hydrocarbons) for both species: *Menidia* - 3.5 mg/L (3.4-3.7); *Mysidopsis* 2.7 mg/L (2.5-3.0). As a benchmark for LSC, only the mean values were used. For the NCP-PS dataset separate LC_{50} values were reported for each dispersant. The geometric mean of #2FO LC_{50} values (after removal of the outliers) was used as the benchmark value. As the data were lognormally distributed, the geometric mean was used as the best measure of central tendency; it also provided a more conservative benchmark value in comparison to the arithmetic mean. (Solomon and Takacs 2002). The #2FO benchmarks were: *Menidia* 12.0 mg/L; *Mysidopsis* 6.9 mg/L.

To evaluate and compare the CTDs for the acute toxicity of the dispersants and dispersant:oil mixtures, various parameters were calculated. The centile associated with the oil benchmark for each CTD was calculated to provide a measure of comparative aquatic toxicity of dispersant and dispersant:oil mixtures. The LC_{50} estimates at specific centiles (1^{st}, 5^{th}, 10^{th}, and 25^{th}) were also developed. Lastly, the probabilities of LC_{50} values being within established toxicity categories, ranging from practically non-toxic (LC_{50} >100 mg/L) to very highly toxic (LC_{50} < 0.1 mg/L), were calculated for each CTD (http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm#Ecotox).

Results and Discussion

To justify the use of dispersants in the environment, ideally they should exhibit low toxicity (slightly toxic or practically non-toxic on the U.S. EPA aquatic toxicity scale) and not generate markedly greater toxicity in combination with oil. This consideration is critical because oils in combination with dispersants may become more toxic, through a variety of mechanisms (Judson et al. 2010; Swannell and Daniel 1999; Ramaxhandran et al, 2004; Barron 1999; Carls et al. 2007; Middaugh and Whiting 1995). Evaluating the difference in effects between the dispersant alone, the oil alone, and the two in combination can make comparative toxicity between individual dispersants difficult. The application of the CTD approach simplifies the evaluation, as examined here considering only the resultant acute toxicity (LC_{50}).

Eight CTDs were developed (Figure 11) based on the two datasets, two species, and two dispersant conditions (alone and in mixture with oil). The acute toxicity data was well characterized by CTD regressions, with r^2 values ranging from 0.80 to 0.97 (Table 9). Individual CTDs were utilized to develop probabilistic parameters: centile at the benchmark, LC_{50} at specific centiles, and probability of LC_{50} being within a certain aquatic toxicity category (Table 9). The NCP-PS (Figure 11 A-B) and DHOS (Figure 11 C-D) datasets exhibited similar, but not identical, trends in their distributions. For each CTD, distinct differences were observed between the distributions of dispersants alone and dispersant-oil mixtures, with increased toxicity of the mixture generally identified (Figure 11). The shrimp model (*Mysidopsis*), in comparison to the fish model (*Menidia*), was more sensitive to oil, but less sensitive to the dispersants alone. Fish and shrimp models did exhibit similarity in terms of their dispersant:oil toxicity distributions (NCP-

PS and DHOS datasets evaluated separately), considering both the toxicity estimates at specific centiles and the likelihood of LC_{50}s occurring within an aquatic toxicity category (Table 9). Comparing the dispersants alone to the dispersant:oil mixture, there was a general shift away from the practically nontoxic towards moderate to highly toxic categories, a trend that held for both datasets and both species. Another important similarity, seen in both datasets and both species, was that the distributions of the dispersant-oil mixtures crossed their specified oil benchmark in all cases at or above the 50^{th} centile (range 50.1- 54.9; Figure 11). This observation suggests that more than 50% of the time the dispersant:oil mixture is more toxic than the estimated toxicity of the oil alone.

Based on the methods used to generate these datasets, it is unclear what might be causing the changes in toxicity between dispersant alone and dispersant:oil mixtures, but dilution, antagonism, additivity, or perhaps potentiation are potential possibilities. Based on our observations with CTDs, it would seem that response to the mixture was likely not a simple additive model. If this were the case, the mixture CTDs would resemble the oil benchmark, with the generally lower toxicity of dispersant alone contributing little in a 1:10 mixture. Thus, the data modeled by CTDs in the present study were generally consistent with previous reports, which suggested that it may not be the direct toxicity of the dispersant, but rather the dispersants action on the oil that may change the toxicity of oil constituents (Judson et al. 2010; Swannell and Daniel 1999; Ramaxhandran et al, 2004; Barron 1999; Carls et al. 2007; Middaugh and Whiting 1995).

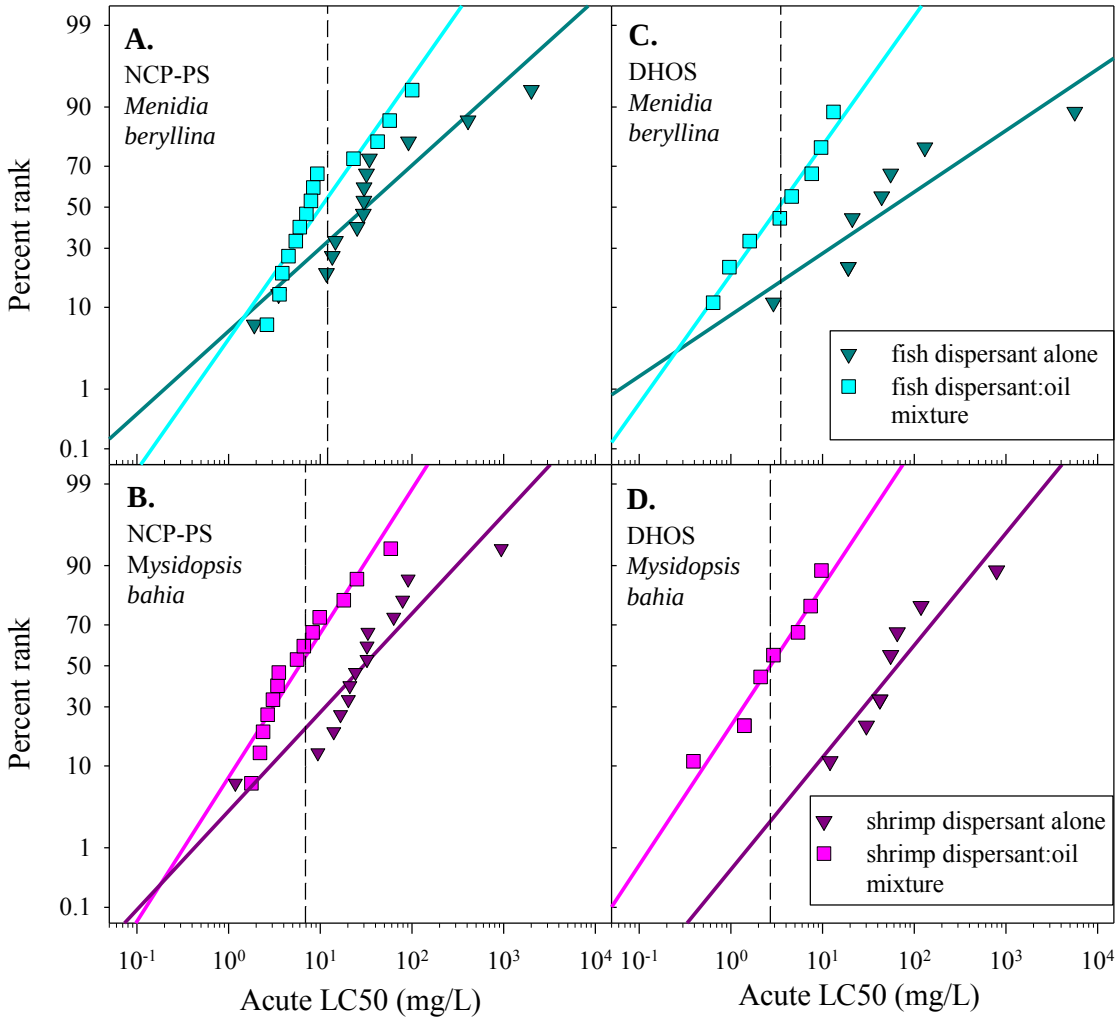


Figure 11. Chemical toxicity distributions of oil dispersants currently on the National Contingency Plan - Product Schedule (NCP-PS), based on acute median lethal concentration (LC50) values of dispersant alone (▼) and in mixture with oil (■) for Menidia beryllina (fish) and Mysidopsis bahia (shrimp). The original NCP-PS dataset (A and B) contains 14 dispersants and utilizes #2 fuel oil as the mixture oil (US EPA 2010). The second data set (C and D) contains eight dispersants re-tested in the wake of the Deepwater Horizon oil spill in the Gulf of Mexico and reevaluated using Louisiana sweet crude (LSC) as the mixture oil (Hemmer et al. 2010 a, b). Benchmark LC50s for oil toxicity (#2FO - A, B; LSC - C, D) for each species are shown (dash line).

The purpose of a CTD is to evaluate the response of a group of chemicals. With small data sets (n = 14 NCP-PS; n = 8 DHOS), it is imperative to utilize all the data points to be conservative in estimating responses. The value of the CTD approach is that it represents all chemicals (real and potential) within the group (Dobbins et al. 2009; Brain et al. 2006; Berninger and Brooks 2010; Williams et al. 2011; Dobbins et al. 2008). The determination of the responses at conservative centiles (e.g., 1^{st} or 5^{th}) provides a way to estimate the likelihood of a encountering a chemical within a group with a response (e.g., LC_{50}) at or below this concentration. Though extreme values (high or low) may influence the probabilistic distribution (Solomon and Takacs 2002), the possibility that other untested dispersants might have similar responses suggests that all available empirical data should be utilized in the development of CTDs.

The CTD approach, like all models, is only as good as the data used to populate it. The NCP-PS dataset exhibited a great deal of variability in terms of response to substances referred to as standardized toxicants (#2FO and DSS; Supplemental Data, Table S1). There are several potential root causes to this variability: intra/inter-laboratory and intra-species variation, the reporting of nominal values for concentrations (rather than analytically verified), and potential differences among batches of reference toxicants (#2FO or DSS). Variability between laboratories and strains of test organisms is generally expected and accounted for with a range of acceptability criteria; however, no such range has been explicitly stated for NCP-PS criteria.

Table 9. Probabilistic evaluation of oil spill dispersants from two different acute toxicity (LC50) datasets: National Contingency Plan – Product Schedule (NCP-PS) and dispersants reevaluated following the Deepwater Horizon Oil Spill (DHOS). Predicted LC50s at the 1st, 5th, 10th, and 25th centiles are show as potential toxicity benchmarks.

						Predicted LC_{50} (mg/L) at specific centiles				Probability (%) of LC50 within range (mg/l)[4]				
										very highly toxic	highly toxic	moderately toxic	slightly toxic	practically non-toxic
Species	Data source[1]	CTD[2]	*n*	r^2	COTB[3]	1st	5th	10th	25th	*<0.1*	*0.1 - 1.0*	*1.0 - 10*	*10-100*	*>100*
Menidia beryllina	NCP-PS	Mixture	14	0.90	54.9	0.41	1.06	1.74	4.01	0.04%	4.66%	45.0%	45.6%	4.8%
		Dispersant	14	0.88	33.0	0.20	0.88	1.93	7.21	0.4%	5.3%	24.3%	40.4%	29.6%
	DHOS	Mixture	8	0.97	51.5	0.13	0.33	0.56	1.30	0.6%	18.9%	59.1%	20.6%	0.8%
		Dispersant	8	0.80	17.1	0.06	0.46	1.32	7.83	1.5%	6.9%	19.3%	30.0%	42.3%
Mysidopsis bahia	NCP-PS	Mixture	14	0.92	54.7	0.32	0.76	1.20	2.56	0.05%	7.65%	58.4%	32.7%	1.2%
		Dispersant	14	0.86	21.3	0.42	1.47	2.85	8.63	0.1%	3.1%	24.4%	47.2%	25.2%
	DHOS	Mixture	8	0.94	50.1	0.13	0.32	0.51	1.13	0.5%	21.6%	62.4%	15.2%	0.3%
		Dispersant	8	0.86	2.3	1.56	4.67	8.37	22.2	<0.01%	0.5%	11.6%	48.3%	39.6%

[1] Reference oil - #2Fuel Oil for NCP-PS, Louisiana sweet crude for DHOS; [2] CTD – chemical toxicity distribution; [3] COTB – Centile at oil toxicity benchmark; [4] Ranges are associated with US EPA aquatic toxicity categories (http://www.epa.gov/oppefed1/ecorisk_ders/toera_analysis_eco.htm#Ecotox)

Reference toxicant data for two dispersants were identified as outliers: NOKOMIS 3-F4 and NEOS AB3000 (Supplemental Data, Table S1). Whereas the removal of the outliers in the present study greatly reduced the variability of the #2FO benchmarks, removal of the accompanying data for dispersant alone and dispersant:oil mixtures changed the CTD very little. The outlier removal changed the predicted probability of toxicity less than 0.3 and 0.6 mg/L at the 5^{th} and 25^{th} centiles, respectively in both species. This exemplifies the robustness of the CTD approach seen in other studies (Dobbins et al. 2009; Brain et al. 2006; Berninger and Brooks 2010; Williams et al. 2011; Dobbins et al. 2008). that even with relatively small datasets, removal or adjustment of data points has only minimal influence on the overall distribution. The NCP-PS methodology does not require analytical verification of any of the toxicants, which may contribute to variability. Additionally, dispersants are manufactured blends of chemicals, and as such are subject to slight variations in composition that could have an influence the magnitude of toxicity. Lack of analytical verification and batch variability mean that single point estimates should likely be coupled with some default uncertainty factor to assess the risk. By using the CTD approach, the risk/hazard associated with using dispersants can be conservatively estimated in a way that is more scientifically robust than a default uncertainty factor coupled to a point estimate. While a less variable, analytically-verified dataset would be ideal, the NCP-PS represents a dataset currently available to risk assessors and those involved in oil spill cleanup to aid in decision making.

