ABSTRACT

Advancing an Understanding of Ecological Risk Assessment Approaches for Ionizable Contaminants in Aquatic Systems.

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Freshwater is increasingly becoming a finite resource in many regions of the world. Gaps between estimated water supply and demand continue to narrow and the prospects of acquiring additional sources of freshwater remain limited. Furthermore, economically efficient water resource management practices are perplexed by increasing urbanization and changing land-use in semi-arid regions. Although repeated use of water is a practical and effective means for easing strain on water supplies, there is concern that unnecessary contamination may diminish future value of this important resource. Some surface waters in semi-arid regions of the U.S. are effluent-dominated as flow is comprised of >90% treated wastewater. Ionizable compounds are chemicals often associated with urban development and examples include pharmaceuticals, agrochemicals, natural toxins, and other common contaminants (e.g. ammonia). Because continued population growth and urbanization are likely to increase contaminant release and alter dilution capacity of receiving systems, it is important that best management approaches are developed at the watershed scale to limit water quality degradation

associated with ionizable compounds. Current methods for prospective and retrospective ecological risk assessments of ionizable compounds seldom consider site-specific conditions during the analysis of effects of phase. Ionization state is largely controlled by the acid/base dissociation constant (pKa) and pH of the solution where a compound resides. Stream water quality can therefore influence ionization state, which is important because the unionized forms a more lipophilic and have a greater propensity to cross cellular membranes. Consequently, the unionized forms are hypothetically more toxic. I completed toxicity tests in the laboratory using various contaminants as model ionizable compounds over a gradient of environmentally-relevant surface water pH and then related measured toxicological endpoints to observed pH of surface waters using both discrete and probabilistic ecological risk assessment approaches. The result of my studies clearly demonstrated that site-specific pH may influence the toxicity of ionizable contaminants. Potential modifications to conceptual frameworks of ecological risk assessment for ionizable contaminants are suggested so that uncertainty can be reduced.

Advancing an Understanding of Ecological Risk Assessment Approaches for Ionnizable Contaminants in Aquatic Systems

by

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A Dissertation

Approved by the Institute of Ecological, Earth, and Environmental Science

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DEDICATION

To my family

CHAPTER ONE

Introduction

General Overview

Freshwater is increasingly becoming a finite resource in Texas and at the global scale (Gleick 2003a). The gap between estimated water supply and demand in the state is narrowing, and the prospect of acquiring additional sources of freshwater are limited (Oki and Kanae 2006). Furthermore, rapid urbanization in select regions (Murdock et al. 1997) perplexes economically efficient water resource management practices. Population growth may continue to proportionally increase demand for municipal water use and further strain the state's already tight water budget. To account for some of these shortages, it is imperative that Texas implements policies targeting conservation and water reuse (Gleick 2003b). Although repeated use of water is a practical and effective means for easing strain on the water supply, there is concern that unnecessary contamination may diminish future value of this important resource (Toze 2006 a + b) . Already some surace waters in the state are classified as perennially effluent-dominant and Brooks et al. (2006) described instances when base flow of some rivers in Texas are comprised of >90% treated wastewater. Ionizable compounds are chemicals often associated with urban development and examples include pharmaceutical and personal care products (PPCP), pesticides, fertilizers and ammonia. Because continued population growth and urbanization will likely increase the release of these contaminants into

waterways, it is important that best management approaches are developed at the watershed scale to decrease water quality degradation by ionizable compounds.

Current ethods for prospective and retrospective ecological risk assessments of ionizable compounds seldom consider site-specific conditions during the analysis of effects of phase (Cleuvers 2003, Bound and Voulvoulis 2004, Sanderson et al. 2004, Suter et al. 2007, Suter et al. 2000). This oversight may needlessly increase uncertainty associated with water quality management decisions. Ionization state is largely controlled by the acid/base dissociation constant (pKa) and pH of the solution where a compound resides. Consequentially, instream water quality parameters will influence the proportion of ionized and unionized forms of a compound (Van Wezel 1998). This may have profound implications on aquatic risk assessment as unionized forms are often more toxic because of their greater lipophilicity The U.S. EPA states that site-specific ambient water quality criteria hould be developed if differences in physical and chemical characteristics of water influence the biological availability and/or hazard of a given contaminant of concern (US EPA 1996, 1999). The overlying goal of my dissertation work is to emphasize the need for site-specific pH considerations during ecological risk assessments for both traditional and emerging ionizable contaminants of concern.

Why is Site-Specific pH Important to Ecological Risk Assessment?

Contaminants, such as some pesticides, fertilizers, PPCPs, are designed to be ionizable to maximize efficacy for their intended purpose. In the case of pesticides this may be exemplified by manufacturer's suggestions to adjust the pH of spray water before applying products to crops. The importance of pH for agrichemical use and associated implications for phytotoxicty has long be recognized (Blackman and Robsertson-

Cuninghame 1952) and is exemplified by more recent work by Green and Hale (2005). They described that the efficacy of the weak acid herbicide nicosulfurnon can be optimized by first increasing the pH of spray water to make the compound more soluble, and then reducing the pH of spray water before application to increases its lipophilicity so that it will have a greater propensity for uptake by target plants. Another example of how ionization state may influence biological activity is a pharmaceutical designed to works on the central nervous system. Most drugs are administered orally and therefore must first pass through the digestive tract and then be transported via the blood to specific targets within the body. Various regions of the body have different pH (stomach: pH 1.5, blood: pH 7, CNS: pH 8), which can alter the bioavailability of drugs by influencing their physiochemical properties (Hernandez and Rathinavelu 2006, Kwon 2001). These interactions are summarized in the pH-partition theory of drug absorption, which takes into account three factors that influence the partitioning process of a drug between water and lipid at different pH: 1) the dissociation constant (pKa), 2) lipophilicity of the compound, and 3) pH of the absorption site (Jollow and Brodie 1972, Kwon 2001). Similar to these pharmacokinetic principles, toxicokinetic principles may be applied for aquatic ecosystems and help scientists more accurately infer environmental hazard.

The environmental availability and physicochemical properties of ionizable contaminants could potentially change appreciably over the range of pH for most minimally impacted freshwater ways in the United States (pH 6-9) depending on their dissociation (Van Wezel 1998). Several factors influence ionization state, including temperature, aqueous pH, and the structural configuration of the compound, which actually determines the pKa value. The pKa is merely a reflection of when the

compounds will exist equally (50:50%) as ionized and unionized moieties. Weak bases will increasing become unionized as the aqueous pH approaches and surpasses the compounds pKa value; the opposite is true for weak acids as they are more likely to exist as the unionized form at lower pH. Ultimately, contaminants that have pKa values within the range of surface water pH are more likely to vary in their ionization state at or among sites (Figure 1).

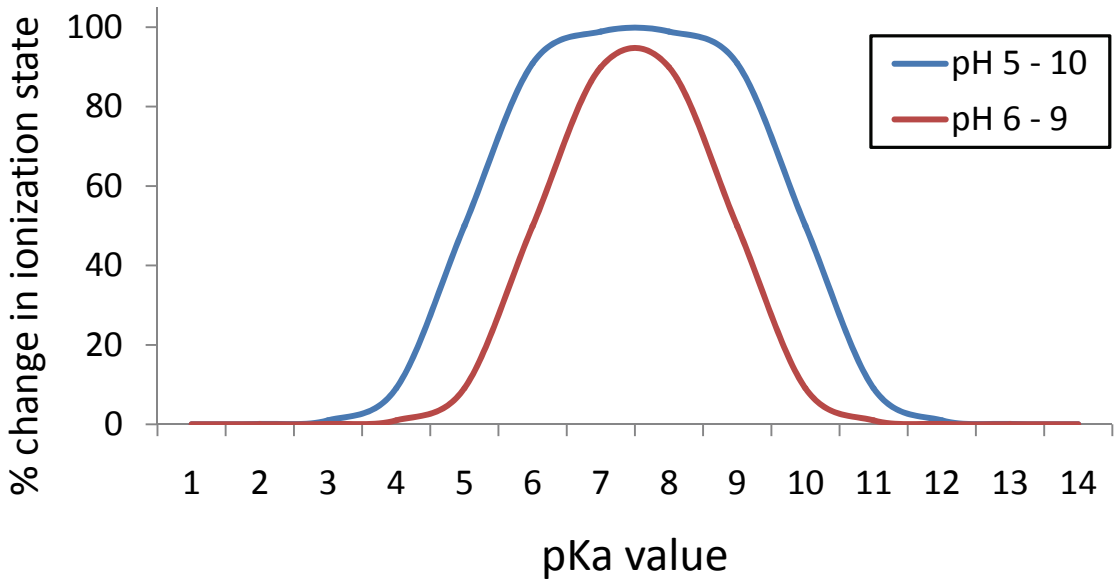

Figure 1. The figure depicts the change in ionization state for hypothetic compounds with different pKa values between environmentally relevant surface pH gradients spanning between pH 6 – 9 and pH 5 - 10.

Ionization state may alter ambient toxicity to aquatic life and the environmental relevance of such is emphasized by the integration of site-specific pH adjustment factors into national ambient water quality criteria in the United States for compounds such as such as ammonia (NH_3) and pentachlorophenol (PCP) (US EPA 1996, 1999). Both the weak base NH_3 ($pK_a = 9.3$) and the weak acid PCP ($pK_a = 4.7$) are more toxic to aquatic

life when they occur in the environment predominately as the unionized forms (US EPA 1996, 1999). To account for these differences, pH adjustment factors are derived by relating site-specific pH to laboratory derived toxicological data from experiments completed with the compound of interest at various ambient pH levels. Site-specific pH consideration can result in markedly different acceptable loads in receiving systems; varying by 13-fold for NH_3 and 60-fold difference for PCP between sites with contrasting surface water pH values of 6 and 9 (US EPA 1996, 1999).

Factors that Influence Site-Specific pH

There can be substantial spatiotemporal variability in surface water pH at sites within and among watersheds due to differences in geomorphology, hydrology, and climate (Allan 1995). The surface water pH at a given site culminates from time-dependent interactions that influence the discharge and chemical composition of base flow, storm flow, and groundwater. Base flow and storm flow are likely more variable in the headwaters and may be influenced substantially by precipitation and/or winter melting, especially in flashy systems. Storm events may cause pulses or dilution of various constituents from surrounding land areas; which may collectively cause temporary shifts in the typically pH at a site (Allan 1995). Groundwater interactions with bedrock are also very important and are controlled by residency time and the type of parent material, soil depth, slope, and water table. The size and shape of a water body may also have connotations for atmospheric interactions that influence site-specific pH. Carbon dioxide (CO_2) exchange between the atmosphere and surface water affects pH as CO_2 in water may be converted to carbonic acid, thus causing a decrease in pH (Maberly 1996). Atmospheric exchange is more likely to have an effect in shallow water bodies

that have large surface area: volume ratio. The impacts of atmospheric exchange are perhaps best evidenced by current problems of acid rain; however, the global increase of CO_2 causes broader concerns about implications associated with global climate change (Fung et al. 2005, Doney et al. 2007). Inevitably, interactions between surface water and either bed rock or the atmosphere are influenced by climate, especially temperature, precipitation, and partial pressure. Consequently, seasonal shifts in pH are often experienced at sites and may be caused by the aforementioned abiotic interactions, as well as biotic interactions that fluctuate throughout the day. In general, CO_2 is the preferred inorganic source of carbon for photosynthesis for aquatic species of plants (Sand-Jensen et al. 1992, Raven 1970, King 1970), and it may be rapidly sequestered from the water column during daylight hours (Talling 1976). High rates of photosynthetic activity may culminate in a rise of pH during the day for freshwater systems if the rate of CO_2 removal exceeds replacement by respiration or diffusion from the atmosphere and sediment (Pearl 1988). The relevance of carbon depletion is evidenced in impaired water bodies by elevated pH conditions (> 9.0) as well as increased diel oscillation of pH (Allan 1995, Maberly 1996, Pearl 1988).

Human alterations to the environment may influence the aforementioned interactions through pollution, eutrophication, altered hydrology, changing land use patterns, and the construction of impoundments. These changes may directly influence pH by altering the biogeochemistry of a site or indirectly by affecting ecosystem processes (e.g., primary production and respiration dynamics). Increased atmospheric carbon dioxide has led to the acidification of some water bodies, and even has connotations for altering the chemistry of the ocean (Doney et al. 2007). Global climate

may also influence the functionality of terrestrial plants, thus altering the availability and transport of anion and cations, as well as macro and micronutrients in the environment (Paerl 1997, Doney et al. 2007, Bowling et al. 2008, Smith et al. 1999).

Scope of Dissertation

The focus of my dissertation is to further expand on an understanding of the importance of site-specific pH as a critical variable during ecological risk assessments of ionizable compounds. The first chapter provides background information that places the importance of pH in the context of ecological risk assessment and briefly summarizes the factors that contribute to spatiotemporal variability in surface water pH. The second chapter details the pH-dependent toxicity of a model pharmaceutical sertraline and then relates how seasonal differences in site-specific pH may alter risk characterization. The third chapter delves more deeply into potential alternative endpoints in fish associated with exposure to sertraline using concepts traditionally used to infer behavioral changes in mammals during drug development as well as automated digital tracking technologies. The fourth chapter details a potential mechanistic explanation for the pH-dependent toxicity of toxins released by *Prymnesium parvum* associated with differences in ionization state. The fifth chapter explores diel oscillation of pH for stream sites that represent a nutrient gradient and then relates how these differences may influence national ambient water quality criteria for both traditional and emerging contaminants of concern.

CHAPTER TWO

Aquatic Toxicity of Sertraline to Pimephales promelas at Environmentally Relevant Surface Water pH

Introduction

NOTE: Chapter two is published in Environmental Toxicology and Chemistry (2009) 28: 2685-2694. Please refer to Appendix B for the licensing agreement.

For over 50 years researchers have recognized that ionization state may alter the biological activity of xenobiotics (Simon and Beevers 1951, Simon and Beevers 1952 a + b, Blackman and Robertson-Cunningham 1953). This observation is attributed to the unionized form crossing cellular membranes more readily than the dissociated moiety due to its lower polarity; hence, it is if often regarded as being more bioavailable for uptake. It is important to consider ionization state during both prospective and retrospective risk assessment because ambient toxicity of some contaminants to aquatic life may vary depending on site-specific pH. Spatiotemporal variability in surface water pH may be a reflection of differences in geomorphology, hydrology, climate, and/or other human disturbances. The relevance of these differences to risk assessment is emphasized by the integration of site-specific pH adjustment factors into national ambient water quality criteria in the United States for ammonia (NH_3) (US EPA 1985, 1999) and pentachlorophenol (PCP) (US EPA 1986, 1996). Both the weak base NH_3 ($pK_a = 9.3$) and the weak acid PCP ($pK_a = 4.7$) are more toxic to aquatic life when they occur in the environment predominately as the unionized forms. To account for these differences, pH adjustment factors have been created by relating site-specific pH to laboratory derived

toxicological data from experiments completed with the compound of interest at various pH levels. Site-specific pH consideration can result in markedly different acceptable loads in receiving systems, varying by 13-fold for NH_3 and 60-fold difference for PCP between sites with contrasting surface water pH values of 6 and 9. Although it is likely that similar mechanisms may influence the behavior of select pharmaceuticals in aquatic systems, ecological risk assessments rarely consider ionization state during exposure and effects analysis.

Many pharmaceuticals are implicitly designed as ionizable compounds to ensure that active components of administered doses reach specific target locations within the body. As well as being detected in surface waters, pharmaceuticals have been demonstrated to bioaccumulate in fish (Brown et al. 2007, Brooks et al. 2005, Ramirez et al. 2009). The pharmacokinetic properties and partitioning behavior of pharmaceuticals are often well understood due to requirements for drug development. Although this information may be extrapolated or modeled to predict environmental fate, it is far more challenging to infer biological effect because other pertinent data is often lacking. For example, uptake rates of some pharmaceuticals and personal care products (PPCPs) will inevitably be influenced by their ionization state as well as simultaneously by other factors, such as the presence of cations and anions, temperature, and species-specific characteristics. The partitioning behavior of drugs is important for ecological risk assessment because exchange between the water column and gills of aquatic organisms will ultimately influence steady-state plasma concentrations, which has direct connotations to the potential culmination of adverse effects associated with exposure.

An increasing body of literature suggests that pharmaceuticals may exert sublethal effects in wildlife due to the evolutionary conservation of human drug targets (Gunnarsson et al. 2008, Kreke and Dietrich 2008). Despite identifying analogous drug targets in humans and other species, it is uncertain whether exposure to organisms will result in the same magnitude or type of effects as those experienced by humans because little is known about potential interspecific differences in uptake, disposition, binding affinity, and functional responses. As pharmaceuticals are being tailored with greater specificity for certain biological targets to improve efficacy at relatively lower concentrations, there is increased concern that environmental exposure may cause adverse effects to non-target species that have similar drug receptors (Kreke and Dietrich 2008). These concerns are further heightened for aquatic organisms because xenobiotics absorbed across the gills directly enter the circulatory system and are therefore not susceptible to first-pass metabolism.

Sertraline was selected as a model compound to investigate how differences in exposure pH may influence ecotoxicological endpoints. Sertraline is a selective serotonin reuptake inhibitor (SSRI) that is being prescribed more prevalently to treat depression and other diseases (Minagh et al. 2009). There is a robust international market for the drug as evidenced by yearly human consumption rates of 122, 157, and 76 g/1000 inhabitants in Denmark, Norway, and Finland, respectively (Christensen et al. 2007). Sertraline was a useful model for the present study because it has a pK_a value of 9.47. Therefore, its ionization state will change markedly between pH 6 and 9, which is a representative range of environmentally relevant surface water pH. As well as being identified in sewage discharge and effluent dominated streams (Schultz and Furlong

2008), Brooks et al. (2005) detected sertraline and its primary metabolite desmethylsertraline in the muscle, liver, and brain of several fish species. In addition, Ramirez et al. (2010) reported sertraline as the pharmaceutical consistently displaying the greatest maximum concentration in both fish tissue fillets and livers during a U.S. national pilot study of PPCPs in fish tissue.

Such observations are important because sertraline elicits its effects on the serotonergic system, which is a highly conserved in the animal kingdom (Gunnarsson et al. 2008, Gould et al. 2007, Smith 1999). In addition to laboratory experiments assessing the toxicity of sertraline at various pH values, continuously monitored quality assured instream pH data for two stream sites in the Brazos River Basin, Texas, USA was obtained for a three year period and then evaluated relative to laboratory derived pH-dependent toxicity relationship for *Pimephales promelas* using both discrete and probabilistic approaches.

Materials and Methods

Experimental Conditions

All experiments were conducted at 25 ± 1º C and had photoperiods of 16:8 h light: dark. Reconstituted hard water (RHW), as described in standard methods (APHA, AWWA, WEF 1998), was used as the dilution water and control. Dissolved oxygen and conductivity were measured using an YSI Model 55 (Yellow Springs, OH, USA) handheld dissolved oxygen meter and salinity, conductivity, and temperature on an YSI Model 30 handheld system. An Orion 720A plus pH/ISE meter (Beverly, MA, USA) was used to determine pH. Alkalinity (mg L^{-1} as $CaCO_3$) and hardness were measured

amperiometrically and by colorimetric titration, respectively, according to standard methods (APHA, AWWA, WEF 1998). Mean (± standard deviation (SD)) water quality parameters for RHW at test initiation were within acceptable ranges described by guidelines (APHA, AWWA, WEF 1998, US EPA 2002) for all parameters, except pH, as dissolved oxygen = 7.7 (± 1.3) mg L^{-1}, specific conductance = 583 (± 8.6) µS cm^{-1}, hardness = 180 (± 5.2) mg L^{-1} as $CaCO_3$, and alkalinity = 116 (± 4.2) mg L^{-1} as $CaCO_3$. Analytical grade nitric acid and sodium hydroxide (VWR Scientific, West Chester, PA, USA) were added to adjust the pH of RHW to desired levels. The pH of exposure water at test initiation were within 0.05 units of the desired level, and drifted by less than 0.5 during any experiment. Sertraline hydrochloride (Ranbaxy Laboratories, New Delhi, India) was used to prepare exposure concentrations, which were based on data from preliminary range finding toxicity experiments.

Pimephales promelas Bioassays

Acute studies Experiments with *P. promelas* were completed concurrently at pH 6.5, 7.5, and 8.5 on three separate occasions according to slightly modified U.S. Environmental Protection Agency (U.S. EPA) protocol (2002a). Each experimental unit consisted of 10 organisms in a 600-ml glass beaker filled with 500 ml of exposure water. To minimize pH drift, experimental units contained a volume of exposure water greater than recommended and were covered with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA). Four replicates were prepared for the control and six sertraline concentrations, including 30, 60, 120, 250, 500, and 1000 µg sertraline L-1. Test solution for each concentration was first prepared, then divided into three aliquots, and each

aliquot was adjusted to the correct pH. This approach was taken to ensure that sertraline concentrations were the same across the three pH treatments. All P. promelas used in experiments were <48 h old. Individuals were fed newly hatched brine shrimp (*Artemia* sp.) 2 h prior to the exposure, but were not fed during experiments. Survival was assessed at 24 and 48 h. A time-to-death study, separate from the aforementioned experiments, was also completed. Individuals were exposed to either control (RHW) or 500 µg sertraline L-1 treatments adjusted to pH 6.5, 7.5, and 8.5. Four replicates of 10 individuals were prepared for each treatment. Survivorship was assessed after 2, 4, 6, 8, 12, 16, 20, 24, 36, and 48 h. Water quality parameters were collected at test initiation and completion for all experiments.

Short-term chronic studies A 7-d short-term chronic experiment was conducted according to slightly modified U.S. EPA protocol (2002b). Five sertraline concentrations, plus a RHW control, were prepared for each pH level. After observing markedly different acute endpoints for the three pH treatments, we choose to use lower concentrations of sertraline for the higher pH treatments. At pH 6.5 concentrations were 60, 120, 250, 500, and 1000 µg sertraline L-1 , at pH 7.5 concentrations were 30, 60, 120, 250, and 500 µg sertraline L-1, and at pH 8.5 concentrations were 15, 30, 60, 120, and 250 µg sertraline L-1. Several concentrations overlapped between the different pH treatments so that comparisons could easily be made; however, we hoped that these concentration series would enable us to capture both effect and no-effect concentrations at pH 6.5, 7.5, and 8.5. Each treatment level had four replicates of 10 individuals and experiments were completed in 600-ml glass beaker filled with 500 ml of exposure water. Beakers were covered with Parafilm, and exposure water was renewed daily.

Survivorship was monitored daily and individuals were fed Artemia sp. twice daily (AM/PM). After 7 d of exposure, survivorship was assessed and individuals from a replicate were pooled minus three individuals used for the feeding behavior trials (see below). The individuals were transferred to previously weighted drying dishes after being euthanized according to Baylor Animal Care Policy and then placed in an oven set at 80º C for 48 h. After drying the fish the weights of the drying dishes were again determined on a Mettler Toledo Model MX5 microbalance (Columbus, OH, USA). Average growth was determined by subtracting the initial drying dish weight from the final drying dish weight containing fish divided by the number of fish.

Feeding behavior. Feeding behavior trials were completed with individuals from treatments that had a sufficient number of survivors after the 7-d exposure. These included the control, 60, 120, and 250 µg sertraline L^{-1} at pH 6.5, control, 30, 60, and 120 µg sertraline L^{-1} at pH 7.5, and control, 15, and 30 µg sertraline L^{-1} at pH 8.5. Experiments were completed based on an approach by Stanley et al. (2007) with slight modifications. Three fish from each replicate were randomly selected and isolated individually in 100 ml glass beakers filled with 100 ml of RHW; thus, a total of twelve fish per treatment level were examined. Food was withheld from fish for 24 h prior to feeding trials. Experiments were initiated by introducing 40 *Artemia* sp. nauplii to an individual fish. Each fish was allowed 15-min to feed, after which time it was removed and the total number of remaining nauplii was enumerated to determine rates of feeding (Artemia $minute^{-1}$). Consumption rates of three individuals for a replicate were determined and then pooled to calculate an overall replicate average, which was used for

subsequent statistical analyses (n = 4 per treatment). Individuals from a replicate were pooled to avoid concerns of pseudo-replication.

Statistical Analyses

Statistical significance of response variables was assigned at $\alpha = 0.05$ for all tests. The 50% lethal concentrations (LC50) values were calculated using Toxstat. The probit method was used if data met assumptions; otherwise, the Trimmed Spearman-Karber method was applied (2002a). Average LC50 values for the three pH ranges were compared with analysis of variance using the computer program Jumpin version 5.0 (SAS institute, Cary, NC, USA). The 50% time-to-death (LT50) values were calculated based on equations derived from best-fit line models.

No-observable-adverse effect concentration (NOAEC) and lowest-observable-adverse effect concentrations (LOAEC) were calculated based on the statistical approach described by standard protocols (2002b). Effect concentration values (EC10, EC25, EC50) for chronic experiments were obtained using a reparameterized logistic three parameter model in Sigma Plot (Systat Software, San Jose, CA, USA) according to Brain et al. (2005) based on Stephenson et al. (2000).

Analytical Methods

Nominal concentrations of sertraline were verified for one acute experiment and for select concentrations on days 1, 3, and 6 for the 7-d sub-chronic experiment. A 500 ml sample of the appropriate test solutions was adjusted to pH 4 using high-performance liquid chromatography grade nitric acid and then run through a 90 cc C-18 solid phase extraction column (SPE) (Waters, Milford, MA, USA). Before adding the sample, the

column was activated by first drawing through 10 ml of nanopure water that was adjusted to pH 4, followed by 10 ml of high-performance liquid chromatography grade methanol, and then a final nanopure rinse. The SPE were stored at -20° C until analyses were completed. Just prior to analyses the SPE cartridges were eluted with 10 ml of methanol in 10 ml disposable borosilicate glass culture tubes (VWR Scientific, West Chester, PA, USA). The eluted samples were diluted 10-fold in methanol to reduce the sertraline concentrations to levels quantifiable by instrumentation. Depending on the expected concentration in the samples, different aliquots were taken from this last dilution and were brought into a final volume of 1000µl. Prior to this, a 50 µl of sertraline-d3 5.5 ppm (internal standard [IS]) was added; resulting in a final concentration of 275 ng L^{-1}.

A LC-MS/MS consisting of a Varian ProStar model 210 binary pump equipped with a model 410 autosampler (Varian, Palo Alto, CA, USA) was used in the present study. Sertraline was analyzed on a 15 cm x 2.1 mm (5µm, 80 Å) Extend-C18 column. A binary isocratic elution consisting of 30% of formic acid 0.1% (v/v) in water and 70% of methanol was employed to achieve analyses in 5 min. Additional chromatographic parameters included an injection volume of 10 µl and a flow rate of 350 µl/min. Eluted analyte and isotope were monitored by MS/MS using a Varian model 1200L triple-quadrupole mass analyzer equipped with an electrospray interface (ESI). To determine the best ionization mode (ESI + or -) and optimal MS/MS transitions for the target analyte and isotope, each compound was infused individually into the mass spectrometer at a concentration of 1 µg/ml in aqueous 0.1% (v/v) formic acid at a flow rate of 10 µl/min. Compounds were initially tested using both positive and negative ionization modes while the first quadrupole was scanned from *m*/*z* 50 to [M + 100]. This enabled

identification of the optimal source polarity and most intense precursor ion for each compound. Once these parameters were defined, the energy at the collision cell was varied, while the third quadrupole was scanned to identify and optimize the intensity of product ions for each compound. Additional instrumental parameters held constant for all analytes were as follows: nebulizing gas, N_2 at 60 psi; drying gas, N_2 at 19 psi; temperature, 300 ^{o}C; needle voltage, 5000 V ESI+, 4500 V ESI-; declustering potential, 40 V; collision gas, argon at 2.0 mTorr.

A solution of IS was added to each calibration point at a concentration of 275 ng L^{-1} to generate a relative response ratio. Analyte concentrations determined using an internal standard calibration procedure. The response factor was calculated by dividing the peak area for sertraline by the peak area for the IS, and a calibration curve was prepared by plotting a linear regression ($r^2 \geq 0.999$) of the response factor versus analyte concentration for all calibrators analyzed. Instrument calibration was monitored through the use of continuing calibration verification samples with an acceptability criterion of ±20%. In a given run, one blank and one continuing calibration verification sample were interspersed between every five samples for quality assurance purposes. Recoveries of the IS were compared with the relative response ratio and a concentration for the unlabeled analyte was calculated.

Continuous Water Quality Monitoring Data

Daily pH data from 2003 to 2005 was obtained for two wadeable streams, CAMS 703 (Gatesville Water Site) and CAMS 704 (Resley Water Site), from the Texas Commission of Environmental Quality (TCEQ) database of surface water quality with quality assurance, quality control review (TCEQ 2008);

www.tceq.state.tx.us/compliance/monitoring/water/quality/data/wqm/swqm_realtime_swf.html.). For continuous water quality data to be considered valid by TCEQ, pre-calibration and post-calibration information must be provided for multiparameter probes. Nutrient enrichment is a concern at both sites as surrounding watersheds are largely agricultural. Resley Creek is influenced by dairy confined animal feeding operations, occurs at higher elevation, and drains into the Leon River, which is the system monitored at Gatesville, Texas, USA. The Gatesville site is located in close proximity to urban development and subsequently receives municipal wastewater discharge and storm water runoff. These sites were selected due to their close spatial proximity to examine how factors other than geomorphology and climate may influence surface water pH. Data flagged by the TCEQ as being invalid during a quality review were removed prior to analysis. Daily as well as seasonal means for the three year period were determined. Cumulative frequency plots of daily instream pH measurements were created so that the likelihood of observing a given pH value at each site could be quantified for both overall as well as seasonal data.

Predictions of Ecotoxicological Responses

The LC50 values for acute experiments completed at different pH were plotted with log LC50 values as the response y-axis and pH as the predictor x-axis. A linear model was fit to the data so that LC50 values could be predicted for any pH. Relating these toxicological relationships with cumulative frequency distributions of pH by season allowed us to examine the likelihood of observing a toxicological response. The lack of analytical data for sertraline precluded specific risk characterization of sertraline at these sites. Alternatively, risk was assessed by examining how variability in site-specific pH

may change predicted toxicological responses. For comparative purposes, a range of potential sertraline concentrations at these sites was examined so that the likelihood of observing adverse effects could be set on a relative scale. The concentrations selected for comparative purposes included 125, 150, 175, and 200 μg sertraline L^{-1} for acute bioassays, while those for growth and feeding were 50, 75, 100, 125, and 150 μg sertraline L^{-1} and 20, 30, and 40 μg sertraline L^{-1}, respectively. These values were selected because they spanned the lower ranges of exposure concentrations and could be used to examine differences in potential effects among the three pH treatments. Additional safety factors were not considered during the analysis. The present study solely focused on how site-specific pH may influence biological responses, and thus was concerned primarily with analysis of effects. The scarcity of reported sertraline concentrations in the environment prevented further investigation of how site-specific pH may influence environmental exposure scenarios.

Results

Analytical Results

Nominal concentrations of sertraline in exposure water were analytically confirmed and values were 96 to 118% of their target concentration (Table 1). Consequently all subsequent data analyses in this study were performed using nominal treatment concentrations because of the similarities with analytically verified values.

Acute Studies

Survivorship was >95% in control treatments, which consisted of RHW adjusted to pH 6.5, 7.5, and 8.5, respectively. Dose dependent responses were apparent as

survivorship was lower at higher sertraline concentrations. Mean LC50 values for experiments completed at pH 6.5, 7.5, and 8.5 were significantly different based on analysis of variance ($p<0.05$) (Figure. 2); and mortality was generally greater at lower sertraline concentrations when individuals were exposed to test water with higher pH.

Table 1. Analytical verification of nominal sertraline treatment levels for acute and short-term chronic experiments with *Pimephales promelas*.

n	Nominal concentration	Measured ± SD (µg/L)[a]	% Recovery ± SD
4	control (0)	0.02	NA[b]
4	15	14 ± 6	96 ± 38
4	30	30 ± 4	100 ± 13
4	60	61 ± 5	101 ± 8
4	125	141 ± 24	118 ± 20
4	250	256 ± 26	102 ± 10
4	500	579 ± 44	116 ± 9
4	1000	1120 ± 69	112 ± 7
1	2000	2111	106

[a] SD = standard deviation.
[b] Not applicable

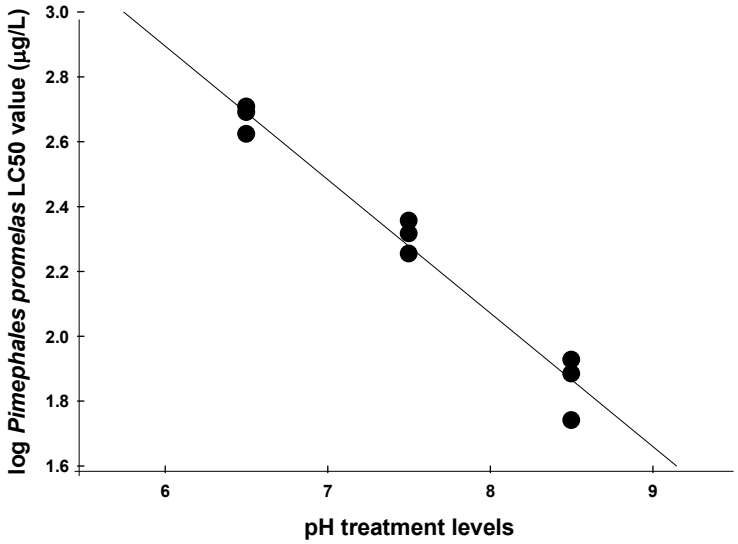

Figure 2. 48-h median lethal concentration (LC50) values for *Pimephales promelas* exposed to sertraline at three pH treatment levels (6.5, 7.5, 8.5). Each data point represents a LC50 value from an independent test. A linear regression model relating LC50 values to pH is also presented.

Results from the time-to-death study showed similar trends as the onset of mortality occurred more rapidly at higher pH (Figure. 3). The LT50 values for individuals exposed to 500 µg sertraline L^{-1} at pH 6.5, 7.5, and 8.5 were >48, 32, and 5 h, respectively. No mortalities were observed in the time-to-death study in control treatments adjusted to either pH 6.5, 7.5, and 8.5.

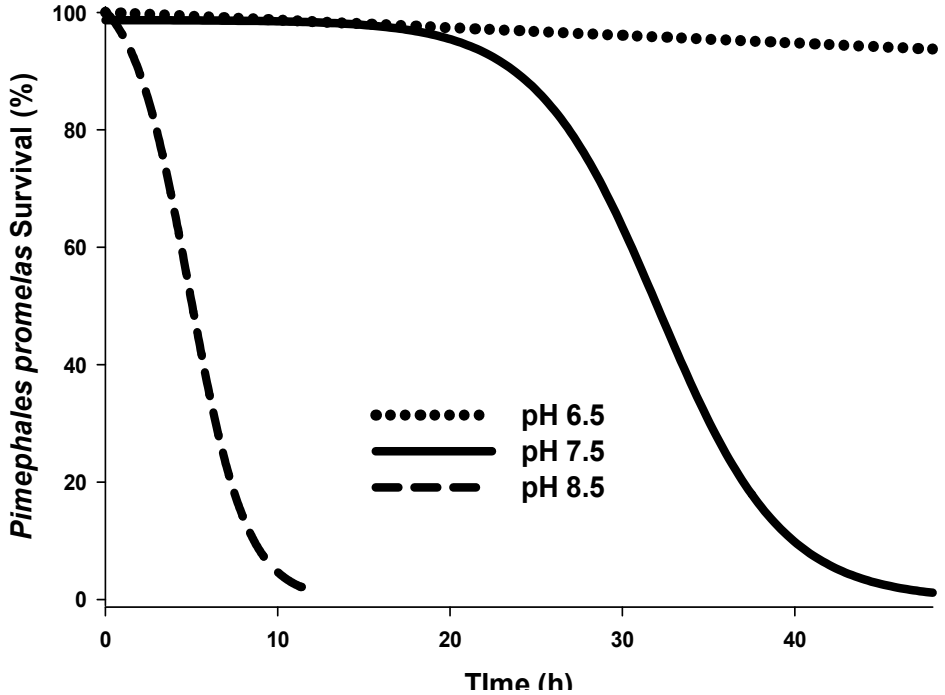

Figure 3. Time-to-death for juvenile *Pimephales promelas* exposed to 500 µg sertraline L^{-1} in reconstituted hard water adjusted to three different pHs. No mortality was observed in control treatments at pH 6.5, 7.5, and 8.5

Short-term Chronic Experiments

Control survivorship was ≥ 90% and dose-dependent responses were apparent in all experiments (Table 2). Similar to the relationship between pH levels and LC50 values, growth and feeding EC10 values were markedly lower at higher pH (Table 2). Feeding rate was generally more sensitive than growth and survivorship. Survivorship, growth, and feeding endpoints were consistently lower for individuals exposed at pH 8.5, relative

to those exposed to lower pH treatment levels (Table 2). At pH 8.5 a NOAEC could not be determined because the lowest test concentration caused a significant reduction in feeding rate relative to the control. The NOAEC and EC10 for feeding rate at pH 8.5 were also substantially lower than similar values for growth (Table 2).

Continuous Water Quality Monitoring Data

The respective overall site pH average (± SD) for Gatesville and Resley from the period of 2003 through 2005 were 7.97 (±0.2, *n*=944) and 7.66 (±0.28, *n*=1033). The frequencies of daily averages pH for each site by season are depicted in Figure 4. Distinctions between seasonal distributions and means are apparent. The respective fall, winter, spring, summer daily means (±SD) for the various sites are presented in Table 3. Also presented in Table 3 are attributes describing the pH distributions of the two sites by season. Daily mean pH values were generally highest during winter and lowest in the summer. Variability in pH was greater for the Resley site than Gatesville site based on differences in standard deviation between and within seasons (Table 3).

Predictions of Ecotoxicological Responses

The LC50 predictions based on discrete overall site pH means were 146 and 204 $\mu g\ L^{-1}$ for Gatesville and Resley, respectively. Seasonal differences were evident for both sites and predictions of toxicity to juvenile fathead minnows were greatest during winter months and the least during summer as respective values for Gatesville were 132 and 156 $\mu g\ L^{-1}$, while those for Resley were 172 and 240 $\mu g\ L^{-1}$. Overall, contrasting the temporal change by calculating the absolute percent difference in the mean predicted LC50 values

for Resley pH distributions resulted in a total of 33.5%; however, total change at Gatesville was only 16%.

Table 3. The average pH by season for two Texas Commission of Environmental Quality continuous water quality monitoring stations (Gatesville, Resley) in the Brazos Watershed (TX, USA). The attributes of the line used to infer the likelihood of a specific pH for each site are also summarized. SD = standard deviation

			Attributes of line fit to pH distributions		
Site	Season	Average pH (SD)	Intercept	Slope	r^2
Gatesville	Fall	7.98 (0.22)	88.95	-80.00	0.994
	Winter	8.06 (0.15)	144.77	-130.78	0.994
	Spring	7.94 (0.16)	96.70	-86.69	0.942
	Summer	7.91 (0.23)	86.24	-77.16	0.991
	All	7.97 (0.2)	93.39	-83.81	0.991
Resley	Fall	7.57 (0.2)	78.38	-68.62	0.981
	Winter	7.82 (0.26)	58.63	-52.00	0.917
	Spring	7.73 (0.28)	66.91	-59.26	0.990
	Summer	7.51 (0.27)	71.62	-62.53	0.988
	All	7.66 (0.28)	65.40	-57.58	0.999

The pH distributions allowed a probabilistic approach that enabled better estimations of seasonal variability and differences between sites. The span of sertraline concentrations used corresponded to respective pH of 8.09, 7.92, 7.78, and 7.66 based on our LC50 pH-dependent toxicity relationship. The respective pH values based on our EC10 model were 8.13, 7.84, 7.63, 7.47, and 7.33. Predictions based on daily average pH distributions suggested trends similar to those described from discrete predictions. Table 4 depicts the likelihood that a LC50 value is predicted to be less than the respective comparative value. For all seasons combined, LC50 values at Gatesville are predicted to be <125 μg L^{-1} 10% of the time, <150 μg L^{-1} 46% of the time, <175 μg L^{-1} 82% of the time, and <200 μg L^{-1} 97% of the time. Analogous comparisons for the same

comparative values at Resley are less indicative of ambient toxicity as respective values are 2, 14, 40, and 68% (Table 4). Seasonal differences were also apparent. For example, an LC50 value is predicted to be less than 150 μg L^{-1} 69% of the time during the winter at Gatesville, but only 37% of the time during the spring. Another example would be that an LC50 value is predicted to be less than 150 μg L^{-1} 23% of the time during winter for Resley, but only 3% during spring months (Table 4). Similar trends were also apparent for comparisons based on growth and feeding EC10 values (Table 4).

Discussion

In this study the sensitivity of *P. promelas* to sertraline changed markedly over a gradient of environmentally relevant surface water pH. Adverse effects were observed at lower concentrations for individuals exposed at pH 8.5 than for those exposed to the lower pH treatments. Furthermore, individuals in control treatments with water adjusted to either pH 6.5, 7.5, or 8.5 had average survivorship ≥90% in both the acute and short-term chronic bioassays. Neither growth or feeding rate were significantly different among pH levels for the sub-chronic bioassay as respective values ranged from 0.36 to 0.41 mg per surviving individual and 1.7 to 1.9 *Artemia* min^{-1}, respectively (Table 2). High survivorship and similar sub-lethal measures for controls in the three pH treatments suggests that differences in sensitivities between treatments containing sertraline was not directly due to physiological stress experienced by organisms as a result of pH adjustment approaches, which followed established U.S. EPA methods. Rather, the observed variability in sensitivities at the different pH levels likely resulted from changes in the physiochemical properties of sertraline as a consequence of different pH environments.

Table 2. Results of short-term chronic experiments with *Pimephales promelas* exposed to sertraline at pH 6.5, 7.5, and 8.5. The average survivorship, growth, and feeding rate for the various sertraline concentrations at different pH are reported and accompanying standard deviations are shown in parentheses. (*) treatments that are statistically significantly different from the control (p=0.05). The no-observable-adverse effect concentration (NOAEC) and lowest-observable-adverse effect concentrations (LOAEC) are shown respectively by A and B for each pH treatment.

pH	Treatment (µg/L)	Survivorship (%)	Growth (mg)	EC10	EC25	EC50	Feeding rate (*Artemia* min^{-1})	EC10	EC25	EC50
6.5	Control	90 (8)	0.37 (0.05)	469	496.4	544.4	1.9 (0.2)	69.6	106.9	199.7
	60	88 (13)	0.36 (0.05)				1.7 (0.2)			
	120 A	98 (5)	0.34 (0.07)				1.3 (0.2)			
	250 B	83 (17)	0.26 (0.04)*				0.8 (03) *			
	500	50 (14) *	0 (0) *				NA			
	1000	0 (0) *	0 (0) *				NA			
7.5	Control	93 (10)	0.41 (0.04)	118.7	124.6	131.4	1.7 (0.2)	65.6	99	149.5
	30	90 (8)	0.4 (0.05)				1.7 (0.3)			
	60 A	95 (6)	0.42 (0.02)				1.6 (0.1)			
	120 B	88 (13)	0.3 (0.06) *				1.1 (0.1) *			
	250	0 (0) *	0 (0) *				NA			
	500	0 (0) *	0 (0) *				NA			
8.5	Control	90 (8)	0.36 (0.06)	30.3	39	50	1.9 (0.1)	8.7	20.7	80.3
	15 B	90 (8)	0.37 (0.04)				1.5 (0.1) *			
	30	75 (13) *	0.33 (0.06) *				1.3 (0.2) *			
	60	20 (18) *	0.11 (0.11) *				NA			
	120	0 (0) *	0 (0) *				NA			
	250	0 (0) *	0 (0) *				NA			

EC = effective concentration; NA = not applicable.

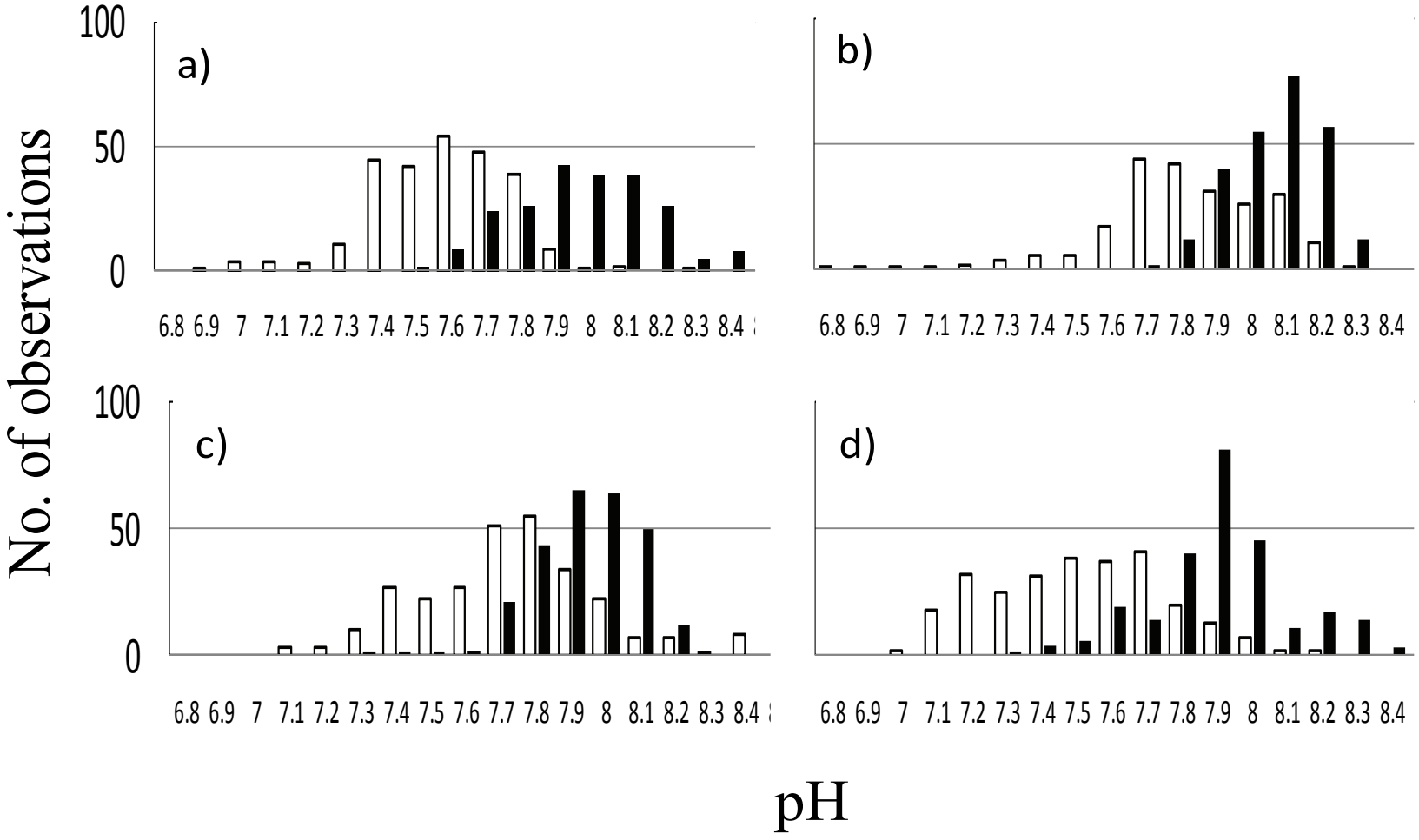

Figure 4. Seasonal frequency distributions of pH by the number of observations at two continuous monitoring stations, Gatesville (■) and Resley (□), in the Brazos River Basis, Texas, USA. Data from 2003 to 2006 was obtained from the Texas Commission on Environmental Quality Surface Water Quality Monitoring Information System.

Table 4. The likelihood that the predicted sertraline median lethal concentration (LC50) and growth and feeding 10% effective concentration (EC10) values for *Pimephales promelas* will be below the comparative value at two sites based on aquatic toxicological models derived during laboratory bioassays and distributions of surface water pH.

Site	Endpoint	Comparative sertraline values ($\mu g\ L^{-1}$)	Fall	Winter	Spring	Summer	All
Gatesville	LC50	125	14	11	7	7	9
		150	62	86	52	46	55
		175	94	100	92	86	93
		200	100	100	100	98	100
	Growth EC10	50	17	17	9	9	12
		75	67	91	58	51	61
		100	93	100	91	85	92
		125	99	100	99	97	99
		150	100	100	100	99	100
	Feeding EC10	20	35	49	24	22	28
		30	94	100	92	86	93
		40	100	100	100	99	100
Resley	LC50	125	0	7	4	0	2
		150	5	30	25	5	16
		175	30	62	60	26	46
		200	67	84	86	60	76
	Growth EC10	50	0	9	5	0	3
		75	7	34	28	7	18
		100	29	60	58	25	45
		125	56	79	80	50	68
		150	79	90	92	72	84
	Feeding EC10	20	1	17	12	1	6
		30	30	62	60	26	46
		40	78	17	91	70	83

Pimephales promelas was specifically selected as a model organism for these experiments because of its ability to tolerate a wide range of environmental conditions. Researchers have previously utilized *P. promelas* in studies over pH gradients similar to that used in our experiments; examples include development of national ambient water quality criteria for NH_3 and PCP (US EPA 1985, 1986, 1995, 1999), as well as U.S. EPA toxicity identification evaluation approaches that employ pH adjustments.

Various studies report that ionization state may influence the toxicity of some xenobiotics to organisms. The difference in toxicity between the ionized and unionized moiety can be attributed to variable rates of diffusion into cells, which are determined by the external concentration and the pH of the exposure water (Simon and Beevers 1951, Simon and Beeves 1952 a + b, Blackman and Robertson-Cunningham 1953, US EPA 1985, Sarrikoski et al. 1986, Sarrikoski and Vilukesela 1981, Whitley 1968). Results from the time-to-death experiment support the hypothesis that differences in ionization state of sertraline may explain aquatic toxicological responses across pH gradients. The unionized moiety is often assumed to be absorbed and delivered to target sites at a higher rate than the ionized from. Therefore, although aquatic organisms may be exposed to the same concentrations, adverse effects should be observed more rapidly in treatments with higher proportions of the unionized moiety; such a relationship was observed during the time-to-death study with fathead minnows (Figure 3).

Nakamura et al. (2008) reported similar pH-dependent toxicological relationships during experiments in which Japanese medaka (*Oryzias latipes*) were exposed to the SSRI fluoxetine, a weak base with a pK_a of 10.1, at pH 7, 8, and 9 as respective LC50 values were 5500, 1300, and 200 μg L^{-1}. These researchers were able to ascertain that the

heightened acute toxicological response at the higher pH was likely due to a greater bioconcentration factor at increased pH as rates varied markedly over the pH gradient. Fisher et al. (1999) exposed zebra mussels (*Dreissena polymorpha*) to PCP and observed significantly different uptake rates at various pH and temperatures that ranged from 0.33 to 2133 L kg^{-1} d^{-1}, and noted that lethal body resides varied at different pH and temperature levels, as respective values for pH 6.5, 7.5, and 8.5 were 69, 245, and 782 μmol kg^{-1} at 25 °C and 863, 1351, and 6253 μmol kg^{-1} at 17 °C. Again, these results suggest that the neutral, unionized moiety is more lipophilic, and that uptake by organisms is favored when this form is more prevalent.