Chemical toxicity distributions may provide a means to support prospective environmental assessments of new product applications. Without prescriptive toxicity

guidelines (i.e., acceptable toxicity ranges) regarding NCP-PS listing, the approach in the present study could be used to anticipate the likelihood of approval of a new dispersant based on a CTD of the currently listed compounds. A specific centile of a CTD could be used to define a cut off value or as a trigger for further testing with additional taxa and chronic studies. For example, based on the CTD of *M. beryllina* (NCP-PS), the 5th centile could be used as the cut off, suggesting that the lowest acceptable LC_{50} for listing be 0.9 and 1.0 mg /L for dispersants alone and in mixture with #2FO, respectively (Table 9). A higher centile (e.g., 25th centile) could then be used as a trigger for additional testing, with any compound with acute values between the lowest acceptable LC_{50} and the 25th centile (7 and 4 mg/L, dispersant and mixture respectively) requiring further investigation and those with values greater than the 25th centile threshold could be approved for NCP-PS listing.

Employing the CTD approach in retrospective assessment and management efforts may provide a way to examine the potential for environmental impacts that various dispersants have on aquatic ecosystems. During environmental management of oil spills, it is critically important to understand the potential risk associated with the use of dispersants and subsequently select the proper amounts and types that might assist in mitigation of those risks (Kirby and Law 2008) with a minimum of negative consequences. The sensitivity of the ecosystem, the volume and duration of the oil spill, and toxicity of the oil itself should be considered. For example, in open ocean (generally considered non-critical habitat) with a large spill of moderately toxic oil, it may be reasonable to select dispersants whose toxicity in mixture might be as low as the 1st centile (DHOS - 0.13 mg/L for both species), whereas the same oil in an estuary may

require a higher centile (perhaps above the oil toxicity bench mark; 2.7 -3.5 mg/L LSC) be selected because of the greater sensitivity of the ecosystem. The CTD approach may also help in assessing the risk associated with using multiple dispersants on a single spill. Using the 1st or 5th centile of the CTD may provide a more conservative toxicity estimate; accounting for the possibility that the interactions between multiple dispersants may increase toxicity beyond the point estimates.

Oil spills usually mean that risk assessments are conducted in crisis mode, with truncated time-tables and no time to conduct additional studies. The CTD approach can provide initial conservative estimates of risk that utilize the available data in a simple, quick methodology supported by sound science. The present study demonstrates the utility of CTDs in analysis of the comparative aquatic toxicity of these products alone and in combination with different oil types. This approach may be useful for regulatory agencies and the regulated community involved in prospective and retrospective environmental assessments of oil dispersants, particularly until robust environmental exposure data and when information on chronic aquatic toxicity becomes available.

Table 10. Dispersant acute toxicity data (LC50) as reported in the National Contingency Plan Product Schedule as on 1/1/2011 (http://www.epa.gov/osweroe1/content/ncp/product_schedule.htm).

	Dispersant LC50 (mg/L)		#2 Fuel Oil LC50 (mg/L)		1:10 Mixture Dispersant-#2FO LC50 (mg/L)		Ratio #2FO : Dispersant-#2FO Mixture		DSS Reference Toxicant LC50 (mg/L)	
Dispersant	*Menidia beryllina*	*Mysidopsis bahia*	*Menidia beryllina*	*Mysidopsis bahia*	*Menidia beryllina*	*Mysidopsis bahia*	*Menidia beryllina*	*Mysidopsis bahia*	*Menidia beryllina*	*Mysidopsis bahia*
BIODISPERS	13.5	78.9	12.4	2.8	6.0	2.7	2.1	1.1	11.8	21.8
COREXIT® EC9500A	25.2	32.2	10.7	16.1	2.6	3.4	4.1	4.7	7.1	9.8
COREXIT® EC9527A	14.6	24.1	10.7	16.1	4.5	6.6	2.4	2.4	7.1	9.8
DISPERSIT SPC 1000™	3.5	16.6	11 .6	11.7	7.9	8.2	1.5	1.5	6.3	11.7
FINASOL® OSR 52	11.7	9.4	6.0	2.4	5.4	2.4	1.1	1.0	8.5	21.8
JD-109	1.9	1.2	9.4	3.1	3.8	3.5	2.4	0.9	2.6	8.1
JD-2000™	407.0	90.5	8.4	2.6	3.6	2.2	2.3	1.2	2.2	10.5
MARE CLEAN 200	1996.0	938.0	10.7	16.1	42.0	9.8	0.3	1.6	7.1	9.8
NEOS AB3000	91.1	33.0	*201.8	11.5	57.0	25.0	3.5	0.5	1.5	9.3
NOKOMIS 3-AA	34.2	20.2	22.5	11.1	7.0	5.6	3.2	2.0	#5.4	#7.8
NOKOMIS 3-F4	29.8	32.2	*100.0	*72.7	100.0	58.4	1.0	1.2	*159.6	*267.7
SAF-RON GOLD	29.4	63.0	16.8	5.9	9.3	3.0	1.8	2.0	15.9	9.8
SEA BRAT #4	30.0	14.0	16.0	14.0	23.0	18.0	0.7	0.8	1.1	1.0
ZI-400	31.8	21.0	18.1	2.7	8.4	1.8	2.2	1.5	16.1	27.8
Descriptive Statistics Max	1996.0	938.0	22.5	16.1	100.0	58.4	4.1	4.7	16.1	27.8
Min	1.9	1.2	6.0	2.4	2.6	1.8	0.3	0.5	1.1	1.0
Range	1994.1	936.8	16.6	13.8	97.4	56.6	3.9	3.7	15.0	26.8
RF	1050.5	794.9	3.8	6.8	38.3	33.0	16.1	10.3	14.7	27.8
Mean	194.3	98.2	12.8	8.9	20.0	10.8	2.0	1.6	7.3	12.6
Standard Deviation	528.8	243.1	4.7	5.8	28.2	15.3	1.1	1.0	5.2	7.4
Coefficient of Vari.	272%	248%	36.6%	64.8%	141%	142%	53.6%	65.9%	71.1%	58.8%
Geometric Mean	31.2	29.5	12.0	6.9	10.1	5.9	1.7	1.4	5.4	10.1

All LC50 values as nominal concentrations; * outliers ($p<0.05$); # CuSO4 reference toxicant; *and #values not included in statistics; RF – range factor

Table 11. Assessment of #2 Fuel Oil acute toxicity (LC50) variability as reported in the National Contingency Plan – Product Schedule (NCP-PS) with (n = 14) and without outlier values ($p<0.05$; Grubb's Outlier Analysis). *CV* of 31.2% for *Mysidopsis* and 38.5% in *Menidia* and a range factor of 3.5 for marine test species are common acceptable variability measures in other EPA datasets (US EPA 2001, US EPA 1981). All values mg/L except *CV* and range factor.

Species		*n*	Mean	Std Dev	*CV* (%)[2]	Geometric Mean	Median	Maximum	Minimum	Range	Range Factor
Menidia beryllina	All Data	14	32.5	54.2	166.7%	17.1	12.0	201.8	6.0	195.9	33.6
	Trimmed[1]	12	12.8	4.7	36.6%	12.0	11.1	22.5	6.0	16.5	3.8
Mysidops is bahia	All Data	14	13.5	17.9	132.6%	8.1	11.3	72.7	2.4	70.3	30.3
	Trimmed[1]	13	8.9	5.8	64.8%	6.9	11.2	16.1	2.4	13.7	6.7

[1] Trimmed – dataset after outlier removal; [2] *CV*- coefficient of variation;

Table 12. Dispersant manufacturers and distributors.

Dispersant	Manufacturer	Primary Distributor
BIODISPERS	Petrobiotech LLC	Petrobiotech LLC
COREXIT® EC9500A	Nalco Energy Services, L.P.	Nalco Energy Services, L.P.
COREXIT® EC9527A	Nalco Energy Services, L.P.	Nalco Energy Services, L.P.
DISPERSIT SPC 1000™	U.S. Polychemical Corp.	Maritime Solutions, Inc.
FINASOL® OSR 52	Total Fluides	Total Petrochemicals USA, Inc.
JD-109	GlobeMark Resources Ltd.	GlobeMark Resources Ltd.
JD-2000™	GlobeMark Resources Ltd.	GlobeMark Resources Ltd.
MARE CLEAN 200	Taiho Industries Co., Ltd.	Klinview Corporation
NEOS AB3000	NEOS Company Limited	NEOS Company Limited
NOKOMIS 3-AA	Mar-Len Supply, Inc	Mar-Len Supply, Inc
NOKOMIS 3-F4	Mar-Len Supply, Inc	Mar-Len Supply, Inc
SAF-RON GOLD	Sustainable Environmental Technologies Inc.	TRK Enterprises, LLC
SEA BRAT #4	Alabaster Corp.	Garner Environmental Services
ZI-400	Z.I. Chemicals	Z.I. Chemicals

CHAPTER FIVE

A Novel Probabilistic Approach for Developing Acute to Chronic Uncertainty Factors for Biological Active Compounds

Introduction

In environmental (human health, ecological) risk assessment, uncertainty factors are commonly used when data is limited, data quality is questionable, or when it is necessary to extrapolate between species or sensitive subpopulations. For many chemicals, very limited toxicology data is available, with the majority of information existing for acute mortality benchmark concentrations. In aquatic toxicology, uncertainty factors are routinely applied to acute benchmarks (median lethal dose, LC_{50}) to estimate chronic toxicity thresholds (e.g., No Observed Effect Concentrations, NOEC). These extrapolation factors are so critical that their magnitude and usage criteria are even embedded in regulatory documents. Some documents use specific derived data values for uncertainty factors, like the value 18, utilized in the U.S. Environmental Protection Agency (U.S. EPA) water quality guidance for the Great Lakes (US EPA 1995). The majority of regulatory documents, however, including guidelines within REACH (Williams et al. 2009) and Organization for Economic Cooperation and Development (OECD; OECD 1992), use various order of magnitude, default values (e.g., 10, 100, 1000; Duke and Taggart 2000). However, such default values appear to have been simply adopted from human health risk assessment (Dourson and Stara 1983; Forbes and Callow 2002). Often these default values have been in use so long, they are applied without careful consideration of appropriate scientific justification (Chapman et al. 1998).

To reduce this reliance of default uncertainty factors critical evaluations of acute-to-chronic extrapolations have occurred, beginning more than 40 years ago (Mount and Stephan 1967; Calabrese and Baldwin 1993), which initially resulted in an uncertainty factor called the Acute to Chronic Ratio (ACR). Subsequently, a number of researchers have conducted critical evaluations of ACR data, attempting to develop scientific justification for various ACR extrapolation factor values (Table 13). These studies often report both the median ACR value and a 90^{th} percentile ACR value (ACR_{90}), but most utilize the ACR_{90} to optimally ensure the actual response values are captured by the extrapolated estimates. A number of these studies have identified 22-35 as an appropriate ACR_{90} range for most industrial chemicals (Kenaga 1982; Calabrese and Baldwin 1993; Slooff et al. 1986; Lange et al. 1998, Roex et al. 2000; Ahlers et al. 2006). In these same studies, when all chemicals are considered the ACR_{90} values are substantially higher (Table 13).

The most extensive evaluation of the ACR to date analyzed 456 chemicals across a variety of chemical and mode of action (MOA) classes, and compared ACR values for different taxa (Raimondo et al. 2007). In the Raimondo et al (2007) study, and a number of others, some specific groups of chemical have been identified as having ACR values larger (>100) than what would be expected based on ACR_{90} values of all chemicals. Kenaga (1982) found that those chemicals with the highest ACRs were generally associated with some type of biological activity: irreversible target binding - some acetylcholinesterases; limited to no excretion – some metals; or other various highly specific biological action (e.g., creation of methemoglobin). Raimondo et al. (2007) identified a number of specific chemical classes and mode of action groups as having

large ACRs (>100). One unlikely group with a high ACR was the non-polar narcosis MOA. In other studies (Kenaga 1982; Lange et al. 1998; Roex et al. 2000; Ahlers et al. 2006) this group was identified as having a low ACR. The MOA groups used for many of these studies are those derived from Russom et al. (1997) and Verhaar et al. (2000) which derive MOA based on acute toxicity and structural activity relationships of the chemical. Whereas this method works well for grouping chemicals by acute MOA, it is not as well developed for chronic MOA, which may exert specific biological actions, resulting in a larger ACR. In fact, higher ACR values are even used diagnostically to suggest target specific interactions (Rand 1995).

The chronic biotic activity that results in large ACR values generally results from two root causes: different MOAs for acute and chronic responses, and/or the biotic action that over the extended time course of a chronic study manifested at a lower concentration. Biological activity, regardless whether it is caused by irreversible binding, inability to efficiently metabolize or reduce clearance, or some specific MOA, presents a problem for acute to chronic extrapolation because it does not generally fit the assumptions that support standard acute-to-chronic extrapolations. Whereas previous studies have advanced an understanding of specific MOAs with high variability and large ACR, to date these approaches do not robustly address biotic activity within a chemical group.

Berninger and Brooks (2010) suggested that probabilistic approaches may be useful to develop ACR values. Probabilistic approaches are increasingly employed in environmental (human health, ecological) risk assessment. For example, probabilistic ecological risk assessments employ probability distributions of environmental exposure distributions (EEDs) and species sensitivity distributions (SSD; Solomon and Takacs

2002). Previous work by our group has extended similar approaches for probabilistic hazard assessments using chemical toxicity distributions (CTD) of chemicals with common MOAs and for common model organisms and responses (Dobbins et al 2008, 2009; Berninger and Brooks 2010; Berninger et al 2011; Williams et al 2011). Using probabilistic approaches may also, through the use of acute and chronic CTDs, provide an approach to dealing with chemical groups where acute and chronic MOA are so different as to be non-predictive using traditional approaches. By using centiles analogous to those used in SSDs and CTDs (e.g., 1^{st}, 5^{th}, 10^{th}) it may be possible to construct more protective ACR values. In the present study, the primary objective was to apply probabilistic distributions to several sets of biological active compounds to explore the utility and robustness of this approach.

Methods

Acute to Chronic Ratio

The acute to chronic ratio is generally calculated by dividing the acute value (represented by the LC_{50}) by the chronic value. A variety of different chronic responses have been used in the calculations of ACRs. When available, the most appropriate chronic value is the maximum acceptable toxicant concentration (MATC; a calculated value – geometric mean of no-observed effect concentration (NOEC) and lowest-observed effect concentration (LOEC)). Alternatively, ACRs may also be calculated using NOEC or LOEC values. For the purposes of consistency and comparability, in this study all the ACR values calculated within a single dataset were from the same endpoint.

Table 13. Previously reported 90th percentile Acute to Chronic Ratios (ACR) for use in extrapolating from acute response (LC_{50}) to chronic response (no observed effect concentration).

ACR	Chemical or Mode of Action	N	Species[1]	Reference
25 125	Industrial chemicals All chemicals	84	Mixed	Kenaga 1982
26 86	Laboratory response Ecosystem response	164	Mixed Multispecies	Slooff et al. 1986
27 55 265	All chemicals	93	Fish	Suter et al. 1987; Calabrese and Baldwin 1993
73 86 94 128	All chemicals All chemicals Pesticides All chemicals	62 27 9 9	Fish Invertebrate Invertebrate *Daphnia magna*	Länge et al. 1998
42 198	All chemicals All chemicals	102 32	*Daphnia magna* Fish	Ahlers et al. 2006
80 90 68 60 78 28 149	All chemicals All chemicals All chemicals AChE[2] inhibitors Organophosphate Carbamates Narcosis	456 261 195 78 62 16 167	Mixed Fish Invertebrate Mixed Mixed Mixed Mixed	Raimondo et al. 2007

[1]Mixed: fish and invertebrate models used for ACR development, individual ACRs calculated from single species per chemical; multispecies: individual ACR values calculated using lowest LC_{50} and lowest NOEC across all species in a single chemical using. [2] Acetylcholinesterase.

Often different ACRs are presented as a specific percentile (e.g., 10th, 50th, 90th). To avoid confusion when ACRs are referenced herein they aree followed with a subscript of the percentile. For example, a 90th percentile ACR of 75 will be referred to as ACR_{90} 75. In the literature often the ACR is reported in the 90th percentile a means of presenting protective extrapolation factors. To distinguish between reported values and probabilistic values developed in this study all probabilistic ACR values will be designated as pACR with a subscript designating the specific centile value (e.g., $pACR_{95}$).

Data Sets for Analysis

Pesticides. As previous research has indicated that larger ACR values were generally found among acetylcholinesterase inhibitors (AChEI), organophosphate (OP) and carbamate (CB) insecticides were selected for this exercise. Among all available OP and CB data, only *Daphnia magna*, a common model cladoceran, was selected to reduce interspecies variability. The original list of AChEI pesticides was acquired from a previously published dataset acute LC_{50}s for *D. magna* (Williams et al. 2011); the original source data was developed from the ECOTOX (http://cfpub.epa.gov/ecotox) and Pesticide Ecotoxicity Database (Office of Pesticide Programs; http://www.ipmcenters.org/Ecotox/index.cfm) maintained by the U.S. Environmental Protection Agency (U.S. EPA). Database values used in the Williams et al (2011) study were extensively evaluated for quality using the Klimisch scale. Values for chronic response thresholds were acquired from the same database sources. To maintain consistency, only 21d reproduction NOEC values were used for this exercise. As previous studies have done (Raimondo et al. 2007), OPs and CBs were treated separately for all analyses.

Endocrine active compound.s. An initial list of endocrine active compounds was compiled from Dobbins et al. (2008). This list was supplemented with additional data from endocrine active compounds from Berninger and Brooks (2010). All acute and chronic values were from fish studies. All efforts were made to collect acute and chronic data from the same species; however, this was not always possible due to limited data (Table 14). For each value chronic responses were verified from primary literature.

Table 14. Pharmaceutical data used to calculate fish Acute to Chronic Ratios for endocrine active compounds.

	Acute Response (mg/L)		Sub-Lethal/Chonic Response (mg/L)			
Compound	Species	96h	Species	mg/L	Test type	ACR
Bisphenol A[1]	FHM	4.6	FHM	0.160	164d reproduction	28.8
Flutamide[2]	MED	3.6	FHM	0.651	21d reproduction	5.5
Ketoconazole[3]	FHM	3.96	FHM	0.025	21d reproduction	158.4
Methoxychlor[4]	FHM	27.7	FHM	0.005	21d reproduction	5540
Nonylphenol[5]	FHM	0.251	FHM	0.008	21d reproduction	31.0
Pentylphenol[6]	FHM	2.59	MED	0.051	61d reproduction	50.7
Perfluorooctanesulfonate[7]	FHM	5.02	FHM	0.230	21d reproduction	21.8
Triclosan[8]	FHM	0.286	MED	0.013	21d reproduction	22.3
Vinclozolin[9]	FHM	25.7	FHM	0.060	21d reproduction	428.6
Drospirenone[10]	FHM	4.6	FHM	$6.6x10^{-4}$	21d reproduction	6970
Estradiol[11]	MED	3.9	MED	$3.0x10^{-5}$	21d reproduction	$1.3x10^{5}$
Estrone[12]	FHM	52.5	FHM	$3.2x10^{-5}$	21d reproduction	$1.6x10^{6}$
Ethinyl Estradiol[13]	FHM	1.7	FHM	$1.0x10^{-6}$	21d reproduction	$1.7x10^{6}$
Levonorgestrel[10]	FHM	6.53	FHM	$3.3x10^{-6}$	21d reproduction	$2.0x10^{6}$
Methyltestosterone[4]	MED	24.3	FHM	0.005	21d reproduction	4860
Trenbolone[15]	FHM	1.5	FHM	$3.0x10^{-5}$	21d reproduction	$5.0x10^{4}$

FHM- Fathead Minnow (*Pimephales promelas*), MED- Japanese Medaka (*Oryzias latipes*);
[1] Sohoni et al. 2001; [2] Jensen et al. 2004; [3] Ankley et al. 2007; [4] Ankley et al. 2001; [5] Harries et al. 2000; [6]Seki et al. 2003; [7]Ankley et al.2005; [8]Ishibashi et al. 2004: [9] Marinovic et al. 2008; [10]Zeillinger et al. 2009; [11] Kang et al. 2002; [12]Thorpe et al 2007; [13]Jobling et al 2004; [15] Jensen et al. 2006

This is a preliminary approach for evaluating compounds believed to have specific MOAs within the hypothalamic-pituitary-gonadal (HPG) axis (Ankley et al. 2009). Whereas the included studies evaluated various endocrine response endpoints (e.g., male vitellogenesis, female estrogen reduction or testosterone increase) the more general term ―endocrine active compounds" was selected to represent this dataset. To maintain consistency for the purposes of this evaluation, only fish reproductive endpoints were selected as the chronic response. While the fish reproductive endpoint was selected all studies included in the evaluation also reported an endocrine specific response. As the common endpoint reported across all datasets, LOEC values were selected as the chronic response. Values for acute responses were taken from Berninger and Brooks (2010) and the US EPA ECOTOX database. Because hormones are more potent than other endocrine active compounds, I divided these chemicals in two groups: chemicals that induce endocrine activity and hormones (natural or synthetic) that induce endocrine activity.

Acute to Chronic Toxicity Relationships

The relationship between available acute and chronic responses was investigated via regression analysis. Datasets were reviewed to determine if acute, chronic, and ACR values were log normally distributed. To establish the relationship between acute and chronic responses data was log transformed. Linear regression analyses were performed on the log transformed data to examine potential data relationships (SigmaPlot Version 11.0, Systat Software, Inc., San Jose, CA, USA).

Acute to Chronic Ratio Distributions

Acute, chronic, and ACR values from each dataset was applied to a probabilistic model, using the same approach that is used in development of species EEDs, SSDs and CTDs. This model allowed for the calculation of selected centile values based on the probabilistic regression. Because of the log normal distribution of toxicity data in general, and these datasets in particular, probabilistic distributions may be considered a more appropriate model for estimating the likelihood of encountering chemical responses at a specific percentile (Solomon and Takacs 2002). Probabilistic distributions for acute, chronic and ACR values were developed based on the CTD methodologies outlined by Solomon and Takacs (2002), modified by Brain et al. (2006) and described further elsewhere (Solomon et al. 2000; Dobbins et al. 2008, 2009; Berninger and Brooks 2010). Each probabilistic distribution included a regression analysis of the distribution, which was used to identify the probability of finding a value at a certain centile. For example, in a CTD of acute values, the point at which the distribution is equal to a value of 1000 might corresponds to the 15^{th} centile, predicting that there is a 15% probability of a class of chemicals having a value of 1000 or less below this point in the distribution. For each analysis, data were numerically ranked in descending order and ranks converted to a probability percentage calculated from the Weibull formula:

$$j = 100 * i / (n + 1) \quad (1)$$

where j is the plotting position, i is the numerical rank, and n is the total number of data points in the data set. Values and ranks were then plotted on a log-probability scale and a regression line fitted to these transformed distributions (SigmaPlot Version 11.0 Systat Software, Inc., San Jose, CA, USA). From each regression, slope, intercept, and r^2 values

were determined. Using intercept and slope values it was possible to calculate centile values (Microsoft Excel 2007 Microsoft Corp, Redmond, WA, USA) using the equation:

$$\text{Centile Value} = \text{NORMSDIST}((m * \log_{10}(x)) + b) \quad (2)$$

where NORMSDIST returns the standard normal cumulative distribution function, x is a selected value (LC_{50}, NOEC, LOEC – mg/L; ACR – unitless), and m and b are the slope and intercept, respectively, of the regression line. For acute and chronic distributions centile values were calculated at the 10^{th} and 5^{th} centiles and for ACR distributions values were determined at the 50^{th}, 90^{th}, 95^{th}, and 99^{th} centiles. This was done so that for each distribution the greatest potential chronic hazard could be evaluated.

Using data from the probabilistic regressions ACR values at the 50^{th}, 90^{th}, 95^{th}, and 99^{th} centiles were calculated. Probabilistic distributions of ACR data were developed for carbamates, organophosphates, endocrine active chemicals, and endocrine active hormones. Using the CTDs of acute and chronic data for each data set ACR values were created by dividing the acute values by chronic values at the 90^{th} and 95^{th} centiles.

Application of Acute to Chronic Uncertainty Factors

To evaluate utility and the likelihood of probabilistically derived uncertainty factors being protective for chronic responses to the study compounds, various factors were applied to acute data to estimate chronic response (Table 15 -15). These predicted chronic values were calculated by dividing the acute values (in each dataset) by the ACR uncertainty factor. A series of 14 different extrapolation factors will be evaluated, across the two datasets. When multiple extrapolation factors were combined, the geometric mean was used. To represent regulatory ACRs (ACR ID – 0), the endpoints the lowest ACR_{90} values for all chemicals or industrial chemicals was combined with the regulatory

ACR value of 18 (US EPA 1995). Several different groups of literature reported values were used to establish ACR uncertainty factors (Table 15). In addition to literature reported values, ACRs from the probabilistic distributions were used (Table 16). For each data set, the 5^{th} and 10^{th} centiles of the acute and chronic CTDs was used to calculate ACRs (Table 16; ACR ID 10-11). Two other extrapolation factors (ACR ID 12-13; Table 16) for each dataset were based on the 90^{th} and 95^{th} centile ACR values based on the probabilistic distributions.

For each dataset nine different chronic values were determined, five literature values and four probabilistic values. These extrapolated chronic values were then compared to the actual measured chronic responses from the dataset to determine if the extrapolated response was lower than the threshold of the actual chronic value (TACV). Extrapolated chronic values with estimated concentrations lower than the empirically derived TACV were considered potentially protective. Within the dataset, the number of extrapolated chronic responses with values below the TACV was calculated and the percent of response above and below the threshold were determined.

Table 15. Acute to chronic extrapolation models derived from literature reported 90th percentile Acute to Chronic Ratio (ACR) values.

ACR ID	Name	Value	Description	Reference	Use
0	$_{low}ACR$	24	Geometric mean of ACR values for industrial chemical	25 – Kenaga 1982 26 – Slooff et al. 1986 27 – Suter et al. 1987 18 – Federal Registry 1995	ALL GROUPS
1	$_{OP}ACR_{90}$	78	90th percentile ACR value for organophosphate in all species	Raimondo et al. 2007	Organophosphate
2	$_{CB}ACR_{90}$	28	90th percentile ACR value for carbamates in all species	Raimondo et al. 2007	Carbamate
3	$_{ACH}ACR_{90}$	60	90th percentile ACR value for AChEI pesticides in all species	Raimondo et al. 2007	Pesticides
4	$_{PEST}ACR_{90}$	94	90th percentile ACR value for pesticides in invertebrates	Länge et al. 1998	Pesticides
5	$_{invert}ACR_{90}$	77	Geometric mean of 90th percentile ACR value for all chemicals in invertebrates	125 – Kenaga 1982 86 – Slooff et al. 1986 86 – Länge et al. 1998 42 – Ahlers et al. 2006 68 – Raimondo et al. 2007	Pesticides
6	$_{fish}ACR_{90}$	107	Geometric mean of 90th percentile ACR value for all chemicals in fish	125 – Kenaga 1982 86 – Slooff et al. 1986 73 – Länge et al. 1998 198 – Ahlers et al. 2006 90 – Raimondo et al. 2007	Endocrine Active
7	$_{fishRP}ACR_{95}$	55	95% prediction interval for ACRs based on fish reproduction	Suter et al. 1987; Calabrese and Baldwin 1993	Endocrine Active
8	$_{fishRP}ACR_{99}$	265	99% prediction interval for ACRs based on fish reproduction	Suter et al. 1987; Calabrese and Baldwin 1993	Endocrine Active
9	$_{narc}ACR_{90}$	149	90th percentile ACR value for narcosis response in all species	Raimondo et al. 2007	Endocrine Active

Table 16. Acute to chronic extrapolation models derived from probabilistic distributions models developed in this study for specific datasets.