Because the pH and total sertraline concentrations at which the exposures occurred were known, we used the Henderson-Hasselbalch equation to determine ecotoxicological endpoints in terms of percent unionized sertraline. At pH 6.5, 7.5, and 8.5 approximately 5, 12, and 28% of the total sertraline should be present in the unionized form. Examining LC50 values in terms of total sertraline by percent unionized in the exposure water produces respective values of 32, 25, and 19 μg L^{-1} unionized sertraline for pH 6.5, 7.5, and 8.5 (Figure 5).

Similarly, growth EC10 values for % unionized were estimated to be 23, 14, and 8.5 μg L^{-1} for pH 6.5, 7.5, and 8.5, respectively. The magnitude of difference in endpoints is substantially less when comparing responses across pH treatment levels in terms of percent unionized concentrations compared to total sertraline concentrations. For example, there is only a 1.5-fold difference in endpoints based on unionized concentrations compared to nearly a 9-fold difference in values based on total concentrations for acute experiments completed at pH 6.5 and 8.5.

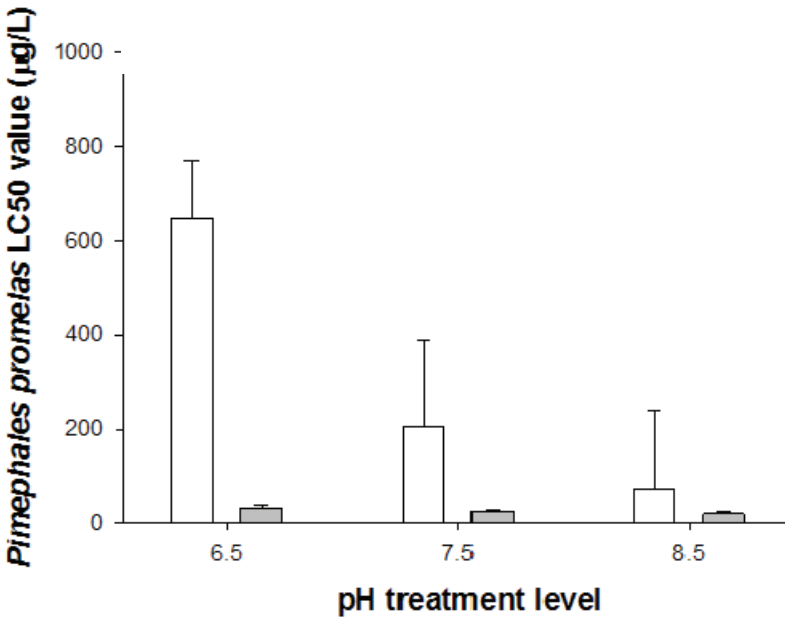

Figure 5. The 48-h mean (±standard deviation) median lethal concentration (LC50) values for Pimephales promelas presented as total and percent unionized sertraline concentrations. Experiments were completed in triplicate for each pH treatment. The % percent unionized (□) was determined by integrating total sertraline (■) concentrations and exposure pH into the Henderson-Hasselbalch equation.

Further, there was a 15-fold difference between growth EC10 at pH 6.5 and 8.5 for total sertraline, but only a 3-fold difference when comparisons are made in terms of unionized concentrations. Nakamura et al. (2008) observed approximately a 28-fold difference in acute endpoints based on total fluoxetine between pH 7 and 9, which equates to less than a 5-fold difference if comparisons are made based on unionized levels of fluoxetine. In experiments examining the toxicity of the ionizable weak acid triclosan to *Ceriodaphnia dubia* over different pH ranges, Orvos et al. (2002) calculated acute endpoints based on total triclosan concentrations that were 4-fold lower at pH 6.8 to 7.0 compared to pH 8.2 to 8.5; however, when calculations were based on unionized concentrations values were 110 and 140 µg L^{-1}, respectively.

Although predicted LC50 values at different pH in terms of unionized concentrations results in less deviation than similar comparison in terms of total concentrations, toxicity may not solely be explained by exposure to the unionized moiety. Saarikoski et al. (1986) concluded that although the rate of absorption is exponentially related to pH in experiments with phenolic and carboxylic acid, the overall rate over a pH gradient is not indicative of what would be predicted based solely on the unionized form in water; suggesting additional mechanism besides internal-external equilibrium of the neutral entity. Erickson et al. (2006) postulated explanations for this discrepancy in fish models, including that the 1) release of excretory products from gills may alter the pH of the area where cells come in immediate contact with contaminant, 2) ionized molecules may be absorbed to support chemical flux and amiable diffusion gradients, and 3) ionized entities are not completely impermeable to uptake across membrane barriers. The latter two suggestions are recognized by site-specific national ambient water quality criteria values for NH_3 in the U.S., which are determined by concentrations of both the ionized and unionized moiety.

Behavioral Responses to SSRIs

Gerhardt (2007) suggested that behavior is among the most sensitive measures of impairment by stressors and thus an important consideration in ecotoxicology. Brooks et al. (2003) described the merit of augmenting traditional standardized ecotoxicological responses with alternative endpoints, particularly for SSRIs because they may result in behavioral modifications. Kreke and Dietrich (2008) further identified that standardized endpoints may be insufficient for ecological risk assessments of SSRIs. In the present study, feeding behavior was more sensitive than standardized mortality and growth

responses and the magnitude of survival, growth and behavioral toxicity was influenced by pH gradients. Stanley et al. (2007) also observed feeding behavior in the fathead minnow model to represent a more sensitive and ecologically relevant response than standardized endpoints (e.g., survival, growth) to the SSRI fluoxetine. Previous studies have reported that behavioral responses can be 10 to 100 times more sensitive than standard toxicological benchmarks, such as survivorship (Gernhardt 2007).

Several factors make feeding behavior a reasonable choice as an alternative endpoint for assessing SSRI exposure; the foremost being the connection between observed effects and potential ecological consequences. Maltby (1999) noted that lowered energy input associated with reduced feeding as a consequence of environmental exposure may be integrated into energy budget models to predict effects at the population level. Furthermore, a plausible cause and effect relationship between reducing feeding behavior and exposure to sertraline can be concluded based on our current understanding of its mode-of-action in mammalian models. The desired human therapeutic effect of sertraline is to inhibit the reuptake of serotonin into presynaptic terminals of the central nervous system (MacQueen et al. 2001). Sertraline blocks the reuptake carrier that normally removes serotonin released from the synapse. Consequently, extracellular levels of serotonin become elevated in mammals when administered sertraline. In addition, sertraline also blocks the reuptake of the carrier on the cell body, which subsequently leads to activations of serotonin autoreceptors that negatively modulate firing rates (Sprouse et al. 1996).

Previous studies have reported the detection of sertraline and its primary metabolite desmethylsertraline, in muscle, liver, and brain tissues in fish residing in an

effluent dominated stream (Brooks et al. 2005); which likely represent worst case scenarios for aquatic exposure to SSRIs in the developed world (Brooks et al. 2006). In order to understand fish responses to SSRIs in these systems comparative pharmacology and read-across approaches can be very useful (Brooks et al. 2009, Ankley et al. 2007). For example, Benmansour et al. (1999) demonstrated down regulation of the serotonin transporter (SERT) in brains of rats treated for 21 d with sertraline. This observation is critical because SERT downregulation is important in the antidepressant action of SSRIs, resulting in swimming activity responses and other behavioral effects in rat models (Holmes et al. 2003). More recently, Gould et al. (2007) reported similar SERT downregulation in zebrafish brains treated for 21 d with sertraline. Such observations and feeding inhibition of juvenile fathead minnows exposed to the SSRIs sertraline in the present study and fluoxetine (Stanley et al. 2007) appear to provide multiple lines of evidence that support a cause and effect relationship between exposure to sertraline and target-mediated reduced feeding behavior in fish.

Although one may anticipate growth responses to be representative of feeding rates, standard test designs may preclude this assumption in the case of SSRIs. Reducing feeding rates may be the function of fewer strikes or lower prey-capture efficiency, and may not be apparent in growth data because of the longevity and abundance at which food is available under typical static, renewal test conditions. The current traditional experimental design described by U.S. EPA (2002b) is advantageous because it may improve statistical power in subsequent analyses by reducing variability among and between replicates; however, it may be limiting as it may not allow for detection of sub-lethal effects that are more subtle, such as strike rate and prey-capture efficiency. Thus,

feeding responses in the fathead minnow model presented here and by Stanley et al. (2007) appear to provide an ecologically relevant measure of effect for fish exposed to SSRIs. Alternative endpoints, such as feeding response, do not preclude the importance of traditional endpoints described in standard protocol, but rather may provide additional data for reducing uncertainty when characterizing environmental effects during risk assessment.

Importance of Site-Specific pH

Using distributions rather than discrete measurements of instream pH, such as overall site averages, affords additional insight and flexibility during both prospective and retrospective risk assessment. Seasonal and overall site mean values are useful for identifying broad distinctions at and among sites during risk assessment; however, such measures often lack the resolution to reflect exposure scenarios to organisms during more finite scales. Consequently, there is a growing impetus that pH should be approached as a dynamic variable that is not accurately quantified with periodic discrete sampling events. Several researchers describe pronounced diel fluctuations in pH and CO_2 concentration that are attributable to photosynthetic and respiratory processes (Howland et al. 2000, Guasch et al. 1998, Rebsdorf et al. 1991) as well as changes in gaseous saturation potential for waters due to increases in temperature during the day. The magnitude of pH change at a site is dependent on abiotic and biotic factors; however, previous researchers have documented daily oscillations that exceed 0.5 pH units (Guasch et al. 1998, Rebsdorf et al. 1991, Allan 1995).

In the present study variability in pH between sites and temporal pH differences at sites suggests potential differences in site-specific ecotoxicological responses to ionizable

contaminants, even in receiving systems located within the same subwatershed. It is reasonable to project that the magnitude of pH differences among receiving systems at broader spatial scales will be more pronounced. Based on the present study findings in the laboratory these changes may influence the dissociation of sertraline and potentially other ionizable compounds in the environment. Thus, it appears relevant that site-specific pH be considered in prospective ecological risk assessments of pharmaceuticals because ambient pH can influence exposure and toxicity of sertraline and potentially other pharmaceuticals to aquatic life residing in receiving systems. Current ecological risk assessment approaches for pharmaceuticals and other contaminants may over- or under-predict instream effects for ionizable compounds if the pH of a receiving system is not considered.

CHAPTER THREE

Sublethal Effects of the Selective Serotonin Reuptake Inhibitor (SSRI) Sertraline on Fathead Minnow Under Potential Worst-Case Environmental Exposure Scenarios.

Introduction

Human pharmaceuticals and personal care productions (PPCPs) have been measured in the discharges of wastewater treatment plants (WWTP) and receiving systems (Koplin et al. 2002, Herberer 2002, Kummerer 2004, Vieno et al. 2005, Brooks et al. 2005, Nikolaou et al. 2007, Williams and Cook 2007). Concerns about the potential environment effects are further heightened by the quantification of drugs in the tissues of aquatic organisms (Larsson et al. 1999, Brooks et al. 2005, Brown et al. 2007, Ramirez et al. 2009, Fick et al. 2010). Selective serotonin reuptake inhibitors (SSRI) are one of the most commonly detected classes of drugs in effluents, and several studies have measured concentrations in the plasma (Citations) as well as the brain tissue (Brooks et al. 2005, Ramirez et al. 2009) of fish collected below wastewater discharges. The SSRIs are medications widely prescribed to treat depression, anxiety, and other mental ailments (Citations), and elicit therapeutic effects by preventing serotonin reuptake from synapses by pre-synaptic serotonin transporter proteins, which triggers increased serotonergic neurotransmission (Frazer 2001). The serotonergic receptors are well described in humans and rodents (Owens et al. 1997) and are largely conserved among vertebrates (Dietl and Palacios 1988). Researchers have demonstrated occupancy of the serotonin transporter by SSRI during *in vivo* studies with mice models and emphasized that competition studies with radiolabelled ligands are useful for identifying binding affinity

of drugs (Scheffel et al. 1994). During *in vitro* studies, Gould et al. (2007) used homogenate binding with [3H] labeled citalopram to compare the pharmacological profiles of central SERT binding sites in several fish species to those of rats and found similar K_D and B_{max} values between the genera. Furthermore, their study also demonstrated *in vivo* down regulation of the SERT transporter in zebrafish following dietary exposure to the SSRI sertraline. The evolutionary conservatism of the SERT transporter site among vertebrates (Dietl and Palacios 1988) suggests that exposure to SSRIs may therefore cause a physiological affect in fish.

Many PPCPs provide ecological risk assessors with a unique set of challenges and researchers have emphasized concerns that traditional ecological risk assessment approaches may be inadequate for hazard characterization (Lange and Dietrich 2002, Huggett et al. 2003, Brooks et al 2009). Environmental hazard assessment frameworks for PPCPs in aquatic ecosystems originally relied heavily on lethality endpoints from short-term bioassays as a starting point because data was generally more available (FDA 1998, EMA 2001). Acute to chronic ratios and safety factors could then be applied to establish environmental concentrations likely to minimal adversely affects on organisms associated with chronic exposure (Huggett et al. 2003). The practical constraint of using this ecological risk assessment framework for PPCPs is that most pose little risk of lethality following acute exposure (Webb 2001), yet many are quite potent and can elicit biological change at low concentration because of their high selectively and specificity. Scott et al. (2004) emphasized that standardized acute lethality tests likely ignore ecological death because exposure to sub-lethal doses may cause organisms to be unable to function in an ecological context. Ultimately, the major dilemma risk assessors face is

that traditional endpoints from toxicity tests (e.g., survivorship, growth, reproduction) only provide a coarse resolution of potential biological effects, thereby perplexing the accurate characterization of risk for some PPCPs to aquatic organisms.

Gerhardt (2007) noted that biochemical, physiological, and behavioral responses can provide greater resolution following short-term exposures because they may become discernable within minutes, whereas life history response and morphological responses may take months or years to manifest. While biomarkers are extremely practical as tools for inferring exposure, they have limited use for identifying biological effects as it is often difficult to elucidate specific causal relationships between biochemical changes and specific ecological consequences. Alternatively, changes in behavior can represent the initial response of an organism to a chemical stressor and may help explain observed reductions in survival, growth, or reproduction (Fernández-Casalderry et al. 1994). Behavior itself is defined as the visual response of an organism to a culmination of biotic and abiotic stimuli. The response of an organism to these cues is dependent upon physiological (internal) signals, as well as environmental or social (external) factors (Gerhardt 2007). Contaminant-induced alterations in behaviors may therefore reveal connections between the biochemical, individual, and population levels of biological organization (Weis et al. 2001). For these reasons, Peakall (1996) emphasized that behavioral endpoints may be more robust and comprehensive for ecological risk assessment than either physiological or biochemical parameters.

Researchers have long recognized the potential applicability of animal behavior as indicators of sublethal stress during laboratory bioassays with fish. For example, Warner et al. (1966) contrasted the behavior of control fish to those exposed to gradients of

various contaminants and showed that exposure may adversely affect organisms at concentrations far below those that cause death or immobility. Since then several other studies have investigated how anthropogenic pollutants affect fish behavior (Marcucella and Abramson 1978; Little et al. 1985; Rand 1985; Atchison et al. 1987; Beitinger 1990; Little and Finger 1990; Døving 1991; Blaxter and Hallers-Tjabbes 1992; Scherer 1992), and Scott and Sloman (2004) emphasized that most of the previous studies focus solely on direct response measures, such as avoidance, coughs, or body tremors. There has been a transition from these pursuits and more recent research has attempted to identify changes in behaviors that are more ecologically relevant, such as foraging (Sandheinrich and Atchison 1990, Kasumyan, 2001, Hahn and Schulz 2007), feeding rates (Grippo and Health 2003, Valenti et al. 2010, Stanley et al. 2007) predator –prey interactions (Weis et al. 2001, Weis et al. 2003), reproduction (Martinovic et al. 2007), and social hierarchies (Perreault et al. 2003). Furthermore, tests with fish to assess anxiety, stress, and fear have been developed as tools to study neuropsychopharmacology, neuropathology, and psychopathology (Baraban et al. 2005, Bass et al. 2008, Blaser et al. 2010).

These efforts have been promoted by advances in digital tracking technologies and statistical approaches for interpreting data, which have absolved criticism concerning the potential subjective nature of behavioral endpoints. Kane et al. (2005) described progress in behavioral sciences and provides a detailed review, as well as future perspectives, for the use of automated techniques during experiments with fish models. One such technology is NOLDUS Ethovision XT, which is a software package that allows automated tracking and analysis of animal movement and activity (NOLDUS website). The system processes video images and has been widely applied to study

behavior in mammalian models, which is often associated with drug development. Tracking swim patterns of individual has proven an effective means to assess sublethal changes in exposed fish (Little and Brewer 2001), and Ethovision XT may prove an effect way to studying sublethal toxic effects of fish. Several studies have used this program to access behavioral responses in larval (Baraban et al. 2005,) and adult zebrafish (Gerlai et al. 2000)

A modified plus maze with both black and white arms is another promising approach for assessing behavioral changes in fish (Sackerman et al. 2010). Several researchers have observed that teleosts have natural preferences to dark environments (Serra et al. 1999, Maximino et al. 2010), and exposure induced change of this behavior could therefore be suggestive of anxiolytic effects of drugs in fish models. Under this premise, fish exposed to agents that reduce anxiety would be more likely to explore and spend time in white arms relative to unexposed individuals. The modified plus maze for fish is based on the elevated plus-maze (EPM) for rats, which is one of the most popular *in vivo* animal tests as over 2500 experiments have been published (Carobrez and Bertoglio 2005). Montgomery (1955) first used the concept and observed that rats were less likely to exhibit exploratory behaviors in 'open' arms compared to 'closed' arms in a Y-shaped test apparatus, which he attributed to an avoidance of open alleys due to the fact that they engender higher levels of fear. A modern version of the EPM has been designed with four arms and has been used to assess how drugs may influence anxiety in rats. For example, Handley and McBlanch (1993) noted that the anti-anxiety drug diazepam increased the ratio of open: total arm entries, while the pro-anxiety drug picrotoxin diminished this ratio.

Another novel approach for assessing anxiety specific to fish models that is gaining in popularity is use of a dive tank (Sackerman et al. 2010). This is a very simplistic approach in which a fish is placed in a novel environment and the time it spends at various depths is monitored. Typically, fish placed in a novel environment will immediately dive to the bottom of a tank, which is likely an anti-predation mechanism. This may also be a useful tool to assess anxiety, which is inherently coupled to predator avoidance, and fish exposed to anti-anxiety drugs would be more likely to spend time away from the bottom compared to unexposed fish. Several researchers have successful used this approach to assess anxiety in zebrafish (Levin et al. 2007, Bencan and Levin 2008, Egan et al. 2009).

In our experiment we examined whether water exposures of the SSRI sertraline caused increases in plasma concentrations that subsequently led to physiological changes in the SERT transporter of adult male *Pimephales promelas* (fathead minnow). Furthermore, we attempted to distinguish whether these changes led to altered behavior by using a barrage of behavioral trials. These experiments included use of the modified plus maze for aquatic organisms, dive tanks, and digital tracking software. The ultimately goal of the study was to relate how increased plasma concentrations of a model xenobiotic could cause physiological changes that cascaded into aberrant behaviors in exposed fish.

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Materials and Methods

Test Organisms

All fish were maintained and examined according to an approved Baylor University animal care protocol. Newly hatched *Pimephales promelas* (<24 h) were purchased from a commercial supplier (Environmental consulting and testing Inc, Superior, WI). Individuals were housed in a flow through system supplied with aged, de-chlorinated tap water at a constant temperature of 25±1º C under a 16:8 light: dark photoperiod. For the first 30 d individuals were fed twice daily solely on a diet of newly hatched *Artemia* spp. and thereafter were fed a mixture of *Artemia* spp. and certified test-grade flake food. Individual were aged to 120 d before exposures were initiated. Only adult male fish that exhibited pronounced reproductive features were used in bioassays.

Experimental Design

Exposures were completed in 5-g experimental units (aquarium) filled with 20-L of test solution. Each aquarium housed 6 adult male *P. promelas* and had 3 shelters (3-in PVC cut in half length wise into sections of 10 cm) to reduce potential altercations. De-chlorinated tap water was used as the control treatment and dilution water for each of the 3 sertraline treatments. A stock of sertraline hydrochloride (Sigma Aldrich, St. Louis, MO, USA) was prepared and used to create the sertraline concentrations. Treatment levels included 3, 10, and 30 µg/L sertraline and each had 3 replicate experimental units, thus a total of eighteen individual adults per treatment. The exposures for each set of replicates were staggered by one day to accommodate for the substantial time that it

would take to complete the behavioral trials. Each set of replicates consisted of four aquariums from each of the treatment levels, including a control.

Exposures were 28-d in duration and fresh exposure water was prepared daily and 75% v/v water renewals were performed. All aquaria were housed in a single walk-in incubator set at 25 ± 1º C with a 16:8 light: dark photoperiod. Temperature in the aquaria was monitored daily. Water quality parameters, including dissolved oxygen, conductivity, pH, chlorine, and ammonia were measured 3 times a week.

Behavioral Trials

Novel Dive Tank. The specific methods for behavioral trials in the dive tank are described in Sackerman et al. (2010) and the overall concept for its use was based on Levin et al. (2008). The dive tank was a transparent, hexagon 19 L fish tank (Marineland Eclipse System 5 Hex combo) filled to a depth of 30 cm. Lines dividing the tank into quarters were drawn on the outside with marker to aid observation (Figure 6). The tank sat on a black countertop, and its back wall was covered with white vinyl to enhance contrast for video recording. Fish in the dive tank were observed and digitally recorded with a SONY Handycam (DCR-SX40) for 300 seconds to determine the amount of time fish spent in each zone. The camera was position on the side at the center height of the aquarium. The plus maze was repeatedly rinsed three times with de-chlorinated tap water before being refilled for the next trial.

Novel light-dark plus maze. The aquatic plus maze test was performed according to methods described by Sackerman et al. (2010). The height, width, and depth of the plus maze were 71 cm, 51 cm, and 10 cm, respectively (Ezra Scientific, San Antonio,

TX). The plus maze module had a 10 cm^2 center section surrounded by four arms (three were10 x 10 cm, while the fourth was 10 x 15 cm). The two shorter opposing arms were lined with black polyethylene, while the other arms were lined with white polyethylene cut from folders and secured to the walls with binder clips. The maze was place on a grey commercial grade cart and the center was left open. An image of the plus maze is shown in Figure 7. The maze was filled to a depth of 8 cm.

Fish netted from the dive tank were released into the center section of the plus maze and observed and digitally recorded for 300 seconds with a SONY Handycam (DCR-SX40). The camera was positioned directly over the center of the plus maze. During the trial, the number of crosses into white arms, total duration of time spent in white arms, and time fish spent motionless in the center section upon introduction (initially frozen) were calculated from digital recordings. This scoring was based on the technique used for rats in the elevated plus maze (Lapiz-Bluhm et al. 2008). The plus maze was repeatedly rinsed three times with de-chlorinated tap water before being refilled for the next trial.

NOLDUS experiments. Once the behavior of 1 individual from each of the treatments was assessed using the dive tank and plus-maze, the 4 fish were allowed one hour of acclimation prior to loading into the NOLDUS behavioral apparatus (Figure 8). The order in which fish were removed from a treatment was determined by using a random number table. The order was also used to determine their position in the NOLDUS apparatus. These experiments were designed to examine shelter-seeking and nest-

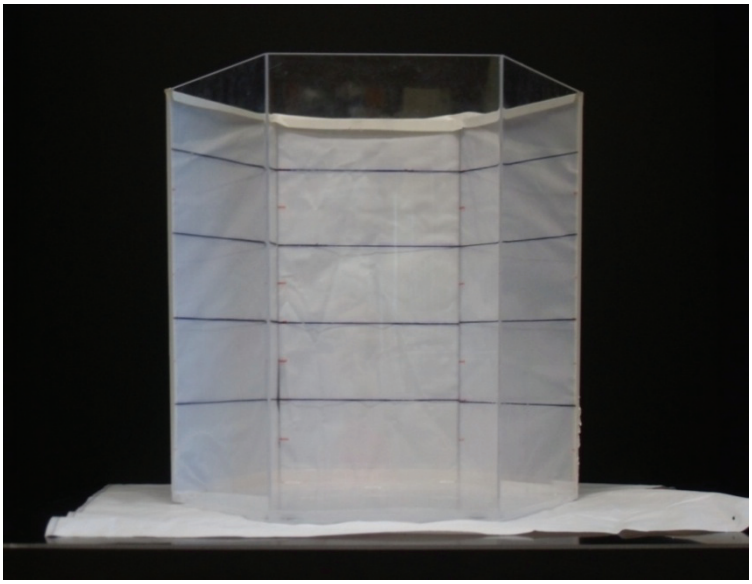


Figure 6.. A photo of the dive tank used to assess differences in the behavior of adult male *Pimephales promelas* unexposed and exposed to sertraline based on work by Levin et al. 2007.