ACR ID	Name	Value	Description	Reference	Use
10	$_{CTD}ACR_{90}$	OrPhos – 304 Carbam – 220 EAC – 1840 Horm – $1.2x10^7$	ACR calculated from the 10^{th} centile of acute and chronic chemical toxicity distributions.	Acute and chronic empirical data	Pesticides and endocrine active
11	$_{CTD}ACR_{95}$	OrPhos – 518 Carbam – 434 EAC – 4560 Horm – $4.3x10^7$	ACR calculated from the 5^{th} centile of acute and chronic chemical toxicity distributions.	Acute and chronic empirical data	Pesticides and endocrine active
12	$pACR_{90}$	OrPhos – 41 Carbam – 42 EAC – 80 Horm – $1.7x10^6$	90^{th} centile ACR calculated from probabilistic distribution of ACR values.	ACRs calculated from empirical data	Pesticides and endocrine active
13	$pACR_{95}$	OrPhos – 39 Carbam – 52 EAC – 81 Horm – $3.6x10^6$	95^{th} centile ACR calculated from probabilistic distribution of ACR values.	ACRs calculated from empirical data	Pesticides and endocrine active

OrPhos – organophosphate; Carbam- carbamate; EAC – endocrine active chemical; Horm – endocrine active hormone.

Results

Acute to Chronic Toxicity Relationships

All datasets were found to be log normally distributed. Of the biologically active groups evaluated in this exercise, only the pesticides showed a statistically significant relationship between acute mortality and chronic reproduction responses (Figure 12 A-B). Based on the linear regression analysis acute and chronic responses were significantly related for both carbamates ($r^2 = 0.74$, $p<0.001$) and organophosphate ($r^2 = 0.84$; $p<0.001$). The r^2 values for carbamates and organophosphates, 0.74 and 0.84, respectively, were slightly smaller than those reported by Roex et al. (2000) in their evaluation of specific acting chemical groups ($r^2 = 0.90$), however, in this analysis no data was excluded. Both carbamates and organophosphates exhibit some degree of irreversible binding to the acetylcholinesterase causing inhibition, and over a short time period (e.g., 48h acute studies) this difference may not be observed. For organophosphates and carbamates, the variability in potency has been well established and connected to affinity to the AChE receptor (Printes and Callaghan 2004). Based on the regression approach employed in this study, it seems plausible that despite differences in potency and irreversibility of AChE binding, that ultimately these pesticides work via the same MOA in eliciting toxicity to both *D. magna* mortality and reproduction endpoints.

Probabilistic Distributions of Acute to Chronic Ratios

For each ACR dataset probabilistic distributions were developed. Data fit well within the regression model with r^2 values ranging from 0.89 to 0.99 (Table 17). The distributions of the two AChE inhibiting pesticides were very similar, particularly for

$pACR_{90}$ and $pACR_{95}$ (Table 17). These values represent a substantial departure from those ACR_{90} previously reported for pesticides or specifically for organophosphate and carbamates (Table 13). Carbamates generally showed a wider spread of the data, but generally organophosphates displayed slightly higher ACRs (Figure 13). CTDs of the acute and chronic data for the two pesticides showed that while exhibiting similar ACR values, the magnitude of carbamate LC_{50} and NOEC values were substantially less than those of organophosphates (Figure 14). The ACR distributions of endocrine active compounds showed a range of values over 9 orders of magnitude (Figure 15). When endocrine actives were separated in two groups (endocrine active chemicals and hormones) they showed distinctly different ACR values, different by four orders of magnitude at the $pACR_{90}$ (Table 17). CTDs were characterized by lower variation of the magnitude of response thresholds for the endocrine active compounds in contrast to hormones (Figure 16)

Application of Acute to Chronic Ratio Uncertainty Factors.

For each of the datasets nine ACR uncertainty factors were applied to the acute data measurements to develop extrapolated NOEC or LOEC (for endocrine agonists) responses. Predicted chronic responses were then plotted with actual measured chronic responses (only the organophosphate plot is shown; Figure 17). Predicted NOEC values were then compared to empirically derived NOECs. The goal of this comparison was to determine if the predicted ACR would provide a potentially protective NOEC when applied to actual data (ACR extrapolated NOECs below the TACV). The results showed that the probabilistic ACR models 12 and 13 provided the greatest potential protection for all compounds considered (Table 5).

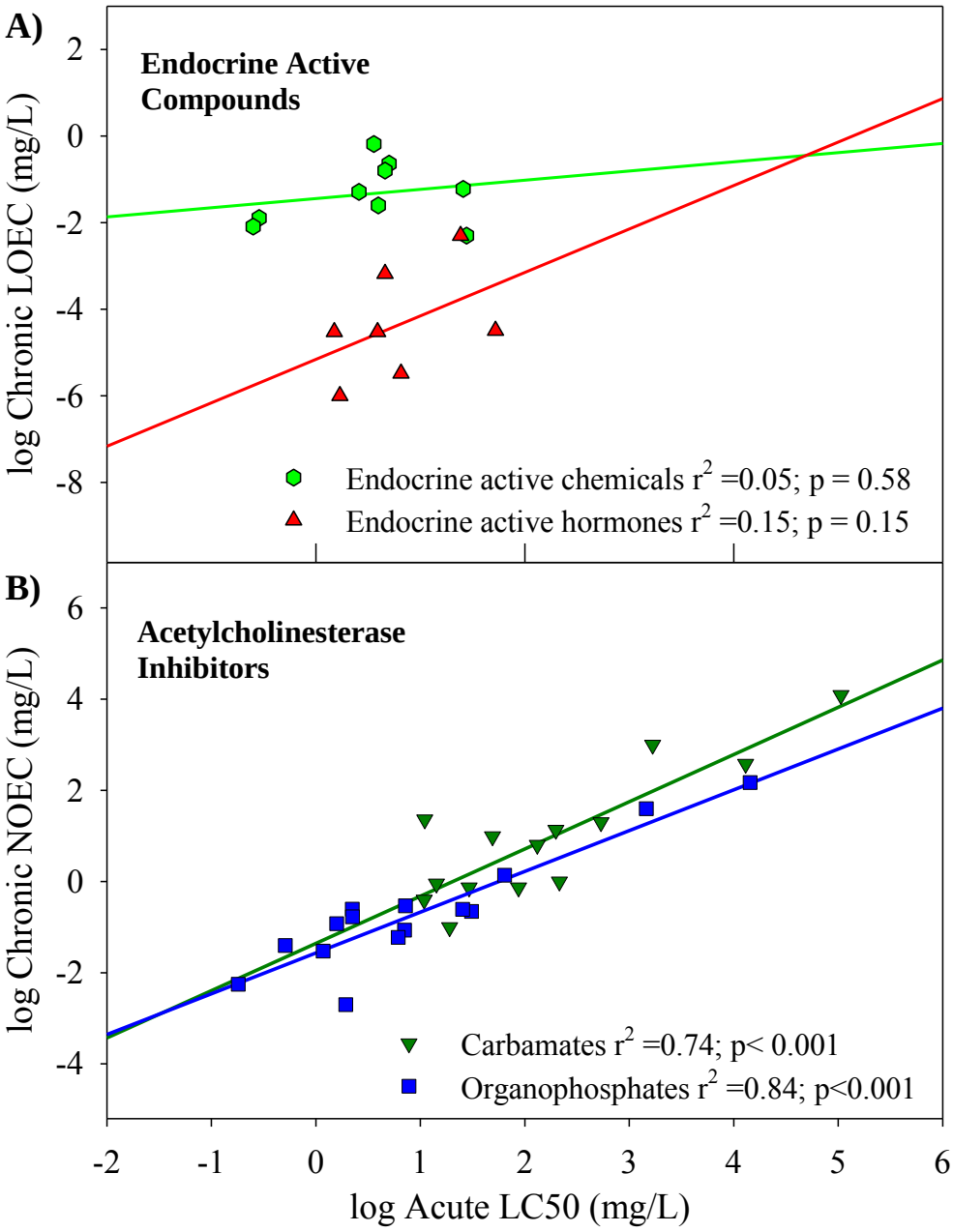


Figure 12**.** Relationship between (A) log acute toxicity (LC_{50}) and log chronic LOEC for fish exposed to endocrine active chemicals and hormones and (B) log acute toxicity (LC_{50}) and log chronic NOEC for *Daphnia magna* exposed to acetylcholinesterase inhibiting pesticides.

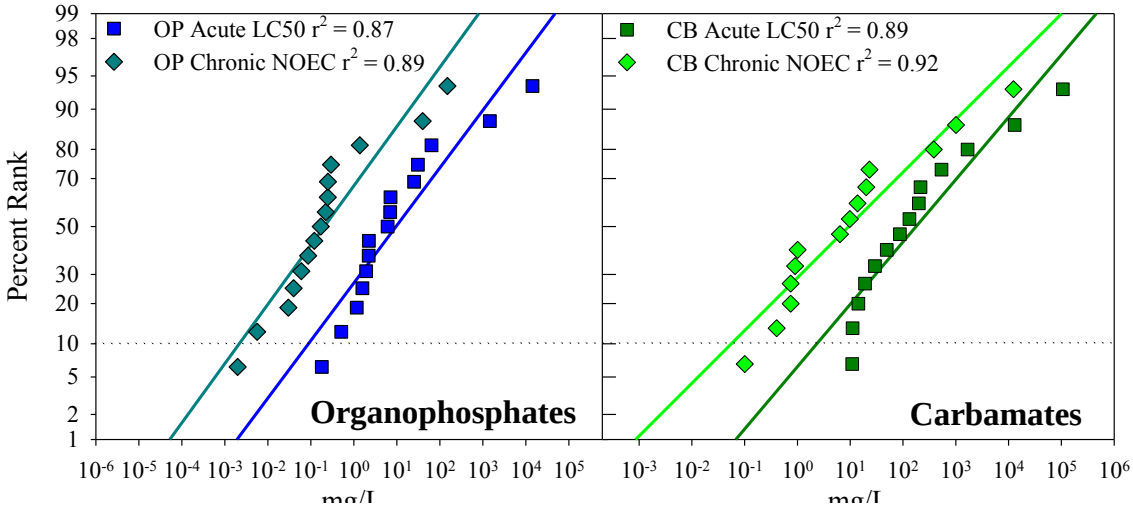


Figure 13. Chemical toxicity distributions of the acute LC50 and chronic NOEC (reproduction) responses for *Daphnia magna* reproduction for two subsets of pesticide data chemicals. Dashed line placed at the 10th centile.

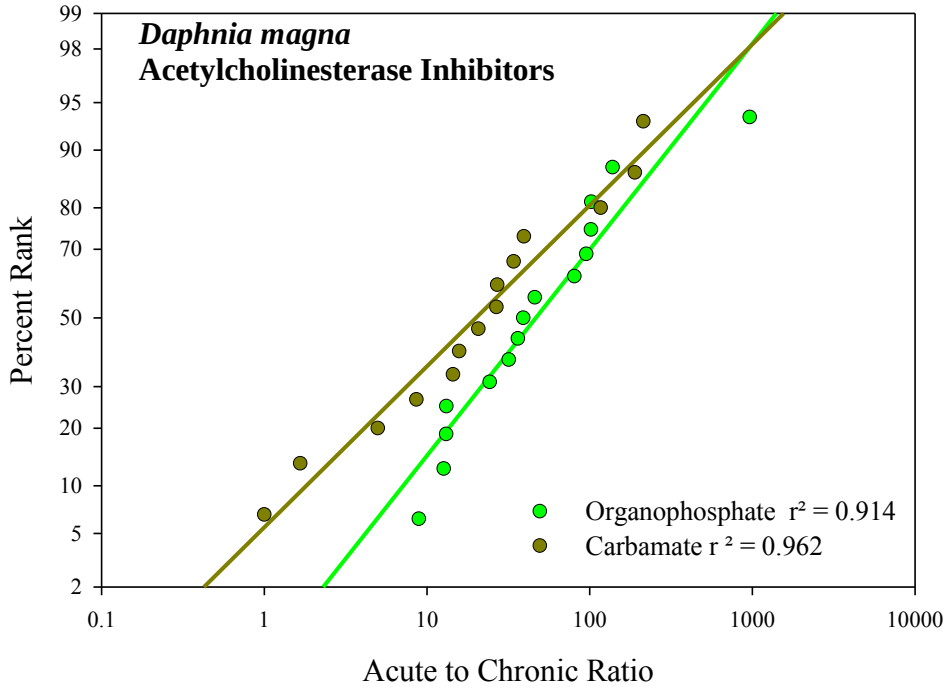


Figure 14. Probabilistic distributions of *Daphnia magna* acute to chronic ratios for the acetylcholinesterase inhibiting pesticides classes: carbamates and organophosphates.