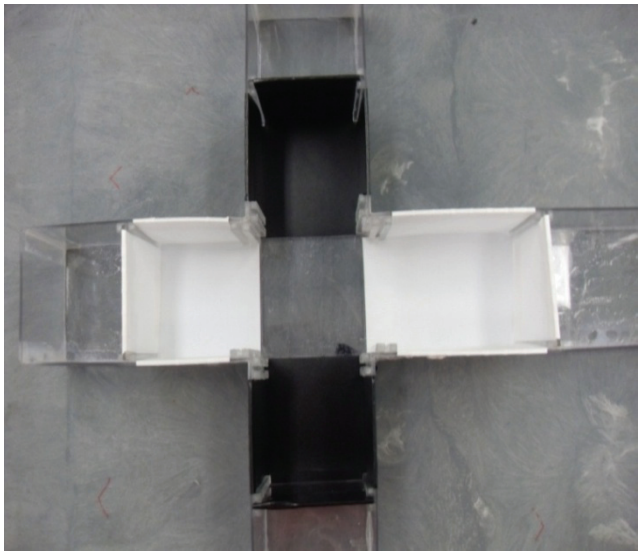


Figure 7. A photo of the plus maze used to assess differences in behavior for sertraline exposed and control adult male *Pimephales promelas*. The overall design is based on similar apparatuses used in drug development to assess anxiety in rats.

guarding behavior under both light and dark conditions. The apparatus essentially consisted of a SONY Handycam (HDR-SR11) that was equipped with a visible light filter (850 nm) positioned over the center of four test areas. The camera was connected to a video port on the back of a computer that had NOLDUS digital tracking software installed using AV cables. Each test area was a white plastic bin (20 L, 35 cm x 40 cm) that housed a shelter and was filled with 15 L of de-chlorinated tap water. The camera and NOLDUS video setting were previously calibrated to optimize tracking with individuals not used in the trials. In addition, the arena was also calibrated by using a ruler placed at several locations in each of the 4 arenas. Two high powered infrared lights (Manufacturer) were positioned below the 4 arenas to allow for indirect illumination with infrared light. These lights remained on throughout the duration of the experiment. Two light fixtures fitted with 60 watt bulbs were position above the areas that were either turned on or off to simulate light and dark.

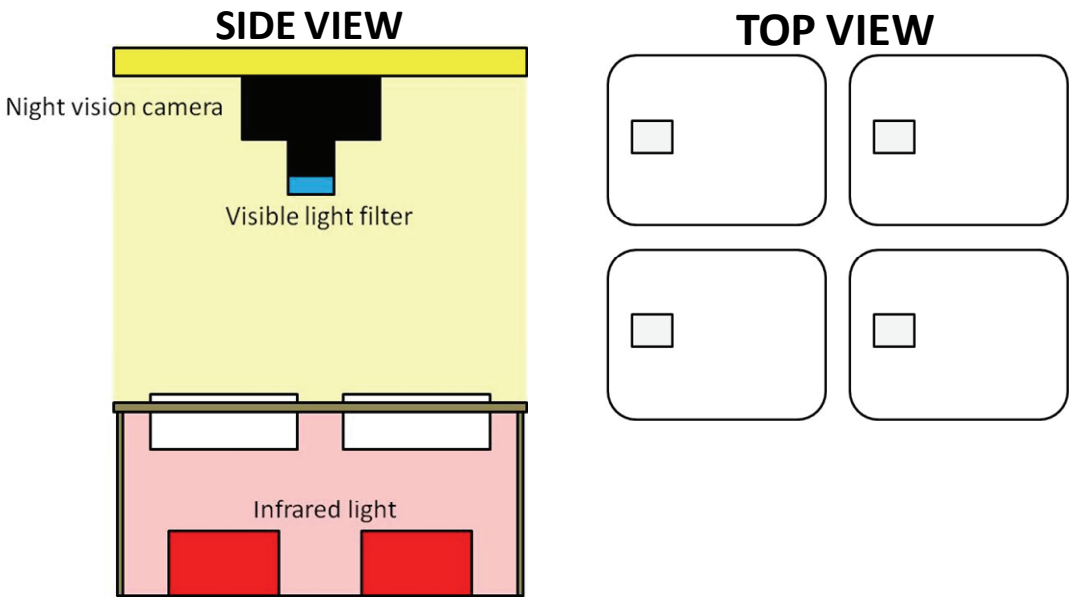


Figure 8. A drawing of the NOLDUS apparatus used to assess differences in the behavior of adult male *Pimephales promelas* unexposed and exposed to sertraline.

Fish were transferred to the areas using an aquarium net and the trial initiated within 10 s once all four fish were loaded. The order that fish were introduced was based on the random number table. The camera was set to record and a trial was initiated on the NOLDUS software program menu. At the beginning of the trial the lights were on; however, after 300 second the lights were then switched off. This processes was repeated a total of 3 times so that during each trial the behavior of the fish could be assessed 3 different times under both light and dark conditions. The NOLDUS software automatically analyzed the total distance traveled, swim velocity, and overall movement for each of the 300 second light and dark time interval for each fish. The time that the fish spent in the shelter was calculated manually by reviewing digital video files to ensure that the fish was actually inside of the shelter and not merely swimming above it.

Following a completed NOLDUS trial the fish were removed, their weight and length measured, and then decapitated. The head was then immediately place inside of a microcentrifuge tube and stored in a -80º C freeze for future brain analysis. A heparinized hematocrit tube from StatSpin (Westwood, MA) was used to collect blood. Once this process was completed for all 4 fish, the blood was spun at 10,000 rpm for 3 min in a microcentrifuge. The resulting plasma was then harvested and placed in a microcentrifuge tube with a micropipette so that a volume could be estimated. The plasma was the immediately diluted 1:10 with 100mM phosphate buffer saline buffer with preservatives pH 7.0 filtered with 0.22 micron (PBS) (Immunalysis Corporation, Ponoma, CA) and placed under refrigeration at -4º C. Another round of plus maze, dive tank, and NOLDUS apparatus trials was completed as previous described using individuals from the same group of tanks.

Analytical Quantification of Sertraline

Sertraline direct ELISA kits (Immunalysis Corporation, Pomona, CA) were used to quantify plasma sertraline concentrations in control and exposed fish. The Immunalysis sertraline direct ELISA kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen in proportion to the concentration of the reaction mixture. The plasma of individuals in the same tank was pooled to ensure adequate volumes for analytical measurements. A calibration curve was developed by collecting plasma from fish not used during the exposure, which was then diluted with PBS. This mixture was then loaded in triplicate in the ELISA kit as the control. Next, the mixture was spiked with sertraline to a final concentration of 800 ng/ml. Then 100 ul of the 800 ng/ml plasma sertraline standard was added with a micropipette to the ELISA kit in triplicate. The 400 ng/ml standard was achieved by adding 50 ul of the 800 ng/ml mixture in triplicate to the ELISA kit. The 200, 100, 50, and 25 ng/ml concentrations were prepared similarly by adding half of the volume of the next higher concentration.

Plasma samples from exposed fish were then added to the wells of the ELISA kit. To ensure that absorbance readings were within the calibration curve range, three dilution of the plasma were prepared. This was achieved by adding 25, 50 and 100 ul of exposed fish plasma in triplicate to the ELISA kit to establish respective dilutions of 0.25, 0.5, and 1. After incubation the kit was then placed on a calibrated microplate reader and absorbance was measured at 450 nm and 630 nm within 30 min of loading the last sample. A detailed description of the directions for the Sertraline direct ELISA kit can be found at the Immunalysis website. Sertraline direct ELISA kits were also used to

confirm nominal concentrations of sertraline in exposure water using methods similar to those previously described.

Comparing Measured versus Predicted Fish Plasma Concentrations

Measured plasma concentrations of sertraline were compared to predictions from a slightly modified model developed by Fitzsimmons et al. (2001). For calculations, Log K_{OW} value (Eq.1) was substituted for the predicted Log D value of 3.77 (Scifinder scholar) and the $LogD_{lipwater}$ value (Eq. 2) of 4.06, which was based on the equation described in Escher and Schwarzenbach (2000). The exposure concentrations of 0, 3, 10, and 30 µg/L were substituted in for the environmental concentrations (EC) values used in Eq.3. The human therapeutic plasma concentration (H_TPC) was defined as 142 ng/mL (Thomson 2008) and used to calculated Eq 4.

$$\text{Log } P_{\text{Blood:Water}} = 0.73 \text{ x Log } D_{\text{Oct:water}} - 0.88 \quad (1)$$

$$\text{Log } P_{\text{Blood:Water}} = 0.73 \text{ x Log } D_{\text{Lip:water}} - 0.88 \quad (2)$$

$$FF_{ss}PC = EC \text{ x } P_{\text{Blood:water}} \quad (3)$$

$$ER = H_TPC/F_{SS}PC \quad (4)$$

Saturation Radioligand Binding to Serotonin Transporters in Whole Brain Homogenates

SERT saturation binding to [3H] citalopram in membrane homogenate preparations from fathead minnow whole brains were performed following the methods of D'Amato et al. (1987), with minor modifications. Whole brain from three fish were

pooled into each homogenate preparation, and tissue was dispersed at 30,000 rpm for 20 sec into 25 ml of ice-cold 50 mM Tris, 120 mM NaCl, 5 mM KCl, pH 7.4 at 26ºC buffer using a tissue homogenizer (Polytron 3100, Kinematica, Bohemia, NY). The homogenate was spun for 10 min at 30,600 x g in a 4°C centrifuge (Avanti A-J, Beckman-Coulter, Brea, CA). The supernatant was discarded and the pellet re-suspended in 5 ml buffer on ice using a hand-held tissue homogenizer. Another 20 ml of buffer was added and the homogenate was centrifuged again for 10 min at 30,600 x g. The final pellet was re-suspended to obtain a protein concentration near 1 mg/ml, as determined with Bradford reagent (Sigma, St. Louis, MO) and measured on a spectrophotometer (DU-640, Beckman). The hippocampal homogenate was incubated at 26°C for 1 hour in buffer containing 0.1 – 12 nM of [3H] citalopram (PerkinElmer, Boston, MA). Non-specific binding was defined by 50 μM sertraline (Pfizer, Groton, CT). Incubation was terminated by addition of 4 ml of buffer, pH 7.4 at 4°C. Labeled homogenates were captured by filtration under vacuum with a tissue harvester (Brandel, Gaithersburg, MD) onto Whatman GF/B filter paper strips (Brandel) pre-soaked in 5% polyethyleneimine (Sigma). Filters were washed twice more with 4 ml of buffer. [3H] citalopram trapped in membrane tissue on the filters was determined using a liquid scintillation counter (LS 6500, Beckman) with 40% efficiency.

Statistical Analysis

Statistical significance of response variables was assigned at $\alpha = 0.05$ for all tests. The mean responses of individuals for each replicates were determined (n=6). These means were then used for statistical analysis (n=3). No-observable-adverse effect concentration (NOAEC) and lowest-observable-adverse effect concentrations (LOAEC)

for results of behavioral trials were calculated based on the statistical methodology described by standard protocols. Endpoints for the plus maze and dive tank were compared among the different treatments using analysis of variance (ANOVA).

For results of experiments using NOLDUS, ANOVAs were performed between the various sertraline treatments by each time period. The time that fish spent in shelters was tallied by hand by reviewing video data, while distance traveled and velocity were calculated using NOLDUS software by creating time bins of 300s for each trial. For endpoints that had significant differences among the means, Dunnett's comparisons were then completed between control groups and those exposed to sertraline for each specific time, respectively.

Results

Analytical Quantification of Sertraline

Nominal treatment concentrations for water exposures were very close to those measured (Table 5). The results of ELISA experiments also clearly demonstrated a dose-dependent relationship between concentrations of sertraline in water and plasma concentration in exposed fish. Sertraline was not detected in the plasma of control fish but was quantifiable for the other treatments. The respective mean (±standard deviation) plasma concentrations for the 3, 10, and 30µg/L exposure treatments were 280±110, 720±70, and 1900±50 ng/mL. These measured sertraline plasma concentrations were very similar to those predicted by the fish plasma concentration model based on water column exposure concentrations (Figure 9). Furthermore, the effects ratios (ER) based the ratio measured fish plasma concentrations and human therapeutic plasma

concentrations were all <1, suggestive that potential biological effects would be realized in exposed fish. These measured values were closer to predicted values based on $LogD_{Oct:water}$ compared to $LogD_{lip:water}$ as the relationship was nearly one to one based on slopes of 0.85 and 0.5, respectively.

Table 5. Measured sertraline concentration in treatment water for the 28 d chronic experiments with *Pimephales promelas.*

Nominal Sertraline (µg/L)	Water concentration (µg/L)			
	Week 1	Week 2	Week 3	Mean ± SD
0	<1.5	<1.5	<1.5	<1.5
3	3.5	3.3	3.4	3.4 ± 0.1
10	9.9	12	11	11 ± 1
30	26	29	27	27 ± 1

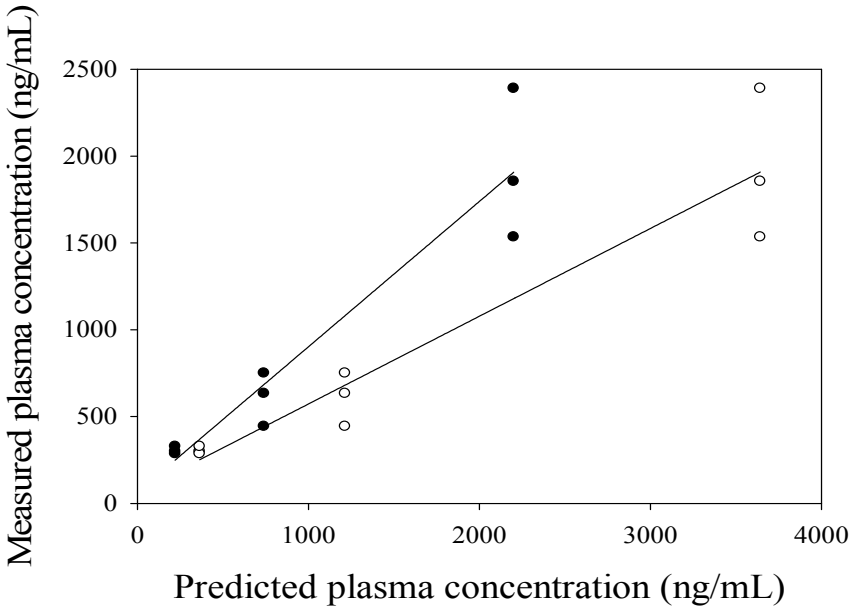


Figure 9. The measured versus predicted fish plasma concentration based on the Huggett et al. model (2003). Closed dots are based on Log D_{octw}, open dots are based on D_{lipw}.

SERT Binding Study

Physiological changes in the brains of fish were also apparent for fish with elevated plasma concentrations of sertraline following water borne exposure. The mean

numbers of low affinity SERT transport sites in fish exposed to sertraline were significantly lower compared to the control (Table 6). However, while all treatments had lower SERT for the high affinity site, only those in the 3 µg/L treatment were significantly different than the control.

Table 6 The results of the SERT bound by radiolabeled citalopram experiments.

Sertraline Treatment (µg/L)	High affinity site mean±SE	Low affinity site mean±SE
0	27 ± 2.1	148 ± 8.2
3	16 ± 3.2*	101 ± 4.1*
10	18 ± 1.5	110 ± 9.4*
30	18 ± 2.4	111 ± 9.3*

Behavioral Trials

There was no significant difference in the behavioral endpoints measured for the plus maze among the different sertraline treatments. Although not significant (p=0.78), the mean number of times (± standard error) that fish exposed to 10 or 30 µg/L crossed into the white arms (4.9 ± 1.4 and 4.8 ± 2.3 crosses, respectively) were greater than the number by individuals in control and 3 µg/L treatments (3.3 ± 1.4 and 3.1 ± 1.5 crosses, respectively) (Figure 10). In addition, the mean number of total crosses between any color were also not significantly different (p=0.73); however, the mean (± standard error) number of total crosses increased with increasing sertraline treatment were 9.3 (±2.1), 11.9 (± 3.8), 12.4 (± 1.6) and 14.6 (± 9.6) for the control, 3, 10, and 30 µg/L (Figure 11). Observed behavioral endpoints from the dive tank experiments were not significantly different between sertraline treatments, although the mean numbers of total crosses were less for the 10 and 30 µg/L treatments (Figure 12). Fish from all treatment levels spent predominantly all time (>95%) in the bottom area of the dive tank (Figure 13).

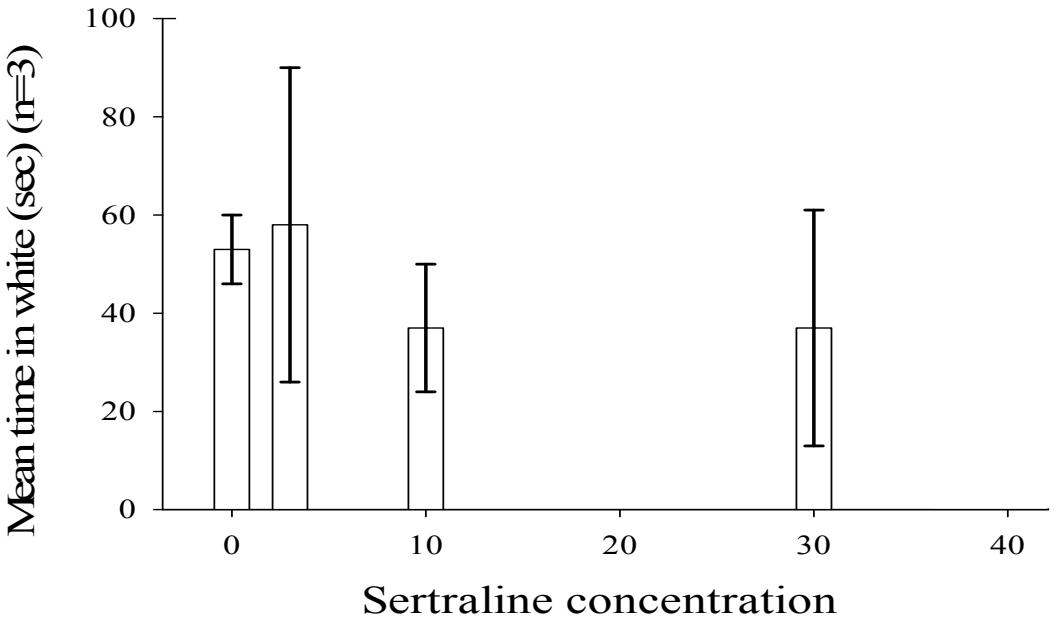


Figure 10. The mean amount of time that *Pimephales promelas* exposed to sertraline spent in white areas of plus maze. Fish spent a total of 300s in the plus maze.

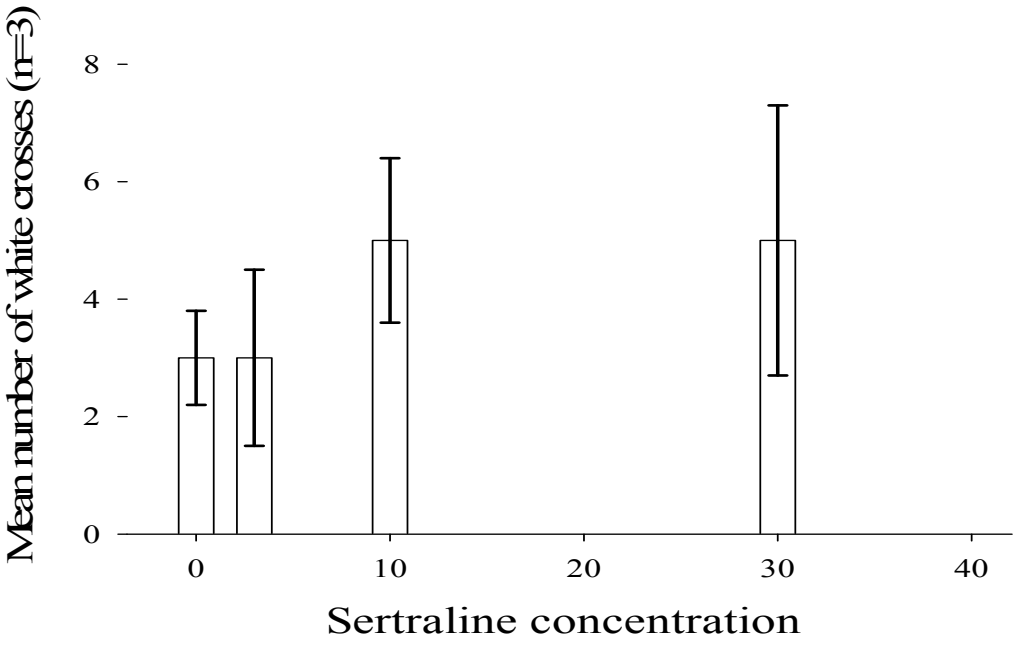


Figure 11. The mean number of times that *Pimephales promelas* exposed to sertraline crossed into white areas of plus maze.

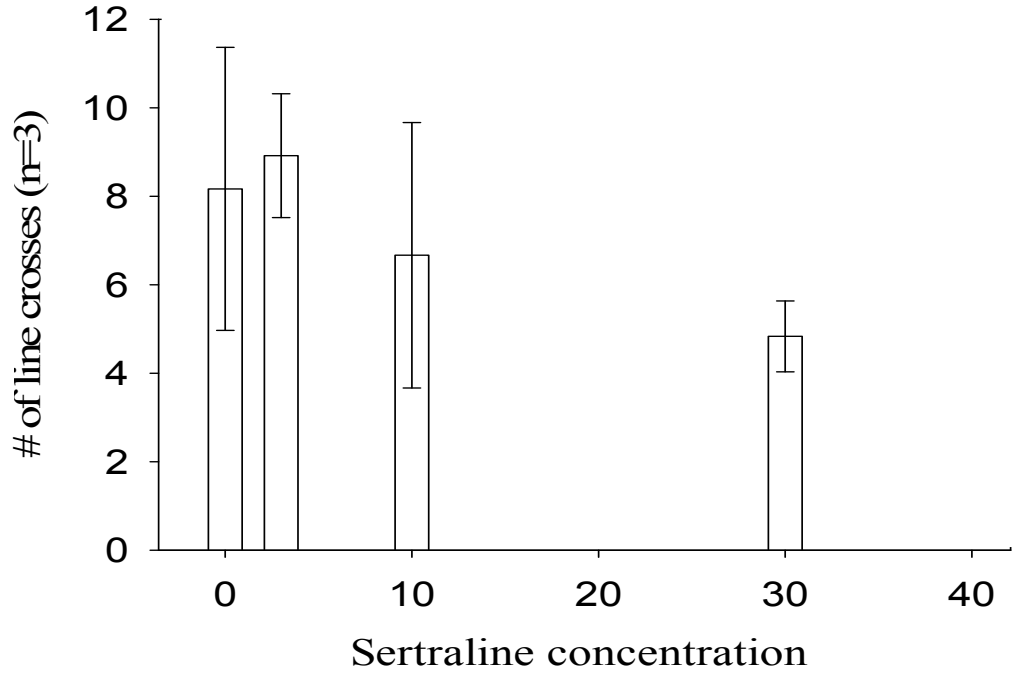


Figure 12 The mean number of times that *Pimephales promelas* exposed to sertraline crossed different areas in the dive tank. Fish spent a total of 300s in the dive tank.

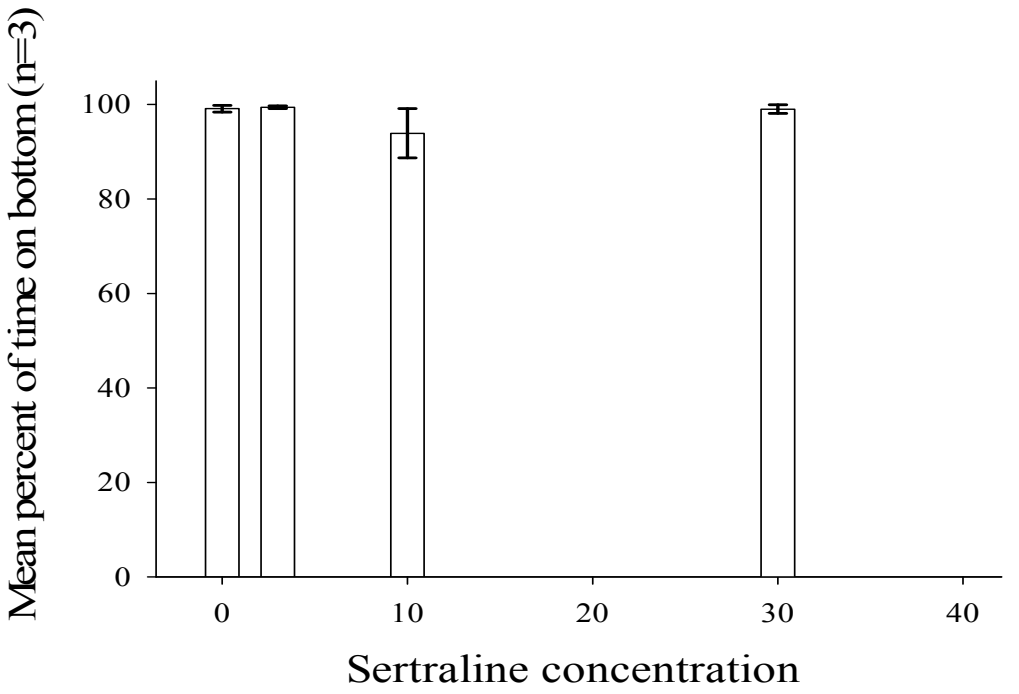


Figure 13. The mean amount of times that *Pimephales promelas* exposed to sertraline spent in the bottom area of the dive tank.

The results of the behavioral trials with NOLDUS clearly demonstrated an appreciable difference among the behavior of exposed and unexposed fish. Control fish spent greater amounts of time in the shelter during light periods (1,3,and 5) compared to the other treatments (Table 7) During light periods 3 and 5, control fish spent more than twice the amount of time in the shelter (mean ± standard error, 217 ± 31 and 239 ± 36 seconds, respectively) compared to those exposed to any level of sertraline as these fish only spent 43-98 seconds in shelters (Table 7). The amount of time that fish spent inside the shelter during dark conditions (time periods 2,4,and 6) were similar for all treatments and means (± standard errors) ranged between 8 ± 3 and 34 ± 29 seconds. In addition, the mean distance of total travel (cm) was also significantly different between control and fish exposed to sertraline during the 2nd and 3rd light periods (Table 7). Exposed fish travelled significantly higher distances during these light periods. Notably, the mean velocities that fish travelled (cm/second) were not significantly different between any time periods (Table 7). Therefore, differences in the mean total distance travelled during light periods may have been merely manifested by the fact that the fish were spent less time in shelters.