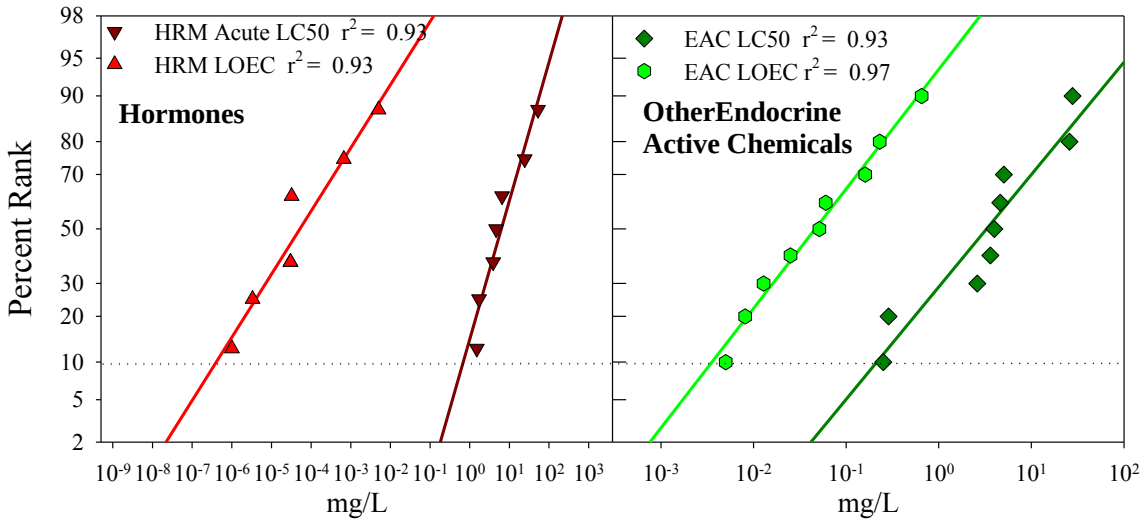


Figure 15. Chemical toxicity distributions of the acute LC50 and chronic LOEC responses for fish reproduction for two subsets of endocrine active substances: hormones and other endocrine active chemicals. Dashed line placed at the 10th centi

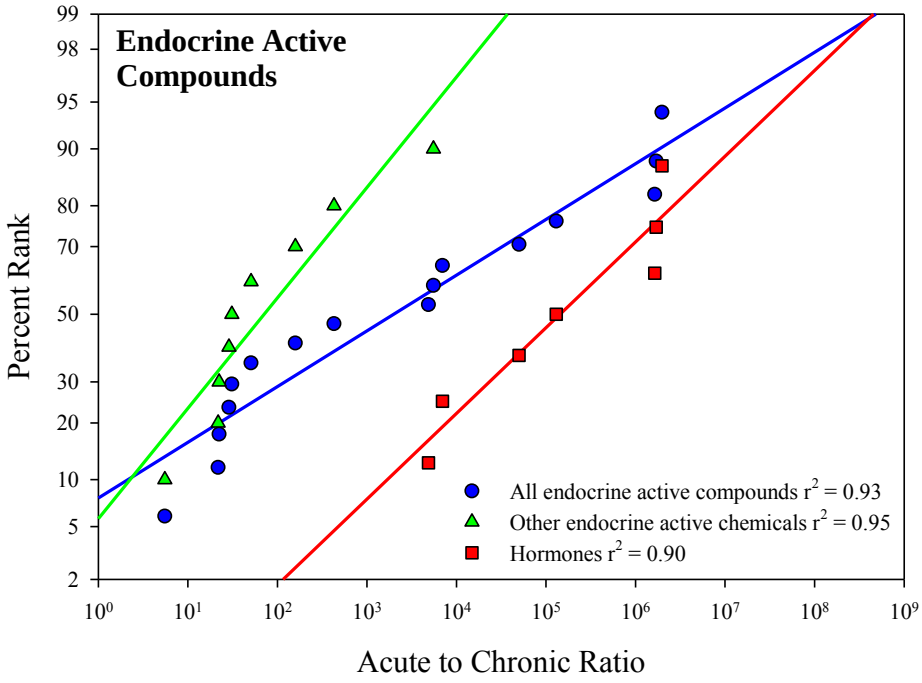


Figure 16. Probabilistic distribution of acute to chronic ratio for fish species exposed to endocrine active compounds. ACR values were developed from acute toxicity (LC50) and chronic LOEC (reproduction) values. Endocrine active compounds a shown as one group (●) and separated into other endocrine active chemicals (▲) and hormones (■).

Table 17. Data from the chemical toxicity distributions of acute and chronic data and probabilistic distributions of acute to chronic ratios from organophosphate and carbamate pesticides, and endocrine active chemicals and hormones.

Distribution	*n*	Chemical Toxicity Distribution 90th Centile			Chemical Toxicity Distribution 95th Centile			Probabilistic ACR Centile value		
		Acute	*Chronic*	*ACR*	*Acute*	*Chronic*	*ACR*	*50%*	*90%*	*95%*
Organophosphates	15	0.088	0.0022	40.8	0.023	0.0006	39.3	47	304	518
Carbamates	14	2.31	0.056	41.6	0.676	0.013	52.0	20	220	434
Endocrine active chemicals	9	0.206	0.0026	79.5	0.098	0.0012	80.6	76	1840	4554
Endocrine active hormones	7	0.689	4.1×10^{-7}	1.7×10^{6}	0.368	1.0×10^{-7}	3.6×10^{6}	1.4×10^{5}	1.2×10^{7}	4.3×10^{7}

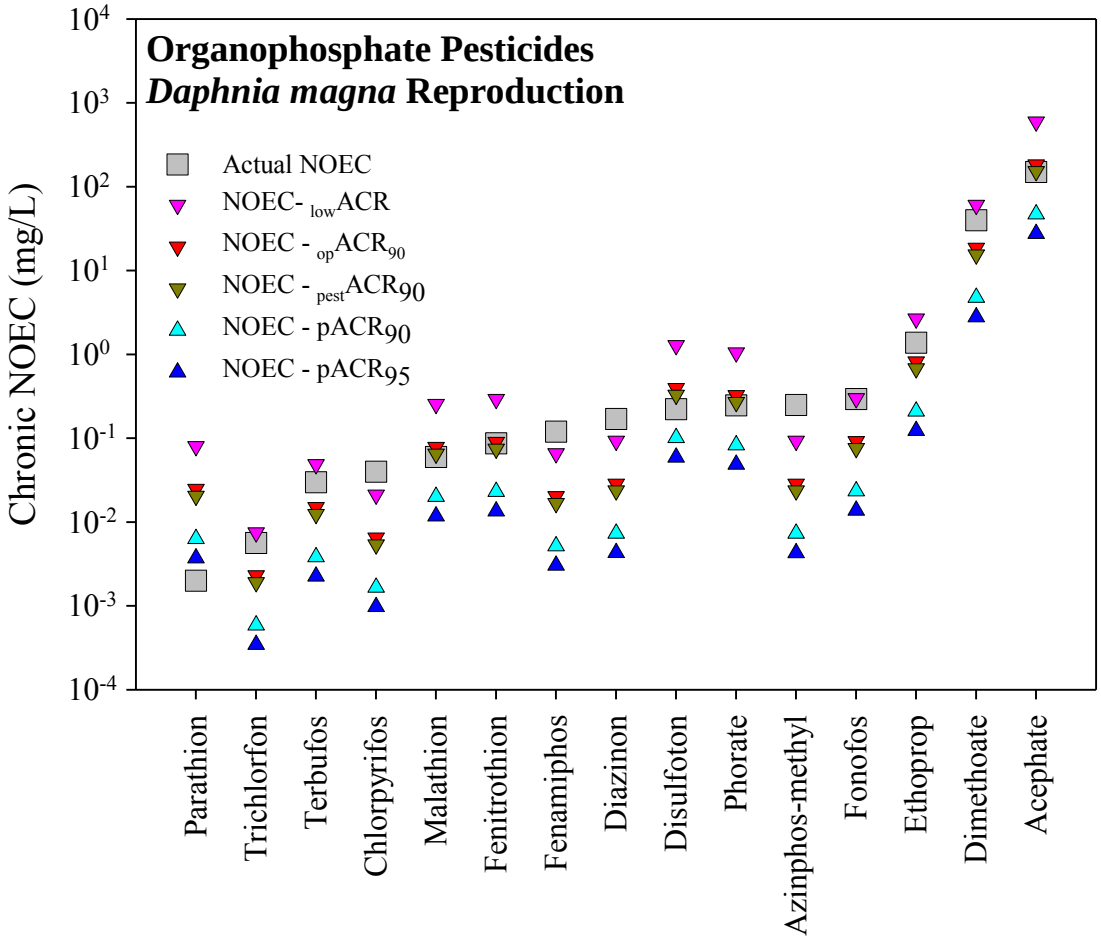


Figure 17. Comparison between the actual chronic threshold NOEC concentrations (■) and ACR predicted NOEC values for organophosphate pesticides. ACR extrapolation values were based upon $_{low}$ACR (24; ▼), $_{OP}$ACR$_{90}$ (78; ▼), $_{pest}$ACR$_{90}$ (94; ▼), and probabilistic estimations pACR$_{90}$ (304; ▲) and pACR$_{95}$ (518; ▲).

Table 18. Evaluation of various ACR extrapolation models from the literature and probabilistic distributions. Evaluation based on the percent of extrapolated ACR chronic responses at or below the actual chronic threshold concentration. For model explanations see Tables 2-3.

Chemical/MOA Group	TAXA	ACR model Name	ACR ID	Percent Below TACV[1]
Organophosphate Pesticides	*Daphnia magna*	$_{low}ACR$	0	26.7%
		$_{OP}ACR_{90}$	1	60.0%
		$_{ACH}ACR_{90}$	3	60.0%
		$_{PEST}ACR_{90}$	4	66.7%
		$_{invert}ACR_{90}$	5	60.0%
		$_{CTD}ACR_{90}$	10	53.3%
		$_{CTD}ACR_{95}$	11	46.7%
		$pACR_{90}$	12	93.3%
		$pACR_{95}$	13	93.3%
Carbamate Pesticides	*Daphnia magna*	$_{low}ACR$	0	50.0%
		$_{CB}ACR_{90}$	2	64.3%
		$_{ACH}ACR_{90}$	3	78.6%
		$_{PEST}ACR_{90}$	4	78.6%
		$_{invert}ACR_{90}$	5	78.6%
		$_{CTD}ACR_{90}$	10	78.6%
		$_{CTD}ACR_{95}$	11	78.6%
		$pACR_{90}$	12	100.0%
		$pACR_{95}$	13	100.0%
Endocrine Active Chemical	Fish	$_{low}ACR$	0	0.0%
		$_{fish}ACR_{90}$	6	60.0%
		$_{fishRP}ACR_{95}$	7	60.0%
		$_{fishRP}ACR_{99}$	8	80.0%
		$_{narc}ACR_{90}$	9	70.0%
		$_{CTD}ACR_{90}$	10	60.0%
		$_{CTD}ACR_{95}$	11	60.0%
		$pACR_{90}$	12	90.0%
		$pACR_{95}$	13	90.0%
Hormones	Fish	$_{low}ACR$	0	0.0%
		$_{fish}ACR_{90}$	6	0.0%
		$_{fishRP}ACR_{95}$	7	0.0%
		$_{fishRP}ACR_{99}$	8	0.0%
		$_{narc}ACR_{90}$	9	0.0%
		$_{CTD}ACR_{90}$	10	100.0%
		$_{CTD}ACR_{95}$	11	100.0%
		$pACR_{90}$	12	100.0%
		$pACR_{95}$	13	100.0%

[1]TACV – threshold of the actual chronic value

Discussion

The objective of the study to was to examine the utility of probabilistic distributions in the development of acute to chronic ratios for biological active compounds. Data from two different groups of chemicals, well known in the literature to have specific toxicological MOAs, were fitted to probabilistic distributions. Two different approaches were used. One applied the chemical toxicity approach to acute and chronic responses with the available data, and then calculated ACRs based on acute and chronic estimates at 5^{th} and 10^{th} centiles. The second approach used the same methodology, but it was applied to ACR values calculated from the data to establish 90^{th} and 95^{th} centile ACR values for each dataset. All of the data fit well within the distributions, as was expected based on the log-normality seen among the acute, chronic, and ACR data, with r^2 values for the regressions between 0.87 and 0.97.

Previous investigators demonstrated that the utility of these probabilistic approaches are that they take an entire available dataset into account in developing a distribution regression (Solomon et al. 2000; Berninger and Brooks 2010; Williams et al. 2011). A group of chemicals, regardless of the level of biological activity, generally represent a continuum of responses. When only percentile is calculated (as in the median value or 90^{th} percentiles reported in the literature) it is possible that the dataset of ACR values is not accurately reflected. A percentile is based upon its numerical position within the dataset; in contrast the probabilistic method uses a regression of all the data to calculate specific centiles. When 90^{th} percentile ACR is calculated for organophosphates and carbamates in this study the values were 124 and 134 respectively, while the probabilistic ACR_{90}s were 304 and 220. In addition to developing estimates based on an

existing dataset, the probabilistic approach allow for estimating the probability of encountering an ACR value for any other AChE inhibitor, now or in the future.

The use of the probabilistic model allows for further analysis of selected centiles employed in previous work (Table 13). If the data used in the model truly represent a group (based on chemicals structure or MOAs) then ACR estimates of a group should be reflective of its variability. As with any model, the quality of the data used to create it is reflected in the quality of the outcome. For example, while Roex et al. (2000) found significant relationships among different MOA groups; for some groups this came at the cost of excluding certain data points. In that study, data points were selected a priori, based on MOA classes. The exclusion of certain data, while improving the regression model, makes the model less inclusive of the actual responses within that MOA class. The objective of this study was to provide ACR estimates that were inclusive of all selected available data for specific MOA classes. With the AChE inhibiting pesticides for example, inclusion of available data also resulted in a statistically significant relationship between acute and chronic endpoints.

Calculating an ACR value for a dataset where only a few chemicals exhibit large ACR values may lead to some misestimation, particularly for biological active chemicals like endocrine active contaminants. However, as more and more MOA specific data is considered, it may be possible to examine other datasets for specific chemical classes. This can be seen in the analysis by Länge et al. (1998) and Raimondo et al. (2007) (Table 13), where larger datasets were divided in specific groups based on chemical classes or likely MOAs (Russom et al. 1998; Verhaar et al. 2000), more specific ACR_{90} values were developed that were more reflective of the specified group. When Raimondo et al.

(2007) focused on specific groups ACR_{90}s became generally better estimations of chemicals within that group regardless if ACR_{90} changed or variability increased. In the present study, endocrine active compounds were considered first as a single group. The resulting probabilistic ACR_{90} was very large ($1.3x10^6$), creating a large over-estimation of ACR values for many of the chemicals within the group. However, when the group was separated to hormones and other endocrine active chemicals, the $pACR_{90}$ for each group was much more reflective of the individual compounds within the groups. However, it is also important to note that due to data scarcity, the present study combined endocrine active compounds with multiple MOAs in fish, but result in the same adverse reproduction outcome (Ankley et al 2009). Clearly future studies should develop pACR90 values based on specific MOAs such as estrogen agonists, androgen agonists, or anti-estrogenic compounds.

In comparing the two different probabilistic methods used in this study an interesting observation was made. For the two pesticide datasets, where the comparison of log transformed acute and chronic data suggested a significant degree of relation between the two responses, using the CTD approach produced low $_{CTD}ACR_{90}$ values (~41) while the $pACR_{90}$ values where much higher (carbamates - 220; organophosphates - 304). In contrast, hormones, which showed no significant relationship between acute and chronic responses, had very similar responses for both $_{CTD}ACR_{90}$ and pACR90. This suggests that when acute and chronic responses are significantly related the probabilistic ACR may be a more appropriate model. For both datasets the distributions developed from data-derived ACR values appeared to be adequately reflective of the data. Such observations highlight a major concern in calculating ACRs for biologically active

compounds: there are generally different MOA for acute and chronic response, which is commonly observed with mammalian data (Calabrese and Baldwin 1993). However, with mammalian data, chemical specific uncertainty factors have been developed that much more sophisticated, incorporating elements of toxicokinetics and toxicodynamics (Meek et al. 2002; Dourson et al. 2007).

Ahlers et al. (2006) noted that the MOA of a chemical may change with exposure concentration, where at acutely toxic exposure levels aquatic mortality may result from a narcosis MOA, while lower exposure levels may result in responses mediated by interaction with a specific target (e.g., receptor, enzyme). The $_{CTD}$ACR approach presented here may provide a tool for this uncoupling of acute and chronic MOA responses. Of course, it also has the greatest potential utility in circumstances for existing chemicals where no paired acute and chronic responses are available, or for chemicals that may be developed in the future. Determining the thresholds of toxicity at the 10^{th} and/or 5^{th} centiles in both acute and chronic CTDs may provide an interim approach for such limited datasets until more appropriate empirical values can be developed.

Probabilistic Acute to Chronic Ratio Uncertainty Factors Appear Useful

The key element in the development of any ACR uncertainty factor is that it must be provide a protective estimation of chronic response. Based on the data available and the approach taken in the present study, probabilistic ACR models seem provide estimations of chronic response that are generally lower than the actual chronic response. The literature reported ACR uncertainty factors (Table 18) in general were not as likely to result in estimations below the threshold, particularly when considering hormones,

where the actual ACR values are very large (>4000) and chronic response thresholds are generally very low (<1 μg/L). Unlike previous approaches the use of a regression type model allows for the inclusion of all available data, rather than just using a few designated points (e.g., median or 90th percentile). In previous approaches the highest values were either eliminated (Roex et al. 2000) from the analysis, or used the 90th percentile, which was more inclusive than the median, still ignored any large ACR data above that point estimate. Berninger and Brooks (2010) previous identified that these large ACR values represent critically important considerations in aquatic toxicology, particularly for chemicals with inherent biologically activities. Using the two different probabilistic approaches described in this exercise appears to provide a means to extrapolate from acute to chronic toxicity that is data-driven, rather than relying on judgment and default uncertainty factors.

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REFERENCES

1) Ahlers J, Riedhammer C, Vogliano M, Ebert RU, Kuhne R, and Schuurmann G. 2006. Acute to chronic ratios in aquatic toxicity - variation across trophic levels and relationship with chemical structure. Environ. Toxicol. Chem. 25: 2937 – 2945

2) American Public Health Association, American Water Works Association, and Water Environment Foundation [APHA]. 1998. Standard methods for the examination of water and wastewater, 20th ed. Amrican Public Health Association, Washington, DC.

3) Ankley GT, Bencic D, Breen M, Collette TW, Conolly R, Denslow ND, Edwards S, Ekman DR, Jensen KM, Lazorchak J, et al. 2009. Endocrine disrupting chemicals in fish: Developing exposure indicators and predictive models of effects based on mechanisms of action. Aquat. Toxicol. 92: 168 – 178.

4) Ankley GT, Black MC, Garric J, Hutchinson TH, and Iguchi T. 2005. A framework for assessing the hazard of pharmaceutical materials to aquatic species. In: Williams RT (Ed.) *Science for Assessing the Impacts of Human Pharmaceutical Materials on Aquatic Ecosystems*. SETAC Press, Pensacola, FL. pp. 183 – 238.

5) Ankley GT, Brooks BW, Huggett DB, and Sumpter JP. 2007. Repeating history: pharmaceuticals in the environment. Environ. Sci. Technol. 41: 8211 – 8217

6) Ankley GT, Jensen KM, Kahl MD, Korte JJ, and Makynen EA. 2001. Description and evaluation of a short-term reproduction test with the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 20: 1276 – 1290.

7) Ankley GT, Jensen KM, Kahl MD, Makynen EA, Blake LS, Greene KJ, Johnson RD, and Villeneuve DL. 2007. Ketoconazole in the fathead minnow (*Pimephales promelas*): reproductive toxicity and biological compensation. Environ. Toxicol. Chem. 26: 1214 – 1223.

8) Ankley GT, Kuehl DW, Kahl MD, Jensen KM, Linnum A, Leino RL, and Villeneuve DA. 2005. Reproductive and developmental toxicity and bioconcentration of perfluorooctanesulfonate (PFOS) in a partial-life cycle test with the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 24: 2316 – 2324.

9) Au-Yeung SCS, Rurak DW, Gruber N, and Riggs KW. 2006. A pharmacokinetic study of diphenhydramine transport across the blood-brain barrier in adult sheep: Potential involvement of a carrier-mediated mechanism. Drug Metab. Dispos. 34: 955 – 960.

10) Barron M. 1999. Are aromatic hydrocarbons the primary determinant of petroleum toxicity to aquatic organisms? Aquat. Toxicol. 46: 253 – 268.

11) Beijersbergen van Henegouwen GMJ, Van de Zijde HJ, Van de Griend J, and De Vries H. 1987. Photochemical decomposition of diphenhydramine in water. Int. J. Pharm. 35: 259 – 262.

12) Berninger JP and Brooks BW. 2010. Leveraging mammalian pharmaceutical toxicology and pharmacology data to predict chronic fish responses to pharmaceuticals. Toxicol. Lett. 193: 69 – 78.

13) Berninger JP, Du B, Connors KA, Eytcheson SA, Prosser KN, Valenti TW, Chambliss CK, and Brooks BW. 2011. Effects of the Antihistamine Diphenhydramine to Selected Aquatic Organisms. Environ. Toxicol.Chem. Accepted manuscript online: 6 June 2011

14) Berninger JP, Williams ES and Brooks BW. 2011. An initial probabilistic hazard assessment of oil dispersants approved by the United States National Contingency Plan. Environ. Toxicol.Chem. 30: 1704 – 1708

15) Beyenbach KW. 2004. Kidney san glomeruli. Am. J. Physiol. Renal Physiol. 286: F811 – F827

16) Bird SB, Gaspari RJ, Lee WJ, and Dickson EW. 2002. Diphenhydramine as a protective agent in rat model of acute, lethal organophosphate poisoning. Acad. Emerg. Med. 9: 1369 – 1372.

17) Boreen AL, Arnold WA, and McNeill K. 2003. Photodegradation of pharmaceuticals in the aquatic environment: A review. Aquat. Sci. 65: 320 – 341.

18) Brain RA, Hanson ML, Solomon KR, and Brooks BW. 2008 Aquatic plants exposed to pharmaceuticals: Effects and risks. Rev. Environ. Contam. Toxicol. 192: 67 – 115.

19) Brain RA, Sanderson H, Sibley PK, and Solomon KR. 2006. Probabilistic ecological hazard assessment: Evaluating pharmaceutical effects on aquatic higher plants as an example. Ecotoxicol. Environ. Saf. 6: 128 – 135.

20) Brain RA, Solomon KR. 2007. A protocol for conducting 7-day daily renewal tests with *Lemna gibba*. Nature Protocols. 2: 979 – 987.

21) Brooks BW, Ankley GT, Hobson JF, Lazorchak JM, Meyerhoff RD, and Solomon KR. 2008. Assessing the aquatic hazards of veterinary medicines. In: Crane, M., Barrett, K., Boxall, A. (Eds). *Effects of Veterinary Medicines in the Environment.* CRC Press, Boca Raton FL. pp. 97 – 128.

22) Brooks BW, Foran CM, Richards SM, Weston J, Turner PK, Stanley JK, Solomon KR, Slattery M, and La Point TW. 2003. Aquatic ecotoxicology of fluoxetine. Toxicol. Lett. 142: 169 – 183.

23) Brooks BW, Huggett DB, and Boxall ABA. 2009. Pharmaceuticals and personal care products: Research needs for the next decade. Environ. Toxicol. Chem. 28: 2469 – 2472.

24) Brooks BW, Huggett DB, Brain RA, and Ankley GT. 2010. Risk assessment considerations for veterinary medicines in aquatic systems. In: Henderson, K., Coats, J., (Eds). *Veterinary Pharmaceuticals in the Environment*. American Chemical Society, Washington DC. p 256

25) Brooks BW, Riley TM, and Taylor RD. 2006. Water quality of effluent-dominated ecosystems: ecotoxicological, hydrological, and management considerations. Hydrobiologia. 556: 365 – 379.

26) Brown NJ and Roberts II LJ. 2001. Histamine, bradykinin, and their antagonists. In Hardman JG, Limbird LE, eds, *Goodman and Gilman's Pharmacological Basis of Therapeutics,* 10th ed. McGraw Hill, New York, NY, USA, p 656.

27) Burmaster DE and Hull DA. 1997. Using lognormal distributions and lognormal probability plots in probabilistic risk assessments. Hum. Ecol. Risk Assess. 3: 235 – 255.

28) Calabrese EJ and Baldwin LA. 1993. *Performing Ecological Risk Assessments.* Lewis, Chelsea, MI, USA. p 257

29) Calabrese EJ. 1987. Extrapolation from animal data. In: Tardiff RG and Rodricks JV (eds), *Toxic Substances and Human Risk: Principles of Data Interpretation*. Plenum Press, New York, NY, USA. pp 269 – 280

30) Caldwell DJ, Mastrocco F, Hutchinson TH, Lange R, Heijerick D, Janssen C, Anderson PD, and Sumpter JP. 2008. Derivation of an aquatic predicted no effect concentration for 17α-ethinyl estradiol. Environ. Sci. Tech. 42: 7046 – 7054.

31) Carls MG, Holland L, Larsen M, Collier TK, Scholz NL, and Incardona JP. 2008. Fish embryos are damaged by dissolved PAHs, not oil particles. Aquat. Toxicol. 88: 121 – 127.

32) Carvalho FD, Machado I, Martınez MS, Soares A, and Guilhermino L. 2003. Use of atropine-treated *Daphnia magna* survival for detection of environmental contamination by acetylcholinesterase inhibitors. Ecotoxicol. Environ. Saf. 53: 43 – 46.

33) Chapman H, Purnell K, Law RJ, and Kirby MF. 2007. The use of chemical dispersants to combat oil spills at sea: A review of practice and research needs in Europe. Mar. Pollut. Bull. 54: 827 – 838.

34) Chapman PM, Fairbrother A, and Brown D. 1998. A critical evaluation of safety (uncertainty) factors for ecological risk assessment. Environ. Toxicol. Chem. 17: 99 – 108.

35) ChemIDplus. 2008. Available from: http://chem.sis.nlm.nih.gov/chemidplus/chemidlite.jsp National Library of Medicine, Bethesda MD, USA.

36) Clayton JR, Payne JR, Tsang SF, Frank V, Marsden P, and Harrington J. 1992. Chemical oil spill dispersant update state-of-the-art on mechanism of action and laboratory testing for performance. EPA/600/S-92/065. US Enivironmental Protection Agency, Cincinnati, OH.

37) Clubbs RL and Brooks BW. 2007. Daphnia magna responses to a vertebrate estrogen receptor agonist and antagonist: A multigenerational study. Ecotoxicol. Environ. Saf. 67: 385 – 398

38) Crane M, Watts C, and Boucard T. 2006. Chronic aquatic environmental risks from exposure to human pharmaceuticals. Sci. Total Environ. 367: 23 – 41.

39) Daughton CG and Brooks BW. 2011. Active pharmaceuticals ingredients and aquatic organisms. In Meador J, Beyer N eds, *Environmental Contaminants in Wildlife: Interpreting Tissue Concentrations*, 2nd ed. CRC, Boca Raton, FL, USA, pp 281 – 341.

40) de Wolf W, Siebel-Sauer A, Lecloux A, Koch V, Holt M, Feijtel T, Comber M, and Boeije G. 2005. Mode of action and aquatic exposure thresholds of no concern. Environ. Toxicol. Chem. 24: 479 – 485.

41) Delistraty D, Taylor B, and Anderson R. 1998. Comparisons of acute toxicity of selected chemicals to rainbow trout and rats. Ecotoxicol. Environ. Saf. 39: 195 – 200.

42) Dietrich DR and Prietz A. 1999. Fish embryotoxicity and teratogenicity of pharmaceuticals, detergents and pesticides regularly detected in sewage treatment plant effluents and surface waters. Toxicologist. 48 (1-S): 151.

43) Dobbins LL, Brain RA, and Brooks BW. 2008. Comparison of the sensitivities of common *In Vitro* and *In Vivo* assays of estrogenic activity: Application of chemical toxicity distributions. Environ. Toxicol. Chem. 27: 2608 – 2616.

44) Dobbins LL, Usenko S, Brain RA, and Brooks BW. 2009. Probabilistic ecological hazard assessment of parabens using *Daphnia magna* and *Pimephales promelas*. Environ. Toxicol. Chem. 28: 2744 – 2753.

45) Dourson ML and Parker AL. 2007. Past and future use of default assumptions and uncertainty factors: default assumptions, misunderstandings, and new concepts. Hum. Ecol. Risk Assess. 13: 82 – 87.

46) Dourson ML and Stara JF. 1983. Regulatory history and experimental support of uncertainty (safety) factors. Reg. Toxicol. Pharmacol. 3: 224 – 238.