Discussion

In our study we demonstrated that water borne exposure to sertraline caused elevated concentrations in the plasma of *P. promelas* that affected the SERT-binding characteristics in the brains of exposed compared to unexposed fish (Table 6). These results are supported by previous research, which demonstrated that oral doses of the sertraline in food reduced SERT binding in zebrafish brains (Gould et al. 2007). Furthermore, we related these physiological changes to potentially ecologically relevant

behavioral modification of shelter seeking/ nest guarding (Table 7). In previous studies, the alternative endpoint of feeding rate was observed to be more sensitive than growth or survivorship when juvenile *P. promelas* were exposed to SSRI in the water column (Valenti et al. 2010, Stanley et al 2007). In our current study the sublethal response of shelter seeking in adult males (3 µg/L) was actually more sensitive than those for feeding with juveniles (15 µg/L) during exposures to sertraline. These findings suggest that the measurement of SERT occupancy following exposure to SSRIs could potential provide a better understanding of the pharmacodynamics and pharmacokinetics properties of these drugs and the potential risk they pose to aquatic organisms.

The measured plasma concentrations of sertraline in fish exposed to all of the water exposure concentrations tested in our study exceeded therapeutic plasma concentrations for humans. The predicted calculated effect ratios for the 3, 10, and 30 µg/L sertraline water exposures at pH 8.5 based on $LogD_{Oct:Water}$ values were all < 1 as respective values were 0.65, 0.19, and 0.06. The respective means (±standard deviations) based on measured plasma concentrations were 0.57 (±0.1), 0.29 (±0.03), and 0.09 (±0.01). The modeled and measured ERs are similar and both indicate that potential toxicological effects may occur in fish based on plasma levels, which are further supported by the similarity in SERT transport sites (Gould et al. 2003). However, these relationships are only suggestive and cannot clearly prove that adverse affects of ecological consequence may occur in fish because the functionality of the serotonergic system varies among genera.

For use as a tool in ecological risk assessment, it is imperative that observed behavioral modification have ecological relevance and can be subsequently related to fitness or survival of individuals (Scott and Sloman 2004). The decrease in shelter

Table 7. The results of NOLDUS experimental trials including the mean (± standard error) amount of time adult Pimephales promelas spent in the shelter, the mean total distance(± standard error) traveled, and mean(± standard error) velocity. Time period 1,3, and 5 had lights on while time periods 2,4, and 6 were in the dark. Fish spent a total of 300s in the chamber.

Time Period	Sertraline (µg/L)	Time in shelter ± SE (sec)	Distance ± SE (cm)	Velocity ± SE (cm/sec)
1	0	258 ± 22	327 ± 143	16 ± 13
1	3	173 ± 44	372 ± 107	3 ± 1
1	10	203 ± 17	658 ± 276	11 ± 7
1	30	202 ± 49	568 ± 313	16 ± 12
2	0	30 ± 16	1640 ± 66	15 ± 5
2	3	23 ± 6	1590 ± 106	19 ± 12
2	10	33 ± 10	1728 ± 204	7 ± 0.6
2	30	21 ± 9	1890 ± 128	7 ± 0.7
3	0	217 ± 31	847 ± 290	10 ± 3
3	3	91 ± 42*	1760 ± 84*	8 ± 0.2
3	10	55 ± 14*	1300 ± 172*	8 ± 0.5
3	30	81 ± 26*	1746 ± 240*	9 ± 0.7
4	0	19 ± 1	1697 ± 152	21 ± 26
4	3	8 ± 3	1792 ± 138	7 ± 1
4	10	13 ± 5	2023 ± 225	8 ± 3
4	30	20 ± 15	2044 ± 74	7 ± 1
5	0	239 ± 36	905 ± 274	8 ± 3
5	3	78 ± 34*	1493 ± 6*	9 ± 2
5	10	98 ± 47*	1918 ± 195*	7 ± 0.3
5	30	43 ± 20*	2054 ± 152*	9 ± 1
6	0	34 ± 29	1574 ± 238	9 ± 4
6	3	9 ± 6	1777 ± 117	7 ± 1
6	10	5 ± 3	1870 ± 90	9 ± 3
6	30	4 ± 2	1982 ± 82	7 ± 0.4

seeking behavior we observed by adult male *P. promelas* exposed to sertraline during light periods has implications for both survival and fitness. Individuals willing to spend more time away from shelters may face greater predatory risk and thus their overall survival rate may be reduced. In terms of reproduction, fathead minnows typically spawn adhesive eggs to the underside of aquatic plants or woody debris (Nelson and Paetz 1992). Shelter seeking may be intrinsically linked to nest guarding behavior by male fathead minnows, which is important for defining territories and attracting mates (Jamieson 1995). Martinovic et al. (2007) exposed *P. promelas* to environmental estrogens in the water and noted alterations in nest guarding behavior that ultimately led to complete reproductive failure. Kreke and Dietrich (2008) summarized the effects of serotonin and SSRIs in aquatic vertebrates and observed changes in various reproductively relate pathways often within 30 to 90 minutes following *in vivo* injections. Other researchers have demonstrated that serotonin influences reproduction (Khan and Thomas 1992) and aggression (Adams et al. 1996) in fish, and consequently exposure to SSRI may spur similar behavioral modifications.

The altered behavioral patterns of patterns during the NOLDUS experiments may have been linked to anxiolytic properties associated with SSRIs and exposed individuals may have less reserved about exploring their environment. However, our results from the plus-maze and dive tank do not support this and therefore these behavior responses may not have been related to anxiety, but rather potentially to other physiological changes associated with exposure to SSRIs. Kreke and Dietrich (2008) suggested that following chronic exposure SSRIs could reach the retina and pineal organ in fish, which could affect serotonin reuptake at these sites. This could alter normal patterns of

serotonin and melatonin and consequently lead to changes in photosensitive behaviors, such as hunting, feeding, or reproduction (Kreke and Dietrich 2008). Therefore, the observed changes of behavior of *P. promelas* during light periods may have been caused by differences in the way in which they perceive their environment. Additional research examining the specific receptors in fish that may potential be affected by SSRIs exposure is clearly warranted.

There are several potential advantages associated with incorporating behavioral studies into toxicological assessment of pharmaceuticals to aquatic life beyond the fact that changes in behavior occur more rampantly than gross-levels of toxicity, such as survivorship or life-history characteristics. This is potentially advantageous in that it may reduce cost and time lag associated with evaluating risk of pharmaceuticals using biological entities. Furthermore, because observing behavior can be achieved non-invasively, researcher may complete time-dependent studies that entail both exposure and recovery.

CHAPTER FOUR

A Mechanistic Explanation for pH-Dependent Ambient Aquatic Toxicity of Prymnesium parvum Carter

Introduction

NOTE: Chapter two is published in Toxicon (2010) 55:990-998. Please refer to Appendix B for the licensing agreement.

Harmful algal blooms (HABs) may have devastating impacts on aquatic ecosystems, resulting in severe impacts to fisheries. Increases in the frequency and severity of HABs on the global scale has triggered scientific inquiry to define factors causing these trends (Zingone and Enevoldsen 2000, Anderson et al 2002, Hallegraeff 2003); however, among the greatest challenges for managers is the spread of invasive species. *Prymnesium parvum* is an example of an invasive HAB species that has transitioned from marine origins to inland systems. Identified nearly a century ago as a problem in marine environments because of its toxic blooms (Liebert and Deerns 1920), *P. parvum* is more recently recognized as an invasive species threatening inland systems in the arid and semiarid southwestern and south central United States (Baker et al. 2007, Roelke et al. 2007, Schwierzke et al 2010).

Anthropogenic changes to the hydrologic cycle, eutrophication, and salinization of waterways are associated with the spread of HABs (Anderson et al. 2002). Such changes, intertwined with climatological and geological factors, have rendered some Texas reservoirs to be within the tolerance range of *P. parvum* (Larsen and Bryant 1998, Baker et al. 2007, Baker et al. 2009). Specifically, the species' euryhaline nature has

apparently facilitated its transition from coastal marine and estuarine ecosystems to these weakly saline inland impoundments. Since the first harmful blooms of *P. parvum* in Texas were documented in the Pecos River (James and De La Cruz 1989), *P. parvum* has spread to other systems in Texas resulting in toxic blooms and fish kills (Roelke et al 2007, Schwierzke et al. 2010).

Prymnesium parvum is a mixotrophic haptophyte that can gain energy photosynthetically as well as phagotrophically by feeding on other microorganisms (Skovgaard and Hansen 2003). Exposure to *P. parvum* toxins can lyse cells (Yariv and Hestrin 1961, Tillmann 2003), disrupt cell membrane integrity (Yariv and Hestrin 1961, Padilla 1970, Kim and Padilla 1977, Brooks et al. 2010), and affect gill functions of aquatic organisms (Ulitzur and Shilo 1966). Ecologists have proposed several purposes for the production and release of toxins by *P. parvum*, including acquisition of prey (Stoecher et al. 2006), elimination of algal competitors (Fistarol et al. 2003, Graneli and Hansen 2006, Uronen et al. 2007), or reduced grazing pressure (Rosetta and McManus 2003, Tillmann 2003). A variety of factors, including nutrient limitation, salinity, temperature, and light are known to influence cell growth and the toxicity of laboratory cultures of *P. parvum* (Shilo and Aschner 1953, Padilla 1970, Dafni et al. 1972, Larsen et al. 1993, Larsen and Bryant 1998, Johansson and Granéli 1999, Granéli and Johansson 2003, Baker et al. 2007, Baker et al. 2009). Few studies have focused on factors governing the behavior of the toxins once they are released, or considered how bloom formation might alter the environment (e.g., light attenuation, nutrient availability, dissolved oxygen, pH) in ways that could influence the bioavailability and potency of *P. parvum* toxins.

Shilo and Aschner (1953) proposed that *P. parvum* toxins were proteins with high molecular weights. To date, the only characterized toxins are prymnesin-1 and -2, large chains of 90 carbon atoms and trans-1,6-dioxadecaline units with conjugated double/triple bonds at each terminal end; their respective chemical formulas are $C_{107}H_{154}Cl_3NO_{44}$ and $C_{96}H_{136}Cl_3NO_{35}$ (Igarashi et al. 1999). These compounds are amphiphilic, with uneven distributions of sugars and hydroxyl groups, and three chlorine atoms and one nitrogen atom. Both prymnesins are structurally similar to other HAB toxins such as maitotoxin and ciguatoxin, which are characterized by a network of hydroxylated polycyclic ether units (Murata and Yasumoto 2000). The amine present on the prymnesins suggests that these compounds might be weak ionizable bases with pKa values > 8. Prior studies suggested that some of the toxins released by *P. parvum* are ionizable, becoming more toxic to fish exposed at higher pH, with toxicity eliminated below pH 7 (Shilo and Ashner 1953, McLaughlin 1958, Ulitzur and Shilo 1964). However, these experiments were completed under marine conditions, and prior to the development of standardized aquatic bioassays

This study examines whether pH also influences the toxicity of *P. parvum* toxin in less saline waters representative of Texas reservoirs where blooms have occurred. Simultaneous bioassays were performed at three pH levels with samples obtained during *P. parvum* blooms occurring in 2007 from two reservoirs, and with samples of laboratory cultures and culture filtrates. Further, the chemical structures of prymnesin-1 and -2 were examined to estimate their physiochemical properties. We hypothesized that toxins released by *P. parvum* are ionizable weak bases.

Material and Methods

Bioassays with Samples Obtained from Reservoirs Experiencing Blooms

Lake Whitney. Lake Whitney is a reservoir constructed in 1951 on the Brazos River, with a capacity of 4.68 x 10^8 m^3, surface area of 95 km^2, and shoreline of 362 km (Bailes and Hudson 1982). Two 4-L samples were collected in NALGENE® I-Chem Certified Series™ 300 LDPE Cubitainers™ (Fisher Scientific) from Lake Whitney during a bloom in March 2007, transported to the laboratory on ice, and stored under refrigeration at 4º C. This lake sample contained 61.5 x 10^3 *P. parvum* cells mL^{-1} (enumerated microscopically with a hemocytometer). Ambient pH at the site in Lake Whitney when the sample was collected was pH 8.4. Total ammonia in the samples was <1 mg/L in whole samples, which is below ambient water quality criteria for the temperature and pH at which our experiments were completed. Dilutions in our toxicity experiments at which toxicity was observed further confirmed that dose dependent responses were not due to ammonia. Toxicity tests were initiated within 96 h of sample collection following EPA recommendations for ambient toxicity studies (US EPA 2002).

Acute bioassays with <48 h old *Pimephales promelas* were conducted in 100-mL glass beakers. Three replicates of seven individuals were prepared for each treatment level. Reconstituted hard water (RHW) prepared according to APHA et al. (1999) was used as the diluent and control (treatment consisting of 100% RHW). Treatment levels were prepared by diluting lake water with RHW to the following percentages of lake water: control (RHW), 0.01, 0.1, 1, 5, 10, and 20%. These treatment levels were selected based upon ambient toxicity data from prior water quality monitoring efforts. A volume

of 3-L was prepared for each treatment level, which was then divided into three aliquots of 1-L that were then adjusted to pH units of 6.5, 7.5, or 8.5 ($\pm$ 0.05) prior to dispensing experimental aliquots. The pH adjustments were achieved by slowly titrating 10% HPLC-grade nitric acid, which generally followed U.S. Environmental Protection Agency protocols for pH adjustment in Toxicity Identification Evaluations (US EPA 1991). Test individuals were fed newly hatched brine shrimp (*Artemia sp.*) 2 h prior to the exposure, but were not fed during experiments (US EPA 2002). Survivorship was assessed at 24 and 48 h, and temperature, dissolved oxygen, and pH were measured at test initiation and completion. Exposures were conducted at 25±1ºC under a 16:8 light: dark photoperiod.

A 10-d *Daphnia magna* reproductive study was also completed with Lake Whitney water (US EPA 1994, modified as in Dzialowski et al. 2006). Treatment levels consisted of control (RHW), 12, 25, 50, and 100% lake sample water. Test solutions were prepared and adjusted to desired pH as previously described. Experimental units were 100-mL beakers filled with 80 mL of test solution. Organisms were fed daily with *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) augmented with filtrate from a cerophyll suspension (approximately 2 g / L RHW). The final green algae cell concentration was 30 x 10^6 cells mL^{-1}. One *D. magna* individual < 24 h old was introduced in each beaker and transferred every other day to fresh test solution. Five replicates were prepared for each treatment level. Survivorship and fecundity were monitored daily. The experiment was completed for 10 d at 25±1ºC under a 16:8 light: dark photoperiod.

Lake Granbury. Lake Granbury is a reservoir constructed in 1969, with a capacity of 167 x 10^6 m^3, a surface area of 34 km^2, and an average depth of ~5 meters. Samples were collected during a *P. parvum* bloom that caused fish kills in March 2007 at three fixed monitoring stations and were handled prior to bioassay initiation as previously described. Ambient pH at the sites ranged between pH 8.2 and 8.4. Total ammonia concentrations were <0.25 mg/l in the three samples, which again were lower than levels associated with ammonia toxicity (US EPA 1999). Cell counts for samples from Sites 1-3 were 29 x 10^3, 36 x 10^3, and 36 x 10^3 cells mL^{-1}, respectively.

Experiments were conducted with *P. promelas* similar to those previously described in order to assess acute toxicity at the three sites. Six treatments, including a control (RHW), 6, 12, 25, 50, and 100% lake water were prepared using RHW as the diluent and adjusted to pH 6.5, 7.5 and 8.5. A 96-h acute exposure experiment was initiated with < 24 h old *D. magna* using a composite sample from the three stations. Treatments included a control (RHW), 6, 12, 25, 50, and 100% lake water. Ten replicates were prepared for each treatment level. Exposures were completed in 100-mL glass beakers filled with 80 mL of test solution, and water in experimental units was renewed at 48 h. Organisms were fed daily the same concentration of the mixture described for the 10-d *D. magna* experiment, at which time survivorship was assessed. Exposures were conducted at 25±1ºC under a 16:8 light: dark photoperiod.

Laboratory Culture Preparation

The UTEX LL 2797 (University of Texas, Austin, Texas, USA) strain of *P. parvum* was used to initiate cultures. Cultures were grown in 20-L glass carboys filled with 14-L of an artificial seawater (ASW) prepared according to Berges et al. 2001 and

then diluted to a working salinity of 5.8 g L^{-1} with ultrapure water (18 MΩ cm^{-1}). Afterwards, nutrients ($NaNO_3$ and NaH_2PO_4) were added at concentrations of f/2 and f/8 media (Guillard 1975); vitamins and trace metals were the same for both types of media. Three replicates were prepared for each treatment and all carboys were inoculated with 10^3 cells mL^{-1} of *P. parvum* from stock cultures in late exponential phase grown in the corresponding medium at the stock salinity of 5.8 g L^{-1}. Cultures were maintained in incubators at 20±1°C for a 12:12 light: dark cycle with an irradiance of ~140 μE m^{-2} d^{-1}. Carboys were repositioned and mixed daily by gently swirling.

Bioassays with Samples Obtained from Laboratory Cultures

Several experiments with larval *P. promelas* were completed using these *P. parvum* cultures. An initial experiment examined the toxicity of the three replicate carboys of both the f/2 and f/8 cultures. ASW adjusted to a salinity of 5.8 g L^{-1} used in the media served as the diluent and controls. Bioassays were completed in 100-ml beakers filled to capacity with test solution. Treatments included a control (ASW), 1, 2.5, 10, 25, and 100% culture water containing *P. parvum* cells. Four replicates of five individuals were prepared for each treatment. Experiments were conducted at 25±1ºC under a 16:8 light: dark photoperiod.

For subsequent studies, we separately pooled f/2 and f/8 cultures and then filtered half of each volume through GF/C filters (Whattman GF/C; VWR International, West Chester, Pennsylvania, USA). Acute toxicity to *P. promelas* was determined for cultures (f/2, f/8) that were unfiltered and filtered (cell-free filtrate), then these samples were manipulated to pH 6.5, 7.5 or 8.5 following procedures outlined above. ASW adjusted to a salinity of 5.8 g L^{-1} was used as the diluent and control. An additional RHW treatment

for quality assurance was also prepared. Each acute toxicity study included a control (ASW), RHW, 0.1, 1, 5, 10, 25, and 100% media treatment. Four replicates of five *P. promelas* <48 h old were used for each treatment level. Experiments were conducted at 25±1ºC under a 16:8 light: dark photoperiod.

Statistical Analysis

LC_{50} values for acute toxicity to *P. promelas* and *D. magna* were calculated by Probit analysis if data met assumptions; otherwise, the Trimmed Spearman-Karber method was applied using TOXSTAT computer software (US EPA 2002). SAS (SAS Institute, Cary, NC, USA) was used for other statistical analyses. For the 10-d experiment with *D. magna*, significant differences in survivorship between the control at each of the respective pH treatments and reservoir water dilutions were determined using Fisher's Exact Test. Significant differences in reproduction were assessed by an ANOVA comparing the mean neonate production per female for all treatments (α = 0.05), followed by Dunnett's test comparing the controls to each of the treatments at a respective pH (α = 0.05). In addition, we compared the mean control responses between different pH levels for the respective endpoints using ANOVAs for each series of experiments to confirm health of test organisms.

Estimation of Prymnesin-1 and -2 Physicochemical Properties

Calculation of physicochemical parameters for prymnesin-1 and -2 (Figure 14) was carried out using ACD/Labs (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada) ChemSketch, pKa calculator, LogD calculator, and LogP calculator (Version 9). LogP was calculated for each whole molecule, and percent distribution of

species and pKa values were calculated considering the hydrophobic component of the molecule containing the primary amine group (Murata and Yasumoto 2000). The full molecule could not be handled by the program due to the large number of ionizable sites, especially on the hydrophilic portion of the molecule. Calculated values for LogP (octanol: water partitioning coefficient when the compound is primarily unionized), LogD (coefficient of octanol: water partitioning ratio of ionized to unionized over a pH range), and pKa (acid dissociation constant) are estimates based on the use of an extensive database of fragments and predicted inductive effects based on substituents near ionizable sites. LogD was calculated as the sum of logD for the hydrophobic fragment at different pHs with the LogP of the hydrophilic fragment (neutral form), which remains neutral at all relevant pHs (< 12).

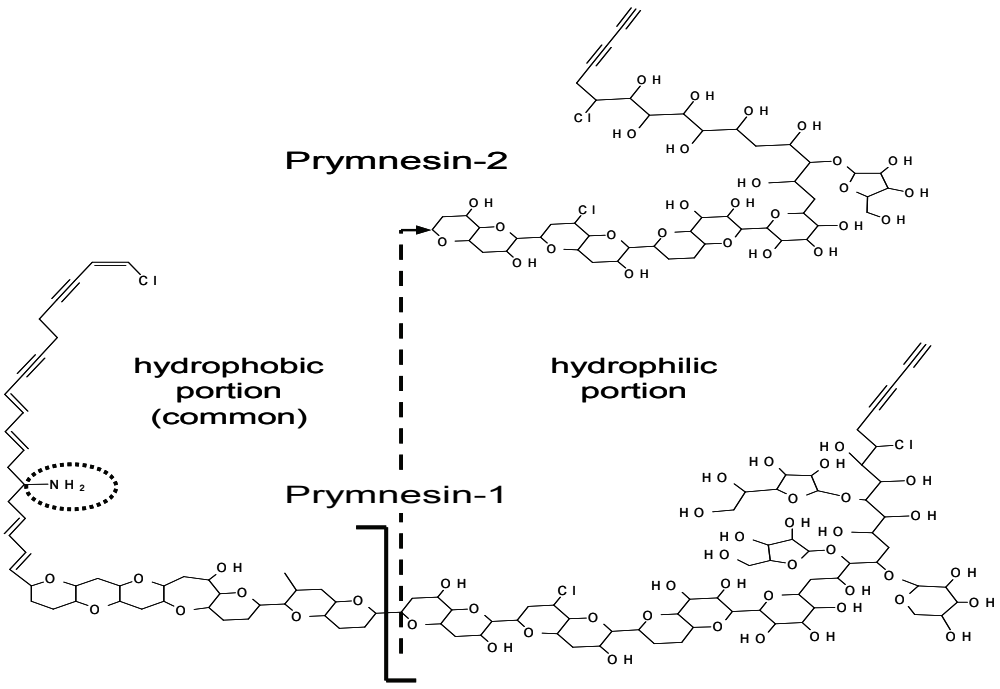


Figure 14. The structures of prymnesin-1 and prymnesin-2 with the hydrophobic and hydrophilic portions of each compound differentiated. The primary amine is highlighted.

Results

pH Dependent Toxicity in Field Studies: Lakes Granbury and Whitney

Ambient toxicity to juvenile *P. promelas* of Lake Whitney samples collected during a Spring 2007 bloom was reduced when pH was adjusted to < 7.5 (Fig. 15). The 48-h LC_{50} value (95% confidence intervals) for experiments completed at pH 8.5 was 1.9 x 10^3 (1.6-2.7 x 10^3) cells mL^{-1}, whereas comparable values at pH 6.5 and 7.5 were 7.8 x 10^3 (7.1-8.8 x 10^3) and 4.1 x 10^3 (2.6-5.3 x 10^3) cells mL^{-1}. There was a similar pH-dependent toxicological relationship during experiments with *D. magna,* as fecundity was significantly reduced at lower densities of *P. parvum* cells when exposure occurred at higher pH (Fig. 16). No reproduction was observed at any pH in 100% Lake Whitney water. There was no significant difference in reproduction between controls at each pH or unmodified RHW.

Susceptibility of *P. promelas* to samples from Lake Granbury during the 2007 bloom also indicated a pH-dependent toxicological relationship. Ambient toxicity to fish was ameliorated in lake samples from two stations and substantially reduced in a third by lowering pH (Table 8). There was insufficient mortality for samples collected from two sites to calculate LC_{50} values at pH 6.5 and 7.5; thus, these values are conservatively reported as > 100%. The sample from Site 2 was the only Lake Granbury sample for which LC_{50} values could be determined for pH 6.5, 7.5, and 8.5. There was approximately a four-fold difference in LC_{50} values between the pH 6.5 and 8.5 treatments, with higher toxicity at higher pH (Table 8).

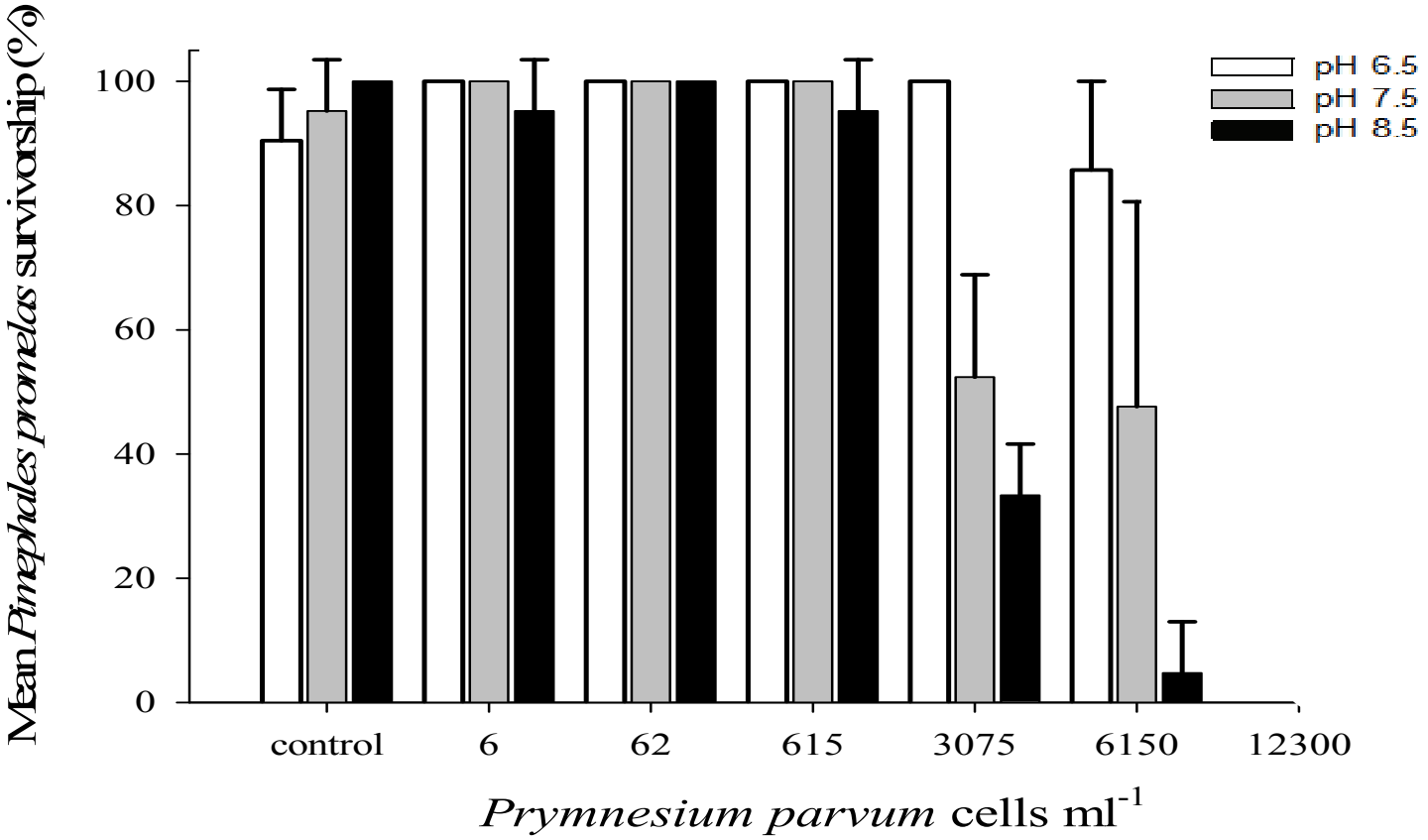


Figure. 15. Average survivorship (±SD, n=4) of *Pimephales promelas* exposed to dilutions of Lake Whitney water collected during a bloom of *Prymnesium parvum* in 2007. Cell density is expressed as the % lake water multiplied by the density of *P. parvum* cells in the undiluted sample. The error bars represent the standard deviation. Missing error bars are due to 100% survivorship in all replicates for a treatment, hence there was no variation to derive a prediction.

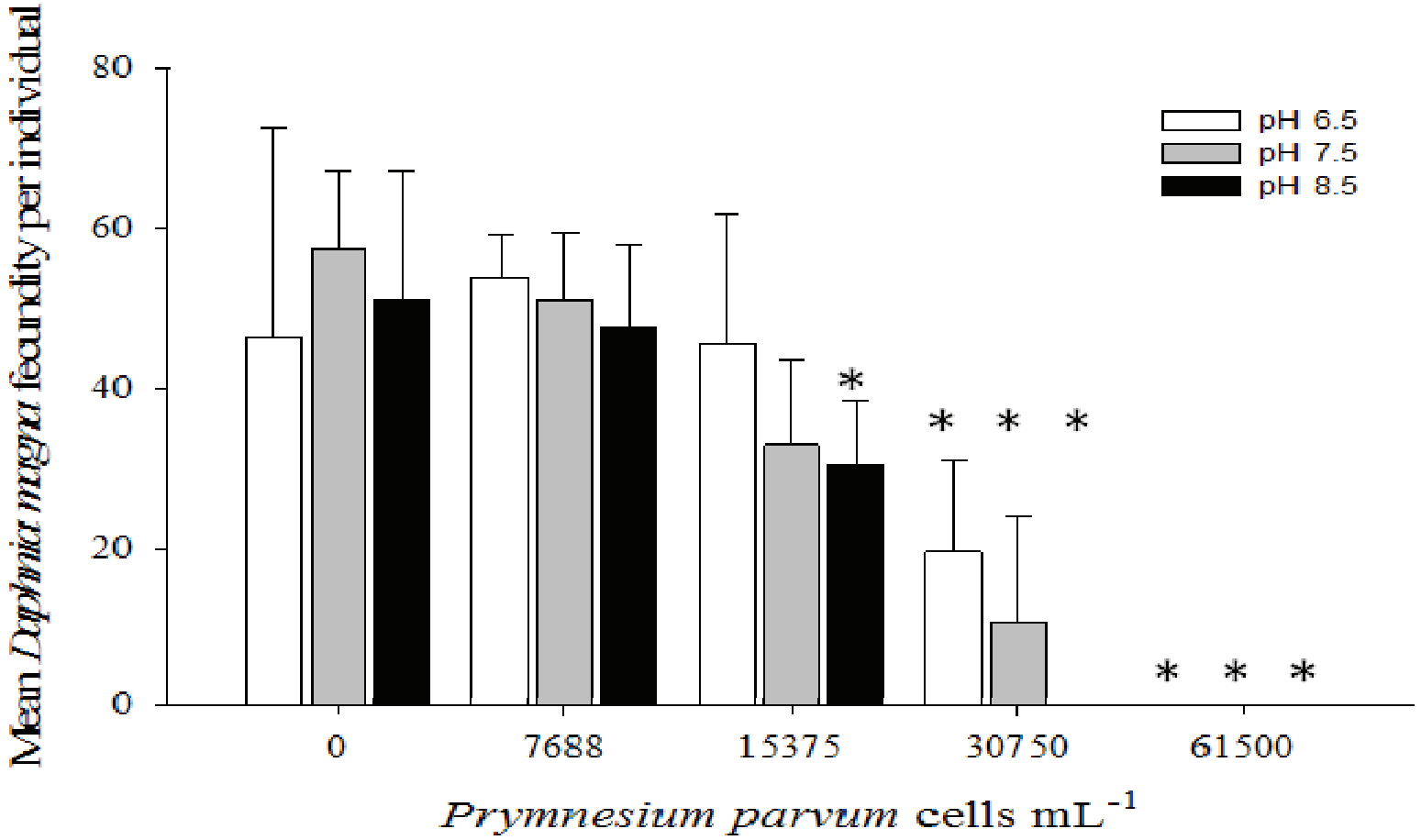


Figure. 16. Mean neonate production for *Daphnia magna* (±SD, n=5) exposed to diluted samples of Lake Whitney water collected during a *Prymnesium parvum* bloom in 2007. Cell density is expressed as the % lake water multiplied by the density of *Prymnesium parvum* cells in the undiluted sample. The error bars represent standard deviations for each value. * represents treatments that are significantly lower than the respective controls ($p<0.05$). Controls did not vary significantly among the three pH treatments ($p>0.05$).

Table 8. The percent survivorship in undiluted samples and 48-h LC_{50} values in terms of percent reservoir water for *Pimephales promelas* exposed to Lake Granbury samples from three stations during a *Prymnesium parvum* bloom in March 2007.

Site	Cells per ml	pH	% Survivorship in undiluted sample	48-h LC_{50} (% lake water)	Upper and lower 95% confidence intervals	48-h LC_{50} (*P. parvum* cell/ml)
1	2.9×10^3	6.5	81	$> 100^A$	nc	nc
		7.5	71	$> 100^A$	nc	nc
		8.5	24	72	61-85	21×10^3
2	3.6×10^3	6.5	38	80	59 - 100	28×10^3
		7.5	5	54	41 -71	19×10^3
		8.5	0	22	15 -31	78×10^2
3	3.6×10^3	6.5	90	$> 100^A$	nc	nc
		7.5	67	$> 100^A$	nc	nc
		8.5	0	43	36 -53	16×10^3

Similarly, toxicity to *D. magna* was reduced in low pH in a 96-h experiment exposing individuals to a composite sample from all three sites (Table 2). LC_{50} values could not be calculated at pH 6.5 due to insufficient mortality, but point estimates for pH 7.5 and 8.5 differed by nearly two-fold, with higher toxicity to *D. magna* at higher pH (Table 9).

Table 9. The 48- and 96-hr LC_{50} values in terms of percent reservoir water for *Daphnia magna* exposed to a composite sample obtained from Lake Granbury during a *Prymnesium parvum* bloom in 2007.

Time	pH	LC_{50} (% site water)	Upper and lower 95% confidence intervals	LC_{50} (*P. parvum* cell/ml)
48	6.5	$>100^A$	nc	34×10^3
	7.5	65.5	42 -100	22×10^3
	8.5	46.7	31- 70	16×10^3
96	6.5	$>100^A$	nc	34×10^3
	7.5	57.4	47-70	19×10^3
	8.5	30.7	26 -37	10×10^3

^{A} = There was insufficient mortality to generate a point estimate.
nc = Not calculable.

pH Dependent Toxicity in Laboratory Cultures

Cultures were terminated on day 28 after reaching late stationary phase, and experiments were immediately performed to assess the toxicity of each replicate culture. Cells were enumerated at this time showing densities in high nutrient (f/2) cultures of 2.0 x 10^5, 1.5 x 10^5, and 2.1 x 10^5 cells mL^{-1}, and densities in low nutrient (f/8) cultures of 1.5 x 10^5, 1.3 x 10^5, and 1.5 x 10^5 cells mL^{-1}. Survival in the ASW and RHW controls was > 90% for all tests at all pH levels. The LC_{50} values for experiments with *P. promelas* were consistently lower for the low nutrient (f/8) treatment compared to those for high nutrient (f/2) (Figure 17). Estimated LC_{50}s were more variable between replicates for the f/2 treatment and increased exposure time resulted in greater toxicity, whereas temporal effects were less evident for the f/8 treatment (Figure 17).

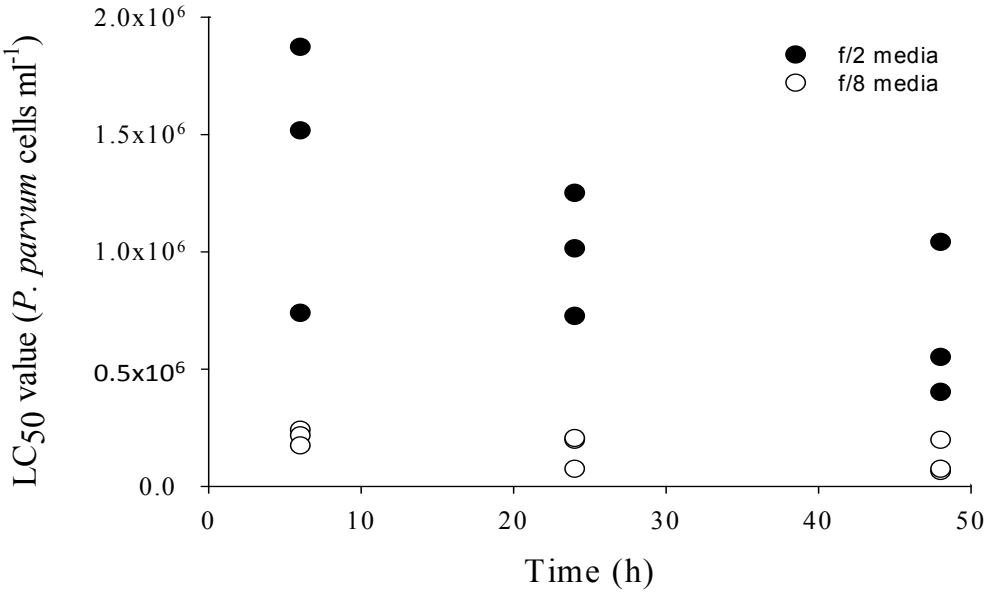


Figure. 17. LC_{50} values for *Pimephales promelas* exposed to cultures of *Prymnesium parvum* grown in the laboratory using two different nutrient conditions (high nutrients – f/2 medium; low nutrients – f/8 medium). Each data point represents the LC_{50} value for an individual culture.

Samples of f/2 and f/8 whole cultures and cell-free filtrate were consistently more toxic to *P. promelas* when exposure occurred at pH 8.5 compared to pH 7.5 or 6.5 (Figure 18). For the f/2 treatment, 50% of exposed individuals died at pH 6.5 in undiluted whole culture; however, only 15% died in the cell free filtrate. Cell free filtrates were also less potent than the whole culture at pH 7.5 and 8.5 for the f/2 treatment; however, differences in toxicity between whole cultures and cell-free filtrates were not as apparent for the f/8 treatment (Figure. 18). The LC_{50} values were markedly lower for filtered and unfiltered cultures grown in f/8 media compared to those in f/2 media; however, endpoints were consistently lower at higher pH for all experiments (Table 10).

Prymnesin-1 and -2 Physicochemical Properties

The structures of prymnesin-1 and -2 (Figure 14) lead to an estimated pKa value of 8.9 for both prymnesin-1 and -2 (Table 11). LogD between pH 5.5 and 8.5 ranged between 2.8 and 5.2 for prymnesin-1, and 2.5 and 4.9 for prymnesin-2, respectively. At pH 6.5 approximately 16% of the prymnesins are predicted to be ionized, whereas at pH 8.5 only 0.002% are predicted to be ionized (Table 11).

Discussion

Our studies with laboratory cultures and samples from reservoirs experiencing *P. parvum* blooms consistently indicate that toxins released by *P. parvum* are more potent when exposure occurred at a higher pH of 8.5 than at lower pH. The predicted physiochemical properties of prymnesins indicate that these toxins are weak bases (pKa =

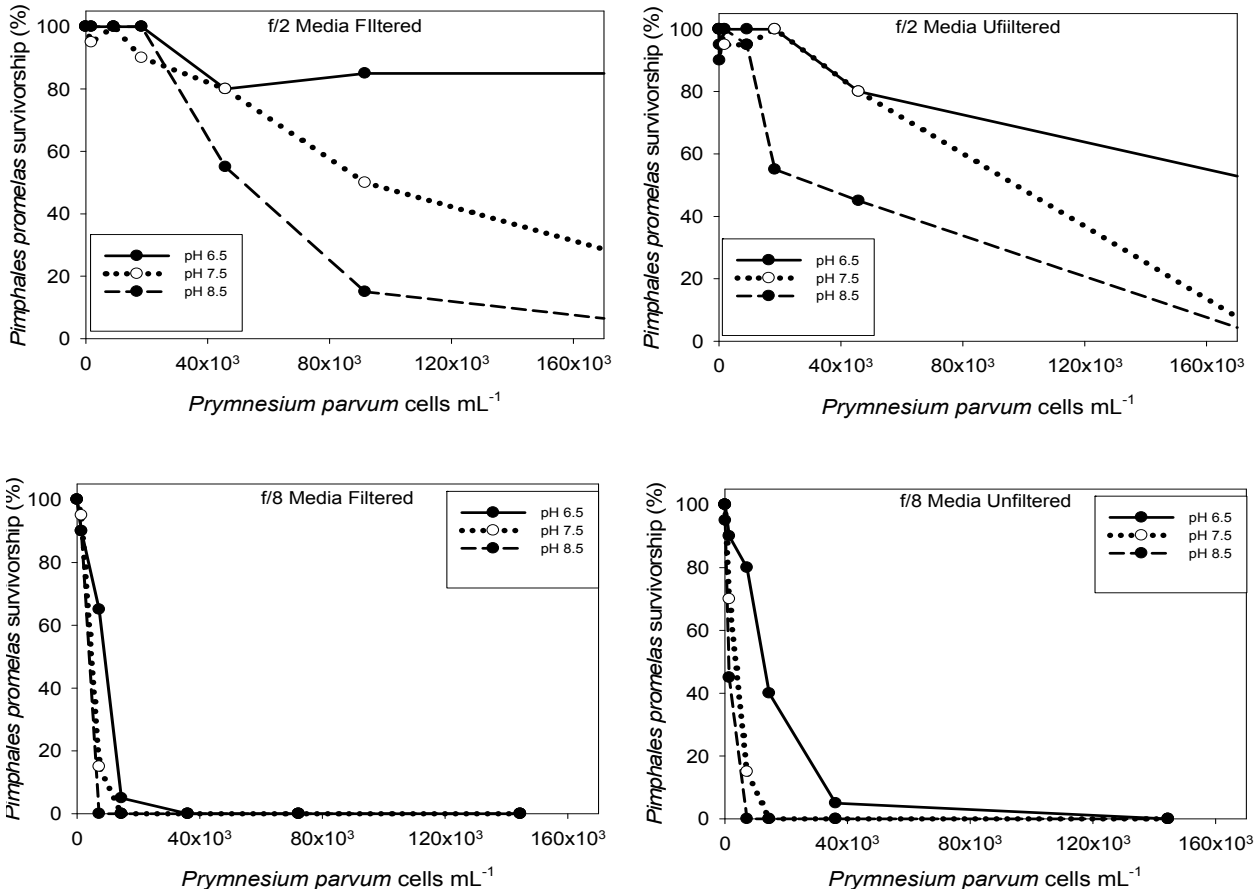


Figure 18. The percent survivorship of *Pimephales promelas* exposed to samples of *Prymnesium parvum* grown under different nutrient conditions with cells (whole culture) and cells removed (filtrate).

Table 10. The LC_{50} value and respective 95% confidence intervals for experiments completed with *Pimephales promelas* and cultures of *Prymnesium parvum* grown in f/2 and f/8 media that were either unfiltered or filtered to remove cells.

Media	Treatment	pH	LC_{50} value (% media)	Upper and lower 95% confidence intervals
f/2	Unfiltered	6.5	>100[A]	nc
		7.5	35	26-46
		8.5	18	12-25
f/2	Filtered	6.5	>100[A]	nc
		7.5	51	36-73
		8.5	30	23-40
f/8	Unfiltered	6.5	7	5-10
		7.5	1.7	1.4-2.4
		8.5	0.7	0.4-1.1
f/8	Filtered	6.5	4.3	3-6
		7.5	2.6	1.8-3.6
		8.5	2	1.1-2.7

[A] = There was insufficient mortality to generate a point estimate.
nc = Not calculable.

Table 11. The predicted physiochemical properties of prymnesin-1 and -2 based on computer modeling and hand computation.

Property	Prymnesin-1	Prymnesin-2
Log P	6.0 ± 1.5	5.6 ± 1.5
Log P (hydrophobic portion)	7.5 ± 0.8	7.5 ± 0.8
Log P (hydrophilic portion)	-1.7 ± 1.5	-2.0 ± 1.5
Log D (pH 6.5)	3.4 ± 1.5	3.1 ± 1.5
Log D (pH 7.5)	4.3 ± 1.5	4.0 ± 1.5
Log D (pH 8.5)	5.2 ± 1.5	4.9 ± 1.5
% ionized (NH_3^+) (pH 6.5)	16	16
% ionized (NH_3^+) (pH 7.5)	0.02	0.02
% ionized (NH_3^+) (pH 8.5)	0.002	0.002
pKa (1º amine)	8.9 ± 0.1	8.9 ± 0.1
pKa (hydroxyl groups)	13-15	13-15

8.9) and, thus, a greater proportion of the prymnesins were likely unionized in higher pH treatment levels (e.g., 8.5). We propose that a higher proportion of prymnesins in unionized forms at pH 8.5 explains the greater toxicity observed in field and laboratory studies. This novel explanation for pH-dependent ambient toxicity associated with *P. parvum* suggests that variability in pH among and within aquatic systems may be an important factor governing the occurrence of fish kills.

Unionized forms of contaminants often have greater propensity to cross cellular membranes due to their lower polarity and thus are more likely to partition into organisms (Simon and Beevers 1951, Sarrikoski et al. 1986, US EPA 1986, Fisher et al. 1999, US EPA 1999, Nakamura et al. 2008, Valenti et al 2010). The importance of ionization state for ambient toxicity and environmental management is evidenced by the integration of site-specific ambient water quality criteria for contaminants such as pentachlorophenol and ammonia (US EPA 1986,1999). Ammonia, like the prymnesins,

has a pKa value of ~9. In addition, ammonia is a weak base and the ionization state of the compound changes appreciably across environmentally relevant surface water pH gradients (US EPA 1999). Consequently, acceptable ammonia loads in stream are 13-fold lower if the receiving system has a pH of 9 compared to a pH 6. Weak bases have a greater propensity to cross cellular membranes if the pH at which the exposure occurs approaches and surpasses the compound's pKa value (US EPA 1999, US EPA 1986, Simon and Beevers 1951, Nakamura et al. 2008, Fisher et al. 1999). Greater interaction with target sites (e.g., gill membranes) would increase the likelihood of adverse effects in exposed individuals; hence, prymnesin-1 and -2 would poses greater risk to aquatic life when these toxins exist predominantly as the unionized form.

In laboratory tests examining the effectiveness of ammonium and barley straw extract to control *P. parvum*, Grover et al. (2007) only observed toxicity in samples with pH > 8. These observations were consistent with Lindholm et al. (1999) who observed fish kills attributed to *P. parvum* in a brackish-water lake when pH ranged between 8.9 and 9.4. Additional studies with *P. parvum* conducted under higher salinity conditions have reported similar pH influences during *in vivo* experiments. Shilo and Ashner (1953) noted that fish were 5-times more sensitive at pH 9 compared to pH 6, and demonstrated that the effects of pH manipulation were reversible during adjustments to and from pH 7 and 6. Ulitzur and Shilo (1964) investigated the toxicity of *P. parvum* toxins, along with various chemicals identified as cofactors, over a range of pH 7 to 9 and consistently noted markedly greater toxicity at higher pH. McLaughlin (1958) observed that high pH shortened the exposure time associated with onset of mortality. The pH-dependent activity of *P. parvum* toxins could also reduce internal damage to cells that are producing

or storing toxins. Extracts of *P. parvum* induced "self-toxicity," reducing growth rates and causing lysis (Olli and Trunov 2007). It is plausible that these ionizable toxins are stored inside cells of *P. parvum* at lower physiological pH, and are thus more ionized than when released outside the cell, where pH values may be higher.

Some previous results from *in vitro* hemolytic experiments with *P. parvum* contradict the results of the *in vivo* experiments reported here and elsewhere. Blood cells rupture more often when exposures are completed at pH < 6 (Igarashi et al. 1996, 1998 Kim and Padilla 1977). Prymnesin-1 and -2 have multiple ionizable groups so that changes in the protonation state could alter the configuration of the toxins. In turn, the interaction of prymnesins with specific binding sites in blood cells and fish gill membranes could depend on the structural configuration, which may be influenced by pH, and thus may be different among such *in vivo* and *in vitro* experiments. Alternatively, prymnesins might not be the only, or even the most important toxins produced by *P. parvum*, and hemolytic activity *in vitro* might have different determinants than lethal activity *in vivo* (Schug et al. 2010).

Cell density alone has been long recognized as a poor predictor of toxicity for samples containing *P. parvum* (Reich and Aschner 1947, Baker et al. 2007, Grover et al. 2007), and this generalization remains apparent during monitoring in Texas reservoirs. Ionization state of the toxins may partially explain some of this variability and reduce uncertainty related to ecological risk assessments and risk management of *P. parvum* blooms. The observed pH-dependent toxicological relationships and the physiochemical properties predicted by computer modeling suggest that the toxins prymnesin-1 and -2 act as weak bases in aqueous solutions. Because their predicted pKa values are within the

range of variation of pH in many surface waters, modest variations in pH could have a large influence on toxicity.

The production of ionizable toxins offers potential advantages to *P. parvum* and may be related to biochemical adaptations associated with its marine origins. The results of our studies and others suggest that the toxins released by *P. parvum* are more potent to gill-breathing organisms when exposure occurs at pH levels representative of those measured in marine systems (e.g. pH > 8). Moreover, blooms of *P. parvum* and other HABs can alter the environment and cause pH to increase through depletion of carbon dioxide during daytime photosynthesis (Pearl 1988). In fish hatchery ponds impacted by *P. parvum*, pH measurements vary by more than one unit between the daylight and evening hours (Shilo and Shilo 1953). Thus, *P. parvum* not only produces toxins during bloom formation, but could also make conditions that increase the potency of their toxins. For example, our research team recently observed high pH levels in Lake Granbury during a *P. parvum* bloom that resulted in ambient toxicity to fish, compared to lower pH levels before and after this bloom (Roelke et al. 2010).

Considering site-specific pH may be especially important for ecological risk assessments of *P. parvum* because of the inherent linkage between physiochemical properties of waters and the organisms that inhabit them. There is far greater spatiotemporal variability in the pH of inland waters compared to marine systems. Some of this variability arises from natural variations in geomorphology, geochemistry, and climate. Anthropogenic activities also influence the pH of inland waters. Inland waters where *P. parvum* blooms have occurred are often affected by altered hydrology, land use changes in the catchment, and increased nutrient loading. In the southwestern and south

central U.S., *P. parvum* blooms and fish kills are often limited to waters where pH is typically high due to an arid climate, limestone bedrock, and sparse vegetation. Consequently, prospective ecological risk assessment approaches may be possible for predicting the occurrence of harmful blooms of *P. parvum* by relating watershed land-use and geography to water quality.

CHAPTER FIVE

Interannual Hydrological and Nutrient Influences on Diel pH in Wadeable Streams: Implications for Ecological Risk Assessment of Ionizable Contaminants.

Introduction

Climate change may reduce future water availability for semi-arid regions of the southwest United States that already experience substantial variability in natural stream flow and prolonged periods of drought (Sun et al 2008, Hurd et al 1999). Flow may be markedly decreased during periods of low precipitation, which may lead to the pooling or elimination of some lotic systems (Carpenter et al 1992, Smakhtin 2001). Drought events may also limit the assimilation capacity of systems receiving point-source discharges or cause deviations in site-specific water chemistry due to altered transient pathways of water, which could change biological and geochemical processes (Carpenter et al 1992, Smakhtin 2001, Brooks et al 2006, Boxall et al. 2010). The most extreme scenario may include suppressed hydrologic regimes in which effluent-dominated streams are losing systems with instream flows strongly influenced or completely comprised by return flows from effluent discharges (Brooks et al 2006). The culmination of effects that reduced flow will have on the aquatic risks of contaminants is difficult to predict and presents a burgeoning challenge for environmental assessment and management efforts of aquatic systems in semi-arid regions.