47) Duke LD and Taggart M. 2000. Uncertainty Factors in Screening Ecological Health Assessments. Environ. Toxicol. Chem. 19: 1668 – 1680

48) Dzialowski EM, Turner PK, and Brooks BW. 2006. Physiological and reproductive effects of beta adrenergic receptor antagonists on *Daphnia magna*. Arch. Environ. Contam. Toxicol. 50: 503 – 510.

49) Escher BI and Hermens JL. 2002. Modes of Action in Ecotoxicology: Their Role in the Body Burden, Species Sensitivity, QSAR, and Mixture Effects. Environ. Sci. Technol. 36: 4201 – 4217.

50) Fent K, Weston AA, and Caminada D. 2006. Ecotoxicology of human pharmaceuticals. Aquat. Toxicol. 76: 122 – 159.

51) Ferrer I, Heine CE, and Thurman EM. 2004. Combination of LC/TOF-MS and LC/ion trap MS/MS for the identification of diphenhydramine in sediment samples. Anal. Chem. 76: 1437 – 1444.

52) Fick J, Lindberg RH, Tysklind M, and Larsson DGJ. 2010. Predicted critical environmental concentrations for 500 pharmaceuticals. Reg. Toxicol. Pharm. 58: 516 – 523.

53) Fitzsimmons PN, Fernadez JD, Hoffman AD, Butterworth BC, and Nichols JW. 2001. Branchial eliminationof superhydrophobic organic compounds by rainbow trout (*Oncorhynchus mykiss*). Aquat. Toxicol. 5: 23 – 34.

54) Foran CM, Weston JJ, Slattery M, Brooks BW, and Huggett DB. 2004. Reproductive assessment of Japanese medaka (Oryzias latipes) following a four-week fluoxetine exposure. Arch. Environ. Contam. Toxicol. 46: 511 – 517.

55) Forbes VE and Calow P. 2002. Extrapolation in ecological risk assessment: Balancing pragmatism and precaution in chemical controls legislation. BioScience. 52: 249 – 257.

56) Gould GG, Brooks BW, and Frazer A. 2007. [3H] citalopram binding to serotonin transporter sites in minnow brains. Basic Clin. Pharmacol. Toxicol. 101: 203 – 210.

57) Gross M, Daginnus K, Deviller G, de Wolf W, Dungey S, Galli C, Gourmelon A, Jacobs M, Matthiessen P, Micheletti C, Nestmann E, et al. 2010. Thresholds of toxicological concern for endocrine active substances in the aquatic environment. Integr. Environ. Assess. Manag. 6: 2 – 11.

58) Gunnarsson L, Jauhiainen A, Kristiansson E, Nerman O, and Larsson DGJ. 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ. Sci. Technol. 42: 5807 – 5813.

59) Haas HL, Sergeeva OA, and Selbach O. 2008. Histamine in the nervous system. Physiol. Rev. 88: 1183 – 1241.

60) Harries JE, Runnalls T, Hill E, Harris CA, Maddix S, Sumpter JP, and Tyler CR. 2000. Development of a Reproductive Performance Test for Endocrine Disrupting Chemicals Using Pair-Breeding Fathead Minnows (*Pimephales promelas*). Environ. Sci. Technol. 34: 3003 – 3011.

61) Hattis DB and Burmaster DE. 1994. Assessment of variability and uncertainty distributions for practical risk assessments. Risk Analysis. 14: 713 – 730.

62) Hazardous Substances Data Bank, 2008. National Library of Medicine, Bethesda MD, USA. Available from: http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB.

63) Heberer T. 2002. Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment: a review of recent research data. Toxicol. Lett. 131: 5 – 17.

64) Hemmer MJ, Barron MG, and Greene RM. 2010a. USEPA dispersant toxicity testing: Comparative toxicity of eight oil dispersant products on two Gulf of Mexico aquatic test species. Office of Research and Development, National Health and Environmental Effects Research Laboratory, Washington DC, pp 1-11.

65) Hemmer MJ, Barron MG, and Greene RM. 2010b. USEPA dispersant toxicity testing: Comparative toxicity of Louisiana sweet crude oil (LSC) and chemically dispersed LSC to Two Gulf of Mexico aquatic test species. National Health and Environmental Effects Research Laboratory, Washington DC, pp 1-13.

66) Hemming JM, Turner PK, Brooks BW, Waller WT, and La Point TW. 2002. Assessment of toxicity reduction in wastewater effluent flowing through a treatment wetland using *Pimephales promelas*, *Ceriodaphnia dubia*, and *Vibrio fischeri*. Arch. Environ. Contam. Toxicol. 42: 9 – 16.

67) Hinton DE, Hardman RC, Kullman SW, Law JM, Schmale MC, Walter RB, Winn RN, and Yoder JA. 2009. Aquatic animal models of human disease: select papers and recommendations from the 4th conference (Editorial). Comp. Biochem. Physio. C: Toxicol. Pharmacol. 149: 121 – 128.

68) Hodge HC and Sterner JH. 1949. Tabulation of toxicity classes. Am. Ind. Hygiene Assoc. Q. 10: 93.

69) Huggett DB, Brooks BW, Peterson B, Foran CM, and Schlenk D. 2002. Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-blockers) on aquatic organisms. Arch. Environ. Contam. Toxicol. 43: 229 – 235.

70) Huggett DB, Cook JC, Ericson JF, and Williams RT. 2003. A theoretical model for utilizing mammalian pharmacological and safety data to prioritize potential impacts of human pharmaceuticals to fish. Hum. Ecol. Risk Assess. 9: 1789 – 1799.

71) Hutchinson TH, Yokota H, Hagino S, and Ozato K. 2003. Development of fish tests for endocrine disruptors. Pure Appl. Chem. 75: 2343 – 2353.

72) Ishibashi H, Matsumura N, Hirano M, Matsuoka M, Shiratsuchi H, Ishibashi Y, Takao Y, and Arizono K. 2004. Effects of triclosan on the early life stages and reproduction of medaka Oryzias latipes and induction of hepatic vitellogenin. Aquat. Toxicol. 67: 167 – 179.

73) Janarda SK, Olson CS, and Schaeffer DJ. 1984. Quantitative comparisons of acute toxicity of organic chemicals to rat and fish. Ecotoxicol. Environ. Saf. 8: 531 – 539.

74) Jensen KM, Kahl MD, Makynen EA, Korte JJ, Leino RL, Butterworth BC, and Ankley GT. 2004. Characterization of responses to the antiandrogen flutamide in a short-term reproduction assay with the fathead minnow. Aquat. Toxicol. 70: 99–110.

75) Jensen KM, Makynen EA, Kahl MD, and Ankley GT. 2006. Effects of the Feedlot Contaminant 17α- Trenbolone on Reproductive Endocrinology of the Fathead Minnow. Environ. Sci. Technol. 40: 3112 – 3117.

76) Jobling S, Casey D, Rodgers-Gray T, Oehlmann J, Schulte-Oehlmann U, Pawlowski S, Baunbeck T, Turner AP, and Tyler CR. 2004. Comparative responses of mollusks and fish to environmental estrogens and an estrogenic effluent. Aquat. Toxicol. 66: 207 – 222.

77) Judson R, Richard A, Dix DJ, Houck K, Martin M, Kavlock R, Dellarco V, Henry T, Holderman T, Sayre P, Tan S, Carpenter T, and Smith E. 2009. The Toxicity Data Landscape for Environmental Chemicals. Environ. Health Persp. 117: 685 – 695

78) Judson RS, Martin MT, Reif DM, Houck KA, Knudsen TB, Rotroff DM, Xia M, Sakamuru S, Huang R, Shinn P, Austin CP, Kavlock RJ, Dix DJ. 2010. Analysis of eight oil spill dispersants using rapid, in vitro tests for endocrine and other biological activity. Environ. Sci. Technol. 44: 5979 – 5985.

79) Kang IJ, Yokota H, Oshima Y, Tsuruda Y, Yamaguchi T, Maeda M, Imada N, Takdokoro H, Honjo T. 2002. Effect of 17B-estradiol on the reproduction of Japanese medaka (Oryzias latipes). Chemosphere. 47: 71 – 80.

80) Kavlock RJ, Ankley GT, Blancato J, Breen M, Conolly R, Dix D, Houck K, Hubal E, Judson R, Rabinowitz J, et al. 2008. Computational Toxicology—A State of the Science Mini Review. Toxicol.l Sci. 103: 14 – 27.

81) Kenaga EE. 1982. Predictability of chronic toxicity from acute toxicity in fish and aquatic invertebrates. Environ. Toxicol. Chem. 1: 347 – 358

82) Kim Y, Choi K, Jung J, Park S, Kim P, and Park J. 2007. Aquatic toxicity of acetaminophen, carbamazepine, cimetidine, diltiazem and six major sulfonamides, and their potential ecological risks in Korea. Environ. Int. 33: 370 – 375.

83) Kirby MF and Law RJ. 2008. Oil spill treatment products approval: the UK approach and potential application to the Gulf region. Mar. Pollut. Bull. 56: 1243 – 1247.

84) Knox C, Law V, Jewison T, Liu P, Ly S, Frolkis A, Pon A, Banco K, Mak C, Neveu V, et al. 2011. DrugBank 3.0: a comprehensive resource for 'omics' research on drugs. Nucleic Acids Res. 39: D1035 – D1041.

85) Kosonen J and Kronberg K. 2009. The occurrence of antihistamines in sewage waters and in recipient rivers. Environ. Sci. Pollut. Res. Int. 16: 555 – 564.

86) Kroes R, Kleiner J, and Renwick A. 2005. The threshold of toxicological concern concept in risk assessment. Toxicol. Sci. 86: 226 – 230.

87) Länge R, Hutchinson TH, Croudance CP, Siegmund F, Schweinfurth H, Hampe P, Panter GH, Sumpter JP. 2001. Effects of the synthetic estrogen 17α –ethinyl estradiol on the life cycle of the fathead minnow (Pimephales promelas). Environ. Toxicol. Chem. 20: 1216 – 1227

88) Länge R, Hutchinson TH, Scholz N, and Solbe J. 1998. Analysis of the ECETOC Aquatic Toxicity (EAT) Database II – Comparison of Acute to Chronic Ratios for Various Aquatic Organisms and Chemical Substances. Chemosphere 36: 115 – 127.

89) Martinovic D, Blake LS, Durhan EJ, Greene KJ, Kahl MD, Jensen KM, Makynen EA, Villeneuve DL, and Ankley GT. 2008. Reproductive toxicity of vinclozolin in the fathead minnow: confirming an anti-androgenic mode of action. Environ. Toxicol. Chem. 27: 478 – 488.

90) Mattes WB. 2006. Cross-species comparative toxicogenomics as an aid to safety assessment. Expert Opin. Drug Metabol. Toxicol. 2: 859 – 874

91) Meek ME, Renwick A, Ohanian E, Dourson ML, Lake B, Naumann BD, and Vu V. 2002. Guidelines for application of chemical-specific adjustment factors in dose/concentration-response adjustment. Toxicol. 181-182: 115 – 120

92) Meinertz JR, Schreier TM, Bernardy JA, and Franz JL. 2010. Chronic toxicity of diphenhydramine hydrochloride and erythromycin thiocyanate to daphnia, *Daphnia magna*, in a continuous exposure test system. Bull. Environ. Contam. Toxicol. 85: 447 – 451.

93) Middaugh DP and Whiting DD. 1995. Responses of embryonic and larval inland silversides, *Menidia beryllina*, to No. 2 fuel oil and oil dispersants in seawater. Arch. Environ. Contam. Toxicol. 39: 535 – 539.

94) Minagh E, Hernan R, O'Rourke K, Lyng FM, and Davoren M. 2009. Aquatic ecotoxicity of the selective serotonin reuptake inhibitor sertraline hydrochloride in a battery of freshwater test species. Ecotoxicol. Environ. Saf. 72: 434 – 440.

95) Mount DI and Stephan CE. 1967. A method for establishing acceptable limits for fish – Malathion and the butoxyethanol ester of 2,4-D. Trans. Am. Fish. Soc. 96: 185 – 193.

96) Munro I. 1990. Safety assessment procedures for indirect food additives: an overview. Reg. Toxicol. Pharmacol. 12: 2 – 12.

97) Munro IC, Renwick AG, and Danielewska-Nikiel B. 2008. The threshold of toxicological concern (TTC) in risk assessments. Toxicol. Lett. 180: 151 – 156.

98) Nakamura Y, Yamamoto H, Sekizawa J, Kondo T, Hirai N, and Tatarazako N. 2008. The effects of pH on fluoxetine in Japanese medaka (*Oryzias latipes*): Acute toxicity in fish larvae and bioaccumulation in juvenile fish. Chemosphere. 70: 865 – 873.

99) National Research Council. 2005. Dispersant-oil interactions and effectiveness testing. In Committee on Understanding Oil Spill Dispersants Efficacy and Effects . *Oil Spill Dispersants: Efficacy and Effects*. National Academies Press, Washington, DC, USA, pp 51 – 134

100) Nunes B, Carvalho F, and Guilhermino L. 2005. Acute toxicity of widely used pharmaceuticals in aquatic *species: Gambusia holbrooki, Artemia parthenogenetica* and *Tetraselmis chuii.* Ecotoxicol. Environ. Saf. 61: 413 – 419.

101) O'Neil MJ. (Ed). 2006. The Merck Index : An Encyclopedia of Chemicals, Drugs, and Biological, 14th ed. Merck, Whitehouse Station, N.J. p 4896.

102) Oakes K, Coors A, Escher B, Fenner K, Garric J, Gust M, Knacker T, Kuster A, Kussatz C, Metcalfe C, Monteiro S, et al. 2010. An environmental risk assessment for the serotonin re-uptake inhibitor fluoxetine: A case study utilizing the European risk assessment framework. Integr. Environ. Assess. Manag. 6: 524 – 539.

103) Organization for Economic Cooperation and Development [OCED]. 1995. Guidance document for aquatic effects assessment. Environmental Monograph 92. OECD Publishing, Paris, France.