Population growth, urbanization, and the intensification of agricultural operations in some semi-arid regions have led to heightened release of nutrients into surface waters (Boxall et al 2009, Heathwaite 2010). Increased nutrient availability may cause shifts in

community composition and standing biomass (Marti et al 2004, Schindler et al 2006, Burcher and Benfield 2006), thereby altering ecosystem metabolism (e.g., production and respiration dynamics) (Connell and Miller 1984, Grimm et al 2000, Walsh et al 2005). Resulting changes in ecosystem interactions may influence surface water quality. Other contaminants, such as pharmaceuticals and personal care products (PPCPs), agrochemicals, and industrial constituents or byproducts, are often associated with the same sources responsible for eutrophication of surface waters (e.g., waste water treatment plants (WWTP), livestock rearing facilities, agricultural fields; Brooks et al 2008). The combination of these stressors makes it challenging to predict environmental hazards because site-specific conditions can alter the physicochemical properties, bioavailability, and toxicity of some contaminants (Farrington 1991, Walsh et al 2005, Van Wezel 1998). Further, nutrient enrichment can modify toxicological thresholds of anthropogenic contaminants, particularly at low trophic levels (Fulton et al 2009).

More than three-quarters of the essential medicinal drugs described by the World Health Organization and approximately one-third of modern pesticides have ionizable groups (Manallack 2007, Franco 2010). Ionization state is important for ecological risk assessment because it can influence the environmental fate and biological effects of some contaminants (Van Wezel 1998). There are inherent connotations between ionization state and lipophilicity that are exemplified by pH influence partitioning of drugs (Jollow and Brodie 1972, Kwon 2001). These relationships are further evidenced by differing Log D and bioconcentration factors (BCF) for ionizable compounds over ranges of environmentally relevant surface water pH (Schwarzenbach et al 1993, Rand 1995, Boethling and MacKay 2000, Hernandez and Rathinavelu 2006, Valenti et al 2010b).

The potential for weak ionizable bases to adversely affect organisms may be heightened in aquatic systems located in semi-arid regions of the United States that experience elevated surface water pH due to altered hydrology and increased primary production. As surface water pH approaches and surpasses the pKa value of a weak base, the compound will increasingly exist in the unionized form, which is often regarded as more toxic because of its greater propensity to cross cellular membranes (Hernaddez and Tathinovelu 2006). The importance of ionization state for ecological hazard is emphasized by the integration of site-specific pH adjustment factors into United States Environmental Protection Agency's National Ambient Water Quality Criteria (NAWQC) for some contaminants. One example is ammonia, a weak base with a pKa value of 9.2. To account for differences in pH between sites that can affect ionization state and biological effects, criteria adjustment factors are determined by relating site-specific pH to toxicological data derived from laboratory experiments completed over pH gradients (USEPA 1986, 2009). Acceptable concentrations of ammonia in the water column, expressed as total nitrogen (TN), may vary appreciably between sites. For example at 24° C, the criterion maximum concentration (CMC) for surface waters at a site with no mussels present and a pH of 6.5 is 31.4 mg TN /L, whereas comparable values for a site with a pH of 9 is only 0.85 mg/L (US EPA 2009).

The practical constraint of implementing criteria based on pH-dependent toxicity is defining site-specific conditions. In fact, it is critical to appreciate that the surface water pH measurement culminates from fluctuating interactions that vary on different spatial and temporal scales among the atmosphere, hydrology, climate, geomineralogy, and physical morphology of watersheds over longitudinal gradients (Santschi 1988, Allan

1995, Rebsdorf et al 1991, Hill and Neal 1997, Fitzhugh et al 1999). Bedrock mineralogy, soil compression, till depth, elevation gradient, precipitation patterns, and vegetation may cause appreciable differences in surface water geochemistry between sites (Omernik and Griffith 1991, Allan et al 1993, Omernik and Bailey 1997, Fitzhugh et al 1999). On more finite spatial scales distinctions between surface water pH at sites may also be realized due to additional factors, such transfers of allochthonous nutrient subsides (Jefferies 2000), groundwater and tributary inputs (Maberly 1996), proximity to wetlands (Hunt et al 1997, Fitzhugh et al 1999), availability of acid-neutralizing materials (Driscoll et al 1987, Fitzhugh et al 1999), and both point and non-point pollution (Heathwaite 2010, Kim et al 2010). Although there is an understanding of how environmental heterogeneity may contribute to spatial variability in surface water pH between sites, less is known about more complex interactions among such factors that may cause temporal variability at a single site.

Interpreting relationships between the spatial dynamics of such variables and surface water pH are challenged by the effects of temporal factors that occur on various time scales. Seasonal and annual variability in precipitation may influence ionic composition of surface waters by controlling inputs of groundwater, subsurface water, and overland flow (Raxcher et al 1987, Carpenter et al 1992, Findlay 1995, Smakhtin 2001). On a more resolute temporal scale, inorganic carbon in surface waters may become uncoupled with concentrations of carbon dioxide in the atmosphere during the day if the rates of biological transformation exceed rates of physiochemical transfer between environmental compartments (Maberly 1996). Diel oscillations in pH will likely have correlates to seasonality as the succession shifts of phytoplankton and macrophyte

communities, coupled with changing in standing biomass, can influence the potential carbon dioxide demand of aquatic systems (Marberly 1996). This disparity may be especially true for effluent-dominated systems in the southwestern and south central United States that already experience low hydrologic regimes (Brooks et al 2006). The reduction or absence of groundwater augmentation in these systems may eliminate important inputs of carbon dioxide. Furthermore, increased ecosystem biomass may cause high rates of respiration while subsidies of organic material from WWTPs, pasture lands, or other terrestrial sources may cause substantial decomposition to occur in surface waters (Moss 2010). These processes may culminate in temporary depression of pH in surface waters as concentrations of inorganic carbon in water exceed rates of degassing (Maberly 1996). Conversely, assimilation of organic carbon driven by photosynthesis can cause elevated pH during light hours as carbon dioxide is removed from the water column and previous research has demonstrated eutrophication of surface waters may facilitate high rates of primary production that potentiate extreme daily oscillations in pH at a site (Halstead and Tash 1982, Maberly 1996, Guasch et al 1998, Kent et al 2005, Tank et al 2009).

Researchers have previously explored the implication of daily change in pH on metal and nutrient availability in aquatic systems (Crumpton and Isenhart 1988, Garban et al 1999, Jones et al 2004, Morris et al 2005, Nimick et al 2007). In this study, we explore interannual variability of diel pH oscillation patterns across 23 stream sites in the Brazos River, Texas, USA watershed during different hydrologic regimes and examine how pH variability throughout the day at these sites may influence risk characterization for select weak bases. Ammonia was be used as a model weak base to determine the

variability of allowable site-specific instream concentration of TN throughout the day due to temporal pH differences. Measured diel changes in pH were also used to develop aquatic toxicity predictions for the weak base sertraline, which is a pharmaceutical from the class of selective serotonin reuptake inhibitors that has been previously shown to exhibit pH-dependent toxicity (Valenti et al 2009). Similar assessments for other contaminants of emerging concern are often prevented because toxicity data over gradients of environmentally relevant pH seldom exists. We further examined relationships between BCF values and site-specific pH to infer the potential bioaccumulation of other model weak bases.

Material and methods

Study Sites

Data were collected from 23 wadeable streams in July-August 2006 and September -October 2007. Sites were selected in the Brazos River basin, Texas, USA within the Cross Timbers Level III Ecoregion (King et al 2009). These sites were purposely selected to span a gradient of phosphorus enrichment, yet still captured a full range of natural variability in geology, drainage networks, stream size, and other physiographic factors (King et al 2009). The permitted wastewater outfalls, land use for the catchment, and potential nutrients inputs for each stream site are presented in Table 12. Several sites (e.g., Bluf-01, Harr-01, Mbos-01, Sbos-01) had >10% crop land. Some sites were located directly downstream of WWTP discharges (e.g., Leon-02, Nbos-01, Nbos-05, Nolc-01, Nolr-01), whereas others likely had at least a portion of flow

comprised of WWTP effluent (e.g., Cowh-01, Nbos-02,-03,-04, Meri-01, Mbos-01, Sbos-01, Leon-01).

Diel Water Quality Monitoring

Diel changes in dissolved oxygen (DO), pH, and temperature were determined by deploying YSI 600 XLM and YSI 6600 (YSI instruments, Yellow Springs, Ohio, USA) multiparameter datasonds at each site for 48-h. Data were collected from 23 wadeable streams in 2006 and 2007 between September and October. Data collected during 2006 was during a near-record drought and flow was minimal or absent at nearly all sites, with effluent-dominated streams as an exception. For example, annual departures from normal precipitation in the studied watershed areas in 2006 were between -102 and -406 mm, while comparable values in 2007 were between 102 and 508 mm (National Weather Service website). Exceptional high stream flows occurred during the summer of 2007 and data collection occurred during a brief period of stream-flow recession (King et al 2009). Instruments were placed in the central channel in areas of discernable flow, if possible. Values for each parameter were collected at 15-min intervals and data was transferred to a computer at the completion of deployment. All instruments were calibrated with all reagents at room temperature within 24-h prior to data collection (TCEQ 2003). For pH calibration, probes were calibrated with buffer solutions at pH 7 and 10. As an additional quality assurance following data collection, post-calibration were completed and using error limits for pH, DO, and temperature of 0.5 standard units, ± 5% error at saturation, and ±1° C, respectively (TCEQ 2003).

Table 12. The location, physical descriptors, dams, wastewater outfall, and land-use breakdown for the 24 sites sampled during 2006 and 2007.

Site	Outfalls (MGD)	Water (%)	Dev. (%)	Forest (%)	Shrub (%)	Grass (%)	Pasture (%)	Crop (%)	Wetland (%)	Imp. (%)	Nutrient input
STEE-01	0	0.8	0.5	14.7	52.4	26.8	2.6	2.1	0.5	0.1	Minimal
LAMP-02	0	0.2	0.7	14	53.6	29.2	1	1.1	0.3	0.1	Minimal
NEIL-01	0	0.2	0.2	35.6	1.4	57.7	0.7	2.2	1.8	0.2	Minimal
PALU-01	0	0.4	1.1	32.9	11	49.8	3.1	0.8	1	0.2	Minimal
ROCK-01	0	0.1	1.3	24.3	39.9	34	0.1	0.1	0.3	0.2	Minimal
CORY-01	0	0	1.5	26.9	5.3	59.2	1.5	3.5	2	0.2	Minimal
DUFF-01	0	0.2	0.4	22.4	13.1	57.1	1.5	4.1	1.1	0.2	Minimal
LAMP-01	0	0.3	0.6	10.9	56.6	28.8	1.5	0.9	0.3	0.1	Minimal
COWH-01	0.06	0.1	0.7	18.8	43	33.8	1.6	1.5	0.4	0.1	2° WWTP
NBOS-02	3.5	0.6	6.5	10.7	20.1	43.9	8.5	7.8	1.9	1.3	2° WWTP
NBOS-03	3.5	0.5	4.3	13.3	22.6	44.6	7.1	6	1.6	0.8	2° WWTP
NBOS-04	3.75	0.5	3.1	18.4	13.5	55.1	4.1	3.5	1.7	0.6	2° WWTP
MERI-01	0.04	0.4	0.3	32.3	0.4	63.7	0.5	1	1.4	0.2	2° WWTP
BLUF-01	0	0.1	0.6	8.8	0	69.1	1.4	17.2	2.9	0	Crop
HARR-01	0	0.1	12	1.6	0	42.6	3	38.6	2.1	2	Crop.
MBOS-01	0.09	0.1	1.6	10.5	0	63.2	2	19.9	2.7	0.1	2° WWTP / Crop.
SBOS-01	1.1	0.2	6.7	3.7	0	48.5	4.9	33.9	2.2	0.9	2° WWTP / Crop.
LEON-01	3	0.8	3.1	12.2	33.1	33.3	8.4	8.5	0.8	0.3	2° WWTP / Pasture
LEON-02	6.08	0.7	2.7	13.5	30.6	37.6	6.8	7.2	0.9	0.3	1° WWTP/ Pasture
NBOS-01	3.5	0.6	8	11	17.6	36.8	12.1	12	1.9	1.6	1° WWTP / Pasture
NBOS-05	5.28	0.5	2.5	23.8	8.5	56.2	3.4	3.1	2	0.5	1° WWTP
NOLR-01	6.73	1.6	12.3	3.2	0.1	65.7	8.8	5.9	2.3	2.5	1° WWTP/Pasture
NOLC-01	33.77	0.5	32.4	24	7.9	30.2	1.4	1.3	1.6	11.7	1° WWTP

Nutrient Measurements

Determination of total phosphorus was conducted using the molybdate-blue method in persulfate digested samples. Total nitrogen in water samples was determined by analysis of nitrate plus nitrite-nitrogen using the cadmium reduction method in persulfate-digested samples. A Lachat Quickchem 8500 Flow Injection Autoanalyzer was use for quantification. 250 ml unfiltered samples were preserved with concentrated H_2SO_4 (pH < 2) and stored in the refrigerator for a period no longer than 28 days.

pH Influences on Aquatic Toxicity

The mean, minimum, and maximum daily pH values and the measured values at 0800, 1100, 1400 and 1700 hrs were determined for each site. In addition, cumulative frequency distributions of daily instream pH measurements were created so that the likelihood of observing a given pH value at each site could be quantified. The pH data for discrete measures (mean, minimum, maximum, specific time points) and the values for each cumulative frequency distribution were then used to examine site-specific NAWQC for ammonia (Eq. 1; (USEPA 2009).

$$\text{CMC} = 0.826 \times \left(\frac{0.0489}{1+10^{7.204-pH}} + \frac{6.95}{1+10^{pH-7.204}}\right) \times MIN\left(12.09, 6.018 \times 10^{0.036\times(25-T)}\right) \quad (1)$$

Similar approaches were used for sertraline to predict acute fish mortality responses based on measured pH at the sites. The mean, minimum, and maximum daily pH applied to the pH-dependent toxicity relationship described by Valenti et al (2010).

Log LC50 µg sertraline/L= (-0.27 × pH) + 4.34

(2)

Daily Oscillation Risk Ratio

In this study, a daily oscillation risk ratio (DORR; unitless) was developed to assess potential change in predicted ecotoxicological responses based on diel fluctuation in pH. DORR was calculated by integrating both the max and min pH for a given site in a previously defined pH-dependent toxicological relationship. Because ammonia and sertraline are weak bases, the predicted endpoint for the lowest daily pH was divided by the respective endpoint for the highest observed pH during a day. Alternatively for a weak acid, the equation would be modified and the predicted endpoint for the highest daily pH would be divided by the endpoint predicted for the lowest observed pH over a day.

Relationship Between High TP and Elevated pH

A pH value of 8.5 was selected as a threshold to investigate the relationship between TP concentrations in the water column and elevated pH at sites. The cumulative frequency of TP and pH > 8.5 were plotted on concurrently on the same graph for all sites. While evaluating this figure it became apparent that several with high TP never exceeded 8.5 and these outliers were those sites that were directly below WWTP outfalls. Considering the substantial decomposition occurring in WWTP and unlikelihood that complete degassing occurred before the location of our study site, we decided to complete analyses again with these sites excluded.

Estimates of BCF and $D_{lip\text{-}water}$ at Stream Sites

The pKa and Log D values for seven model weak base pharmaceuticals (chloprothixene, prochlorperzine, tamoxifen, terfenadine, triflupromazine, promethazine,

and sertraline) at pH 6, 7, 8, 9, and 10 were obtained using ACD/Labs (SciFinder Scholar, American Chemical Society, Washington, DC USA; Table 13).

Table 13. The dissociation constant (pKa value), octanol: water partitioning coefficient (Log D), bioconcentration factors, and K_{lipw} for several weak base pharmaceuticals.

Compound (pka value)	Physicochemical property	pH 6	7	8	9	10
Chlorprothixene	Log D	2.4	3.2	4.1	4.9	5.2
(9.1±0.3)	BCF	24	100	650	2900	4900
	Kliwater	1100	4130	22000	86000	140000
Prochlorperazine	Log D	2.8	3.7	4.4	4.6	4.6
(7.66±0.1)	BCF	46	280	1100	1700	1800
	Kliwater	1900	10000	36000	52000	55000
Tamoxifen	Log D	4.9	5.3	6.2	7.1	7.7
(8.7±0.3)	BCF	2800	6800	37000	220000	710000
	Kliwater	84000	190000	900000	4600000	14000000
Terfenadine	Log D	3.5	4.1	4.9	5.8	6.4
(9.6±0.4)	BCF	200	550	3200	18000	52000
	Kliwater	7600	19000	94000	470000	1200000
Triflupromazine	Log D	2.2	2.9	3.8	4.6	5.1
(9.4±0.3)	BCF	16	54	330	1700	4100
	Kliwater	740	2200	12000	54000	120000
Promethazine	Log D	2.2	3.0	4.0	4.6	4.9
(8.98±0.5)	BCF	15	75	460	1800	2600
	Kliwater	700	3000	16000	55000	80000
Sertraline	Log D	1.9	2.4	3.3	4.2	4.7
(9.47+0.4)	BCF	8	23	140	760	2000
	Kliwater	370	1000	5200	25000	61000

These physicochemical properties were then used to predict BCF values based on equation 3, which was developed by Veith and Kosian (1983).

$$BCF = (0.79 \times \text{Log D}) + 0.4 \quad (3)$$

Escher and Schwarzenbach (2000) suggested that liposome-water systems ($D_{lip\text{-}water}$) are more accurate surrogates for biological systems than octanol-water partitioning predictions (Log D), particularly for membrane-toxic agents and hydrophobic ionogenic

organic compounds. Therefore, $D_{lip\text{-}water}$ values for the pharmaceuticals were estimated using equation 4.

$$\text{Log } D_{lip\text{-}water} = 0.78 \text{ x log } D + 1.12 \quad (4).$$

The calculated BCF and $D_{lip\text{-}water}$ values were then fit by regression in Sigma plot (Version 11.0, SPSS Inc., Chicago, IL, USA) using a sigmoidal, sigmoid, 3 parameter best fit model so that respective values could be estimated for environmentally realistic pH values ranging from 6 to 10. A weighted BCF and $D_{lip\text{-}water}$ for each compound was calculated by using the pH frequency distribution for each site. For the purposes of this study, the pH frequency distribution was also used to predict the percent of the day that the BCF values for each of the model bases was > 1000 at each site. The BCF value of 1000 was selected because it has been previously used to define potential bioaccumulative compounds (US EPA 2000, Fu et al 2009).

Statistical Analysis

The statistical significance of the magnitude of change in DO and pH at sites between low (2006) and high flow (2007) conditions was investigated using a t-test (α=0.05; Jumpin Version 5.0, SAS institute, Cary, NC, USA). In addition, DORR values, mean and lowest predicted LC50 values for sertraline, and weighted mean BCF and $D_{lip\text{-}water}$ values at the sites were also compared between the two years using the same analysis.

In our analysis we examined the influence of surface water TP (predictor) to infer the percent of time that pH at a site was >8.5 (response variable). The percent of time that pH was >8.5 was determined from the pH frequency distribution for each site under low flow (2006) and high flow (2007) conditions. The changepoint analysis uses

bootstrapping (resampling) to estimate a percentile confidence interval around the observed threshold.

Additional ANOVA analyses were completed by comparing the mean CMC ammonia values at 0800, 1100, 1400, and 1700 h for all sites separately for each year ($\alpha = 0.05$). Then Tukey multiple comparisons tests were used to assess significant differences among the mean CMC ammonia values for each discrete time point.

Results

Continuous Water Quality Monitoring and Nutrients

Results from continuous monitoring efforts of water quality at stream sites indicated appreciable differences in daily averages between the two study years (Table 14). In 2006, daily mean DO, pH, and temperature at the sites ranged from 3.1 to 10.2 mg/L, 7.3 to 8.9, and 19.2 to 28.4 ° C, respectively. Daily averages varied considerably less among the sites in 2006 and 2007: DO, pH, and temperature ranged from 6.3 to 8.8 mg/L, 7.5 to 8.1, and 23.6 to 26.7 ° C, respectively (Table 14). Daily oscillation patterns of these variables were apparent in both years; examples for four sites are shown in Figure 19. In general, DO, pH, and temperature all shifted upward during daylight hours, followed by decreases in these variables during the night (Figure 19).

The TN and TP surface water concentrations also varied substantially between sites in both years (Figure 20). At several of the sites located directly below WWTP discharges (Leon-02, Nbos-01, Nolc-01, Nolr-01), TP concentrations were notably greater than other sites, especially during low flow conditions of 2006. The TN concentrations were also elevated during both years at several of these sites (Figure 20).

In particular, relatively high TN concentrations were observed at several sites (Bluff-01, Harr-01, Sbos-01) located in watersheds with between 17.2 and 38.6% row crop agriculture. These sites had appreciably TN concentrations only under greater hydrological conditions in 2007, whereas TN concentrations in 2006 were 3-12 times lower. Inconsistent with the general trend for these agricultural sites, the highest recorded TN concentration occurred at Mbos-01 (19.9 % row crop), during low flow conditions; TN concentrations were appreciably lower during 2007. This disparity may have been caused by storage of TN in sediments at the site during low flow conditions, followed by flushing events that caused transportation of polluted sediment (Heathwaite 2010)

Table 14. Potential nutrient inputs, average dissolved oxygen (mg/L) ± standard deviation (SD), average pH± SD, and temperature ± SD at stream sites in the Brazos River Watershed, TX during low flow (2006) and high flow (2007) conditions.

ITE_ID	Dissolved oxygen (mg/L) ± standard deviation		pH (units) ±standard deviation		Temperature (C) ± standard deviation	
	2006	2007	2006	2007	2006	2007
TEE-01	7.5 + 1.9	6.7 + 0.7	7.7 + 0.1	7.5 + 0.1	25.2 + 1.7	26.2 + 0.7
AMP-02	8.4 + 1.9	7.4 + 1.1	8.7 + 0.1	8.0 + 0.1	21.9 + 1.9	24.8 + 0.7
IEIL-01	8.0 + 1.3	7.7 + 0.7	8.4 + 0.1	7.9 + 0.1	23.7 + 2.2	26.2 + 0.7
ALU-01	8.1 + 2.1	7.2 + 1.1	8.0 + 0.3	7.8 + 0.1	21.9 + 0.9	24.3 + 0.5
OCK-01	8.8 + 0.9	8.4 + 1.4	8.1 + 0.1	7.7 _ 0.1	24.3 + 1.6	25.6 + 0.9
ORY-01	5.3 + 4.2	7.7 + 1.1	7.6 + 0.3	7.9 + 0.1	23.5 + 2.1	24.6 + 0.9
UFF-01	8.9 + 3.1	7.3 + 1.9	8.1 + 0.2	7.6 + 0.1	19.7 + 1.6	26.0 + 0.7
AMP-01	3.1 + 1.2	7.5 + 1.9	7.3 + 0.1	7.7 + 0.1	25.0 + 0.9	25.0 + 1.1
OWH-01	4.7 + 1.7	7.8 + 0.9	7.7 + 0.1	7.7 + 0.1	19.2 + 0.7	23.6 + 0.6
BOS-02	7.4 + 2.2	7.7 + 2.9	8.0 + 0.1	7.9 + 0.2	24.3 + 0.7	25.4 + 0.6
BOS-03	5.4 + 3.0	8.8 + 2.4	8.1 + 0.2	8.1 + 0.1	21.6 + 1.8	24.4 + 1.3
BOS-04	7.9 + 5.3	8.4 + 1.7	8.9 + 0.6	8.0 + 0.1	27.8 + 2.9	24.4 + 1.2
IERI-01	10.2 + 3.7	4.9 + 1.3	7.4 + 0.2	7.7 + 0.1	27.3 + 2.0	23.8 + 0.9
EON-01	7.8 + 2.4	6.4 + 1.1	7.9 + 0.1	8.1 + 0.1	23.3 + 2.4	26.6 + 0.9
BOS-01	9.2 + 2.7	7.2 + 0.3	8.2 + 0.3	8.0 + 0.0	21.7 + 1.0	25.9 + 0.4
LUF-01	5.5 + 1.2	7.8 + 0.8	7.8 + 0.1	8.0 + 0.1	21.5 + 1.6	26.0 + 1.5
ARR-01	9.0 + 2.0	7.3 + 1.4	8.6 + 0.2	7.9 + 0.2	23.2 + 1.7	26.3 + 1.1
BOS-01	10.0 + 2.7	7.1 + 1.1	8.2 + 0.2	7.9 + 0.0	23.7 + 3.7	27.1 + 1.3
EON-02	7.6 + 1.9	7.2 + 0.2	7.3 + 0.1	7.6 + 0.1	23.7 + 1.4	26.1 + 0.2
BOS-01	8.6 + 2.8	7.0 + 1.1	8.2 + 0.3	7.6 + 0.1	28.4 + 2.3	26.1 + 1.0
BOS-05	8.8 + 2.1	7.5 + 0.8	7.8 + 0.2	7.9 + 0.1	25.9 + 1.5	26.7 + 1.0
OLC-01	8.5 + 2.3	7.9 + 0.5	8.2 + 0.2	7.8 + 0.0	22.1 + 4.5	25.4 + 1.2
OLR-01	6.9 + 0.5	6.3 + 0.6	8.1 + 0.1	7.7 + 0.1	21.9 + 1.2	26.1 + 0.8

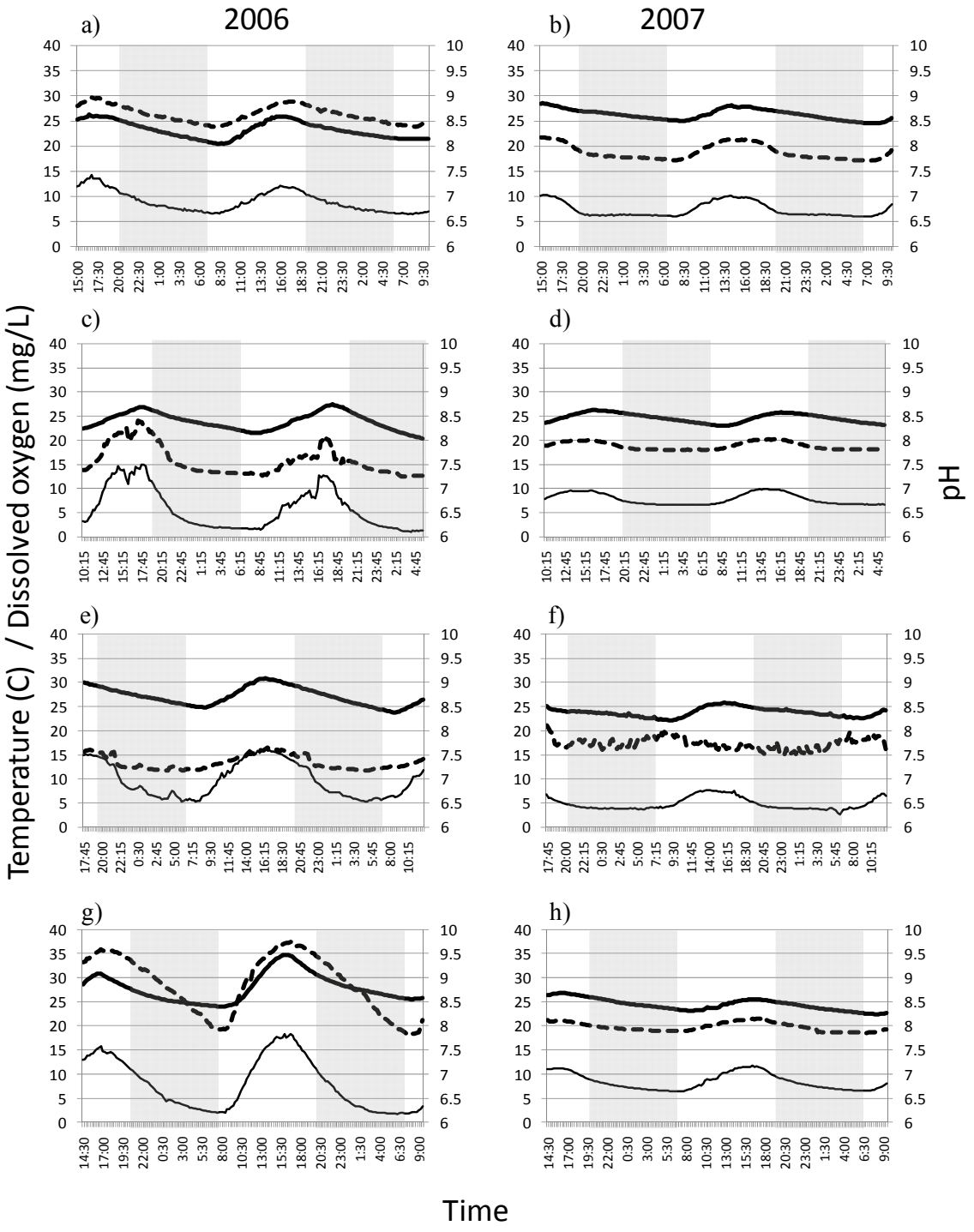


Figure 19. Daily patterns in measured temperature (Thick solid line) and dissolved oxygen (Thin solid line) on the left axis and pH (Dashed lines) on the right axis sampled under low (2006) and high (2007) conditions. All three variables increased during the day (white) and decreased during the night (gray). Duff-01 (a + b) has minimal nutrient input, Bluff-01 (c + d) is in a watershed with 17% row crop agriculture, Mbos-01 (e + f) is in a watershed with 19% row crop agriculture and receives WWTP effluent, and Nbos-03 (g + h) is in a watershed with 7% pasture land and receives WWTP effluent.

There was also a clear relationship between high TP levels and elevated pH at sites in 2006, particularly for analyses that excluded those sites that were directly below WWTP (Figure 21 a,b). The pH was predicted to be > 8.5 10% of the time for sites with TP < 20 µg/L based on cumulative frequency distributions, whereas pH was predicted to be >8.5 between 40 and 100% of the time for sites (Figure 21 a, b). The relationship between TP and pH > 8.5 is particularly apparent for the figure in which sites directly below WWTP were excluded (Figure 21 b).

pH Influences on Aquatic Toxicity

There was substantial variability in allowable ammonia concentrations based on US EPA NAWQC CMC values (2009) due to appreciable shifts in pH at the sites, particularly during the low flow conditions of 2006 (Figure 22). Some sites had higher recommended allowable TN concentrations in 2006, whereas others had higher values for 2007. Interestingly, in 2006 CMC values were significantly higher ($p<0.05$) near sunrise (0800), than during afternoon observations at 1400 and 1700 hrs; respective mean CMCs (±standard deviations) were 14.0 (±1.5) versus 8.4 (±1.6) and 7.3 (±1.3). The average CMC at mid-morning (1100 hr) was not significantly different than any other time (11.3±1.6mg TN/L). Similar comparisons for 2007 indicated that there was no significant difference in CMC values over the course of the day as means for each sequential time point were 7.8 (±0.2), 7.9 (±0.1), 7.9 (±0.2), and 7.9 (±0.2) mg TN/L.

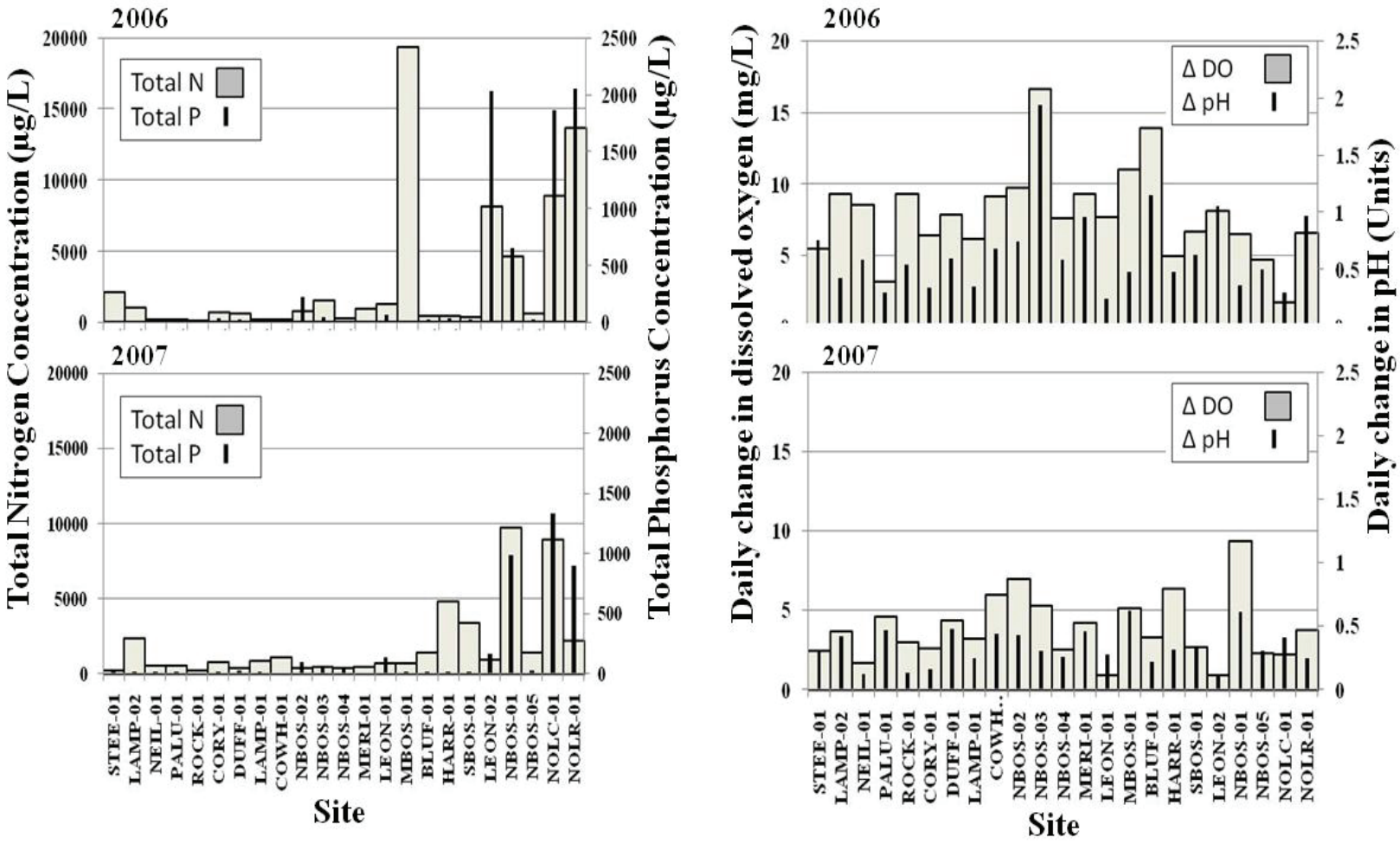


Figure 20. Water column concentrations of total nitrogen (TN), total phosphorus (TP), daily change in dissolved oxygen, and pH at 23 stream sites in the Brazos watershed under low flow (2006) and high flow (2007) conditions

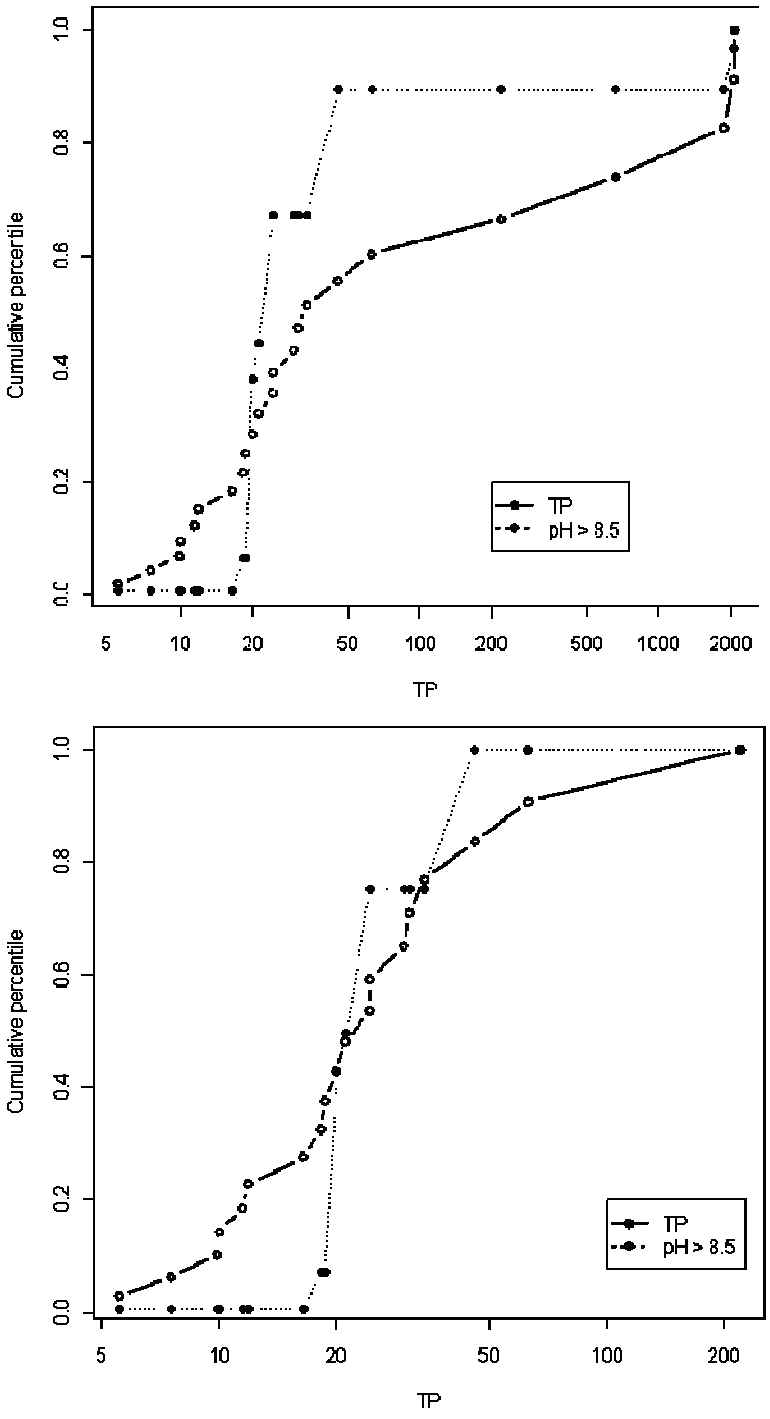


Figure 21. The results of nonparametric changepoint analysis using surface water TP as the predictor variable and % of time that pH >8.5 as the response variable. The open circles represent four sites receiving primary discharge from wastewater treatment plants.

In 2006, the daily mean sertraline LC50 values based on pH-dependent toxicological relationships were predicted to be <150 µg/L at 12 sites, while 19 sites were below this threshold for portions of the day (Figure 23). In 2007, only two sites had daily averages <150 µg/L and eight sites had predicted LC50 values less than this concentration for at least part of the day (Figure 23). In general, there was substantially more variability in acute toxicity predictions during periods of low versus high instream flows (Figure 23). There was not a consistent trend in calculated responses for sites between years as some had lower predicted values in 2006, while others had lower values in 2007. A comparison of daily mean for all sites between years indicated that acute toxicity was lower in 2006, though not significantly different ($p=0.08$) from 2007, as respective daily means (± standard deviation) were 156 (± 39) and 169 (±17) µg/L in 2006 and 2007. Hydrological variability between 2006 and 2007 produced a significant difference ($p<0.05$) of acute toxicity prediction for sertraline based on the lowest predicted acute toxicity value as respective means (± standard deviation) were 125 (±37) and 150 (± 15) µg/L.

Daily Oscillation Risk Ratios

The ammonia DORR values exhibited much greater variability, ranging from 1.7 to 20.7, during drought conditions of 2006, but much lower variability (range of 1.2 to 3.1) was observed with the higher instream flows of 2007 (Table 15). There was a significant difference ($p<0.05$) in ammonia DORR values between the low flow and high flow conditions as respective means (± standard deviation) were 4.0 (± 4.0) and 1.9 (±0.5). Sites with high ammonia DORR values in 2006 included those that received effluent directly (Nolr-01, Leon-02) or indirectly (Nbos-03, Meri-01) from WWTP

discharges, in addition to one site with substantial row cropping (Bluf-01). The sertraline DORR values were smaller than those calculated for ammonia and ranged from 1.2 to 3.3 in 2006, and 1.1 to 1.5 in 2007 (Table 15). The mean (± standard deviation) sertraline DORR values for all sites was significantly higher under low flow conditions (1.5± 0.4) compared to high flow conditions (1.2 ± 0.1).

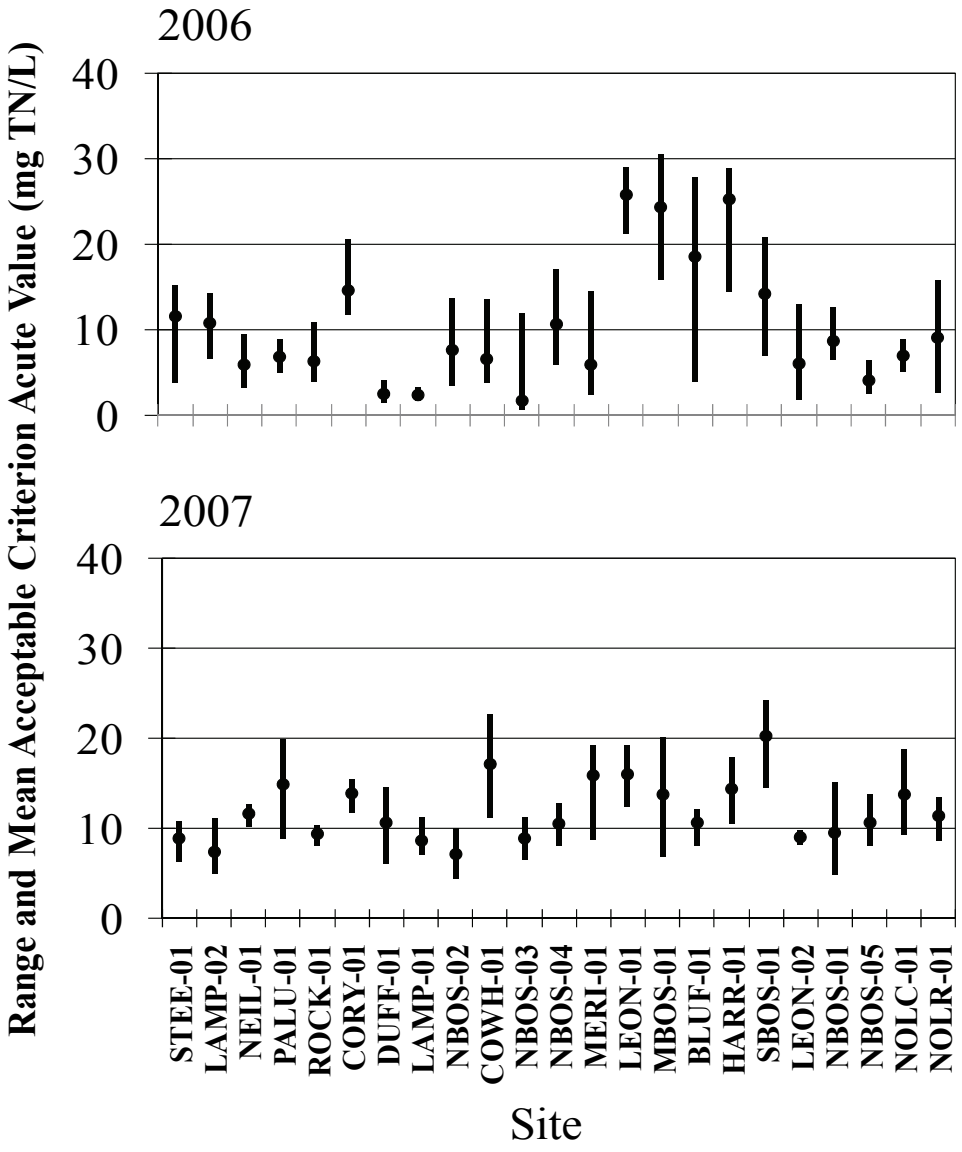


Figure 22. The allowable water column concentrations of TN at sites based on pH-dependent relationships reported in ambient water quality criteria for ammonia (US EPA 2009). The dot represents the daily average and the lines represent the range in predicted values based on daily pH fluctuations at sites.

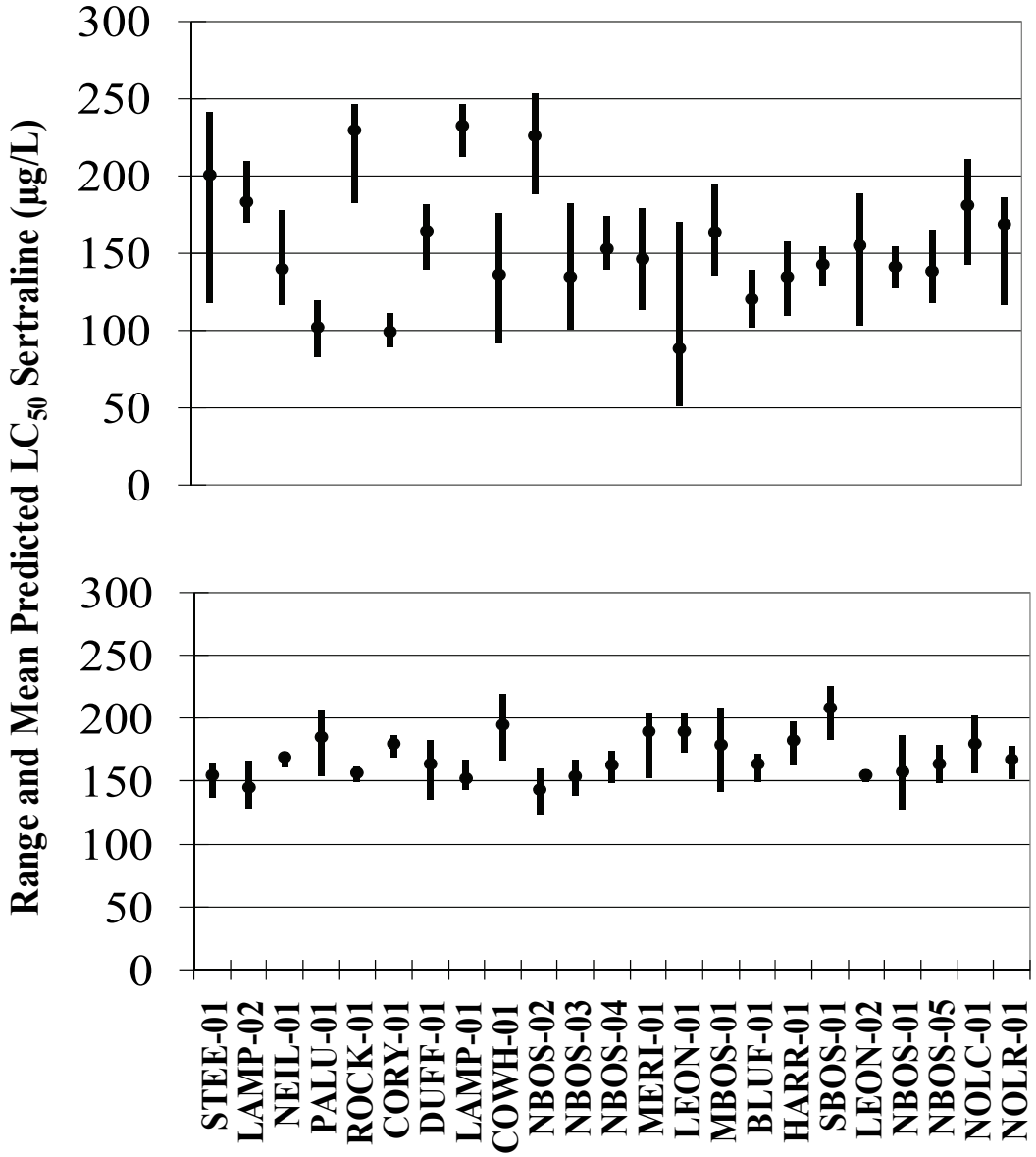


Figure 23. Predicted LC50 value for sertraline based on pH-dependent toxicological reported by Valenti et al. (2009). The dot represents the daily average and the lines represent the range in predicted values based on daily pH fluctuations at sites.

Predicted BCF and $D_{lip\text{-}water}$

The percent of time that BCF values for the compounds were predicted to be > 1000 at the stream sites are shown in Figure 7. The BCF values for tamoxifen were predicted to be >1000 at all sites 100% of the day in both 2006 and 2007. However,

climatological variability was observed to have important influences on BCF predictions for other chemicals. The BCF values at sites were calculated to be >1000 more often in 2006 than in 2007. In fact, several compounds were never predicted to have BCF values >1000 in 2006 (Figure 24). The daily weighted mean $D_{lip\text{-}water}$ values for t chloprothixene, prochlorperzine, tamoxifen, terfenadine, triflupromazine, promethazine, and sertraline varied significantly between 2006 and 2007 (Table 16).

Discussion

The findings of our study clearly demonstrated that interannual variability in hydrology impacted diel cycles of DO and pH in wadeable streams. The magnitude of change related to daily oscillations of these variables at the study sites was significantly greater during periods of low versus high hydrology, despite the fact that data was collected at approximately the same time each year (Figure 20 c,d). For example, fluctuations in DO was > 5 mg/L at 87% of the sites during drought conditions (2006) and several sites had oscillations approaching or surpassing 10 mg/L (Figure 20 c). Similarly, daily change in pH was > 0.5 units at over 60% of the sites during drought conditions and some had oscillations >1.0 pH units over the course of the day (Figure 20 c). Trends in daily oscillations of these variables were less pronounced under high flow conditions (Figure 20 d). In addition, we observed that nutrient concentrations in the water column varied at sites between low flow and high flow conditions, especially for those that received WWTP effluent or where in watersheds with row crop agriculture consisting of >10% of the landuse (Figure 20 a,b; Table 12). Furthermore, we noted that TP concentrations in the water column influenced the likelihood that instream pH exceeded thresholds (Figure 21). The implications of heightened pH at sites for risk

Table 15. The daily oscillation risk ratio (DORR) for the weak bases ammonia and sertraline at stream sites in the Brazos River Watershed, TX during low flow (2006) and high flow (2007) conditions. The DORR is a ratio of the highest predicted threshold response (pH maximum) divided by the lowest predicted threshold response (pH minimum).

Site	Ammonia DORR		Sertraline DORR	
	2006	2007	2006	2007
STEE-01	4.1	1.7	1.6	1.2
LAMP-02	2.2	2.2	1.3	1.3
NEIL-01	3.1	1.2	1.4	1.1
PALU-01	1.8	2.2	1.2	1.3
ROCK-01	2.8	1.3	1.4	1.1
CORY-01	1.7	1.3	1.2	1.1
DUFF-01	2.9	2.4	1.4	1.3
LAMP-01	1.9	1.6	1.2	1.2
COWH-01	3.6	2	1.5	1.3
NBOS-02	4	2.3	1.6	1.3
NBOS-03	20.7	1.8	3.3	1.2
NBOS-04	2.9	1.6	1.4	1.2
MERI-01	6.1	2.2	1.8	1.3
LEON-01	1.4	1.6	1.2	1.2
MBOS-01	1.9	3	1.3	1.5
BLUF-01	7.2	1.5	2	1.1
HARR-01	2	1.7	1.3	1.2
SBOS