104) Organization for Economic Co-operation and Development [OECD]. 2008. Test No. 211: Daphnia magna Reproduction Test. In: *OECD Guidelines for the Testing of Chemicals, Section 2: Effects on Biotic Systems.* OECD Publishing, Paris, France.

105) Orzechowski RF, Currie DS, and Valancius CA. 2005. Comparative anticholinergic activities of 10 histamine H1 receptor antagonists in two functional models. Eur. J. Pharmacol. 506: 257 – 264.

106) Owen SF, Giltrow E, Huggett DB, Hutchinson TH, Saye J, Winter MJ, and Sumpter JP. 2007. Comparative physiology, pharmacology and toxicology of beta-blockers: mammals versus fish. Aquat. Toxicol. 82: 145 – 162.

107) Parrott JL and Blunt BR. 2005. Life-cycle exposure of fathead minnows (*Pimephales promelas*) to an ethinylestradiol concentration below 1 ng/L reduces egg fertilization success and demasculinizes males. Environ. Toxicol. 20: 131 – 141.

108) Physicians' Desk Reference 62nd ed. 2008. Thomson PDR, Montvale, NJ p 3482.

109) Powers DA. 1989. Fish as model systems. Science. 246: 352 – 358.

110) Printes LB and Callaghan A. 2004. A comparative study on the relationship between acetylcholinesterase activity and acute toxicity in *Daphnia magna* exposed to anticholinesterase insecticides. Environ.Toxicol. Chem. 23: 1241 – 1247.

111) Raimondo S, Montague BJ, and Barron MG. 2007. Determinants of the variability in actue to chronic toxicity ratios for aquatic invertebrates and fish. Environ.Toxicol. Chem. 26: 2019 – 2023.

112) Raimondo S, Vivian DN, and Barron MG. 2010. Web-based Interspecies Correlation Estimation (Web-ICE) for Acute Toxicity: User Manual. Version 1.1. EPA/600/R-10/004. United States Environmental Protection Agency, Gulf Breeze, FL.

113) Ramachandran SD, Hodson PV, Khan CW, and Lee K. 2004. Oil dispersant increases PAH uptake by fish exposed to crude oil. Ecotoxicol. Environ. Saf. 59: 300 – 308.

114) Ramirez AJ, Brain RA, Usenko S, Mottaleb MA, O'Donnell JG, Stahl LL, Wathen JB, Snyder BD, Pitt JL, Perez-Hurtado P, Dobbins LL, Brooks BW, and Chambliss CK. 2009. Occurrence of pharmaceuticals and personal care products in fish: Results of a national pilot study in the United States. Environ. Toxicol. Chem. 28: 2587 – 2597.

115) Ramirez AJ, Mottaleb MA, Brooks BW, and Chambliss CK. 2007. Analysis of pharmaceuticals in fish using liquid chromatography-tandem mass spectrometry. Anal. Chem. 79: 3155 – 3163.

116) Rand GM (Ed.). 1995. Fundamentals of Aquatic Toxicology: Effects, Environmental Fate, and Risk Assessment, Taylor & Francis, Washington, DC.

117) Rhomberg LR and Lewanowski TA. 2006. Methods for identifying a default cross-species scaling factor. Hum. Ecol. Risk Assess. 12: 1094 – 1127.

118) Roex EWM, Van Gestel CA, Van Wezel AP, Van Straalen NM. 2000. Ratios between acute aquatic toxicity and effects on population growth rates in relation to toxicant mode of action. Environ.Toxicol. Chem. 19:685 – 693.

119) Russom CL, Bradbury SP, Boderius SJ, Hammermeister DE, and Drummond, RA. 1997. Predicting modes of toxic action from chemical structure: acute toxicity in the fathead minnow (*Pimephales promelas*). Environ. Toxicol. Chem. 16: 948 – 967.

120) Russom CL, Breton RL, Walker JD, and Bradbury SP. 2003. An overview of the use of quantitative structure-activity relationships for ranking and prioritizing large chemical inventories for environmental risk assessments. Environ. Toxicol. Chem. 22: 1810 – 1821.

121) Sanderson H and Solomon K. 2009. Contaminants of emerging concern challenge ecotoxicology. Environ.Toxicol. Chem. 28: 1359 – 1360.

122) Sanderson H and Thomsen M. 2007. Ecotoxicological quantitative structure activity relationships for pharmaceuticals. Bull. Environ. Contam. Toxicol. 79: 331 – 335.

123) Sanderson H and Thomsen M. 2009. Comparative analysis of pharmaceuticals versus industrial chemicals acute aquatic toxicity classification according to the United Nations classification system for chemicals: assessment of the (Q)SAR predictability of pharmaceuticals acute aquatic toxicity and their predominant acute toxic mode-of-action. Toxicol. Lett. 187: 84 – 93.

124) Scherrer RA and Howard SM. 1977. Use of distribution coefficients in quantitative structure-activity relationships. J. Med. Chem. 20: 53 – 58.

125) Schulz M and Schmoldt A. 1996. Therapeutic and toxic blood concentrations of more than 500 drugs. Pharmazie. 52: 885 – 911

126) Schwaiger J, Ferling H, Mallow U, Wintermayr H, and Negele RD. 2004. Toxic effects of the non-steroidal anti-inflammatory drug diclofenac. Part I: histopathological alterations and bioaccumulation in rainbow trout. Aquat. Toxicol. 68: 141 – 150

127) Schweinfurth H, Lange R, and Gunzel P. 1996. Environmental fate and ecological effect of steroidal estrogens. In: *Oestrogenic Chemicals in the Environment.* IBC Conference Proceedings, May 9-10; London. p 1-30.

128) Seki M, Yokota H, Matsubara H, Maeda M, Tadokoro H, Kobayashi K. 2003. Effects of 4-tert-pentylphenol on medaka (Oryzias latipes). Environ. Toxicol. Chem. 22:1487–1496.

129) Slooff W, Vanoers JAM, and Dezwart D. 1986. Margins of Uncertainty in Ecotoxicological Hazard Assessment. Environ. Toxicol. Chem. 5: 841 – 852.

130) Sohoni P, Tyler CR, Hurd K, Cuunter J, Hetheridge M, Williams T, Woods C, Evans M, Toy R, Gargas M, and Sumpter JP. 2001 Reproductive effects of Long-Term Exposure to Bisphenol A in the Fathead Minnow (*Pimephales promelas*). Aquat. Toxicol. 35: 2917 – 2925.

131) Solomon KR and Takacs P. 2002. Probabilistic risk assessment using species sensitivity distributions. In: Posthuma, L., Traas, T., Suter, G. (Eds.), *Species Sensitivity Distributions in Risk Assessment.* CRC Press, Boca Raton, FL, USA p 564

132) Solomon KR, Giesy JP, and Jones P. 2000. Probabilistic risk assessment of agrochemicals in the environment. Crop Prot. 19: 649–655.

133) Stackelberg PE, Furlong ET, Meyer MT, Zaugg SD, Henderson HK, and Reissman DB. 2004. Persistence of pharmaceutical compounds and other organic wastewater contaminants in a conventional drinking-water treatment plant. Sci. Total Environ. 329: 99 – 113.

134) Stanley JK, Ramirez AJ, Chambliss CK, and Brooks BW. 2007. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. Chemosphere. 69: 9 – 16.

135) Stanley JK, Ramirez AJ, Mottaleb M, Chambliss CK, and Brooks BW. 2006. Enantio-specific toxicity of the b-blocker propranolol to *Daphnia magna* and *Pimephales promelas*. Environ. Toxicol. Chem. 25: 1780 – 1786.

136) Swannell RP and Daniel F. 1999. Effect of dispersants on oil biodegradation under simulated marine conditions. *Proceedings,* 1999 International Oil Spill Conference. American Petroleum Institute, Washington, DC, pp 169-176.

137) Thorpe KL, Benstead R, Hutchinson TH, and Tyler CR. 2007. Association between altered vitellogenin concentrations and adverse health effects in fathead minnows (*Pimephales promelas*). Aquat. Toxicol. 85: 176 – 183.

138) Thummel KE and Shen DD. 2001. Design and optimization of dosage regimens: pharmacokinetic data. In: Hardman, J.G., Limbird, L.E. (Eds), *Goodman and Gilman's The Pharmacological Basis of Therapeutics*, 10th ed. McGraw-Hill, New York, USA, pp 1917–2023.

139) Triebskorn R, Casper H, Scheil V, and Schwaiger J. 2007. Ultrastructural effects of pharmaceuticals (carbamazepine, clofibric acid, metoprolol, diclofenac) in rainbow trout (*Oncorhynchus mykiss*) and common carp (*Cyprinus carpio*). Anal. Bioanal. Chem. 387: 1405 – 1416

140) U. S. Environmental Protection Agency [US EPA]. 1995. Water Quality Guidance for the Great Lakes System; Final Rules. Federal Register 60(56) 15366-425

141) U.S. Environmental Protection Agency [US EPA]. 1981. Results: interlaboratory comparison- acute toxicity tests using estuarine animals. EPA-600/4-81-003. Environmental Research Laboratory, Gulf Breeze, FL, pp 1-16.

142) U.S. Environmental Protection Agency [US EPA]. 1997. Swirling Flask Dispersant Effectiveness Test, Revised Standard Dispersant Toxicity Test, and Bioremediation Agent Effectiveness Test. 40 CFR 300 Appendix C 62 FR 35447. U. S. Government Printing Office, Washington, DC. pp 170-188.

143) U.S. Environmental Protection Agency [US EPA]. 2001. Final Report: Inter-laboratory Variability Study of EPA Short-term Chronic and Acute Whole Effluent Toxicity Test Methods, Vol 1. EPA 821-B-01-004. Washington, DC, pp 117-118; 134.

144) U.S. Environmental Protection Agency [US EPA]. 2009a. Web-based Interspecies Correlation Estimation Software. http://www.epa.gov/ceampubl/fchain/webice/index.html. United States Environmental Protection Agency, Washington, DC, USA.

145) U.S. Environmental Protection Agency [US EPA]. 2009b. Estimation Programs Interface Suite™ for Microsoft® Windows, v 4.00. United States Environmental Protection Agency, Washington, DC, USA.

146) U.S. Environmental Protection Agency [US EPA]. 2010. National Oil and Hazardous Substances Pollution Contingency Plan Subpart J. 40 CFR 300. U. S. Government Printing Office, Washington, DC.

147) U.S. Environmental Protection Agency [US EPA]. 1994. 10-day Chronic toxicity test using *Daphnia magna* or *Daphnia pulex*. Compendium of ERT Standard Operating Protocols, SOP #2028. Office of Solid Waste and Emergency Response, Edison, NJ.

148) U.S. Environmental Protection Agency [US EPA]. 2002a. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 5th ed. EPA-821-R-020-012. Office of Research and Development, Washington DC.

149) U.S. Environmental Protection Agency [US EPA]. 2002b. Short-term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA-821-R-02-013. Office of Research and Development Washington, DC.

150) Valenti TW, Perez-Hurtado P, Chambliss CK, and Brooks BW. 2009. Aquatic toxicity of sertraline to Pimephales promelas at environmentally relevant surface water pH. Environ. Toxicol. Chem. 28: 2685 – 2694

151) van Wezel AP and Opperhuizen A. 1995. Narcosis due to enviornmental pollutants in aquatic organisms: Residue-based toxicity, mechanisms, and membrane burdens. Crit. Rev. Toxicol. 25: 255 – 279

152) Verhaar HJ, Solbe J, Speksnijder J, van Leeuwen CJ, and Hermens JL. 2000. Classifying environmental pollutants: Part 3. External validation of the classification system. Chemosphere. 40: 875 – 883.

153) Verispan, LLC. 2008 Top 200 Drugs for 2008 by Sales Vector One® National (VONA), http://www.drugs.com/top200.html (accessed 03/30/2009)

154) Versonnen BJ, Arus K, Verslycke T, Lema W, and Janssen CR. 2003. In vitro and invo estrogenicity and toxicity of o- m- and p- dichlorobenezene. Environ. Toxicol. Chem. 22: 329 – 335

155) Williams ES, Berninger JP, and Brooks BW. 2011. Application of chemical toxicity distributions to ecotoxicology data requirements under REACH. Environ. Toxicol. Chem. 30: 1943 – 1954

156) Williams ES, Panko J, and Paustenbach DJ. 2009. The European Union's REACH regulation: a review of its history and requirements. Crit. Rev. Toxicol. 39: 553 – 575.

157) Williams TD, Caunter JE, Lillicrap AD, Hutchinson TH, Gillings EG, and Duffell S. 2007. Evaluation of the reproductive effects of tamoxifen citrate in partial and full life-cycle studies using fathead minnows (*Pimephaeles promelas*). Environ. Toxicol. Chem. 26: 695-707

158) Winter MJ. Lillicrap AD, Caunter JE, Schaffner C, Alder AC, Ramil M, Ternes TA, Giltrow E, Sumpter JP, and Hutchinson TH. 2008. Defining the chronic impacts of atenolol on embryo-larval development and reproduction in the fathead minnow (*Pimephales promelas*). Aquat. Toxicol. 86: 361 – 369.

159) Wishart DS, Knox C, Guo AC, Cheng D, Shrivastava S, Tzur D, Gautam B, and Hassanali M. 2008. DrugBank: a knowledgebase for drugs, drug actions and drug targets. Nucleic Acids Res.36(Database issue): D901 – D906.

160) Wong DT, Perry KW, and Bymaster FP. 2005. The discovery of fluoxetine hydrochloride (Prozac). Nat. Rev. Drug Discov. 4: 764 – 774.

161) Zeillinger J, Steger-Hartmann T, Maser E, Goller S, Vonk R, and Lange, R. 2009. Effects of synthetic gestrogens on fish reproduction. Environ. Toxicol. Chem. 28: 2663 – 2670.

162) Zhou SN, Oakes KD, Servos MR, and Pawliszyn J. 2008. Application of solid-phase microextraction for in vivo laboratory and field sampling of pharmaceuticals in fish. Environ. Sci. Technol. 42: 6073 – 6079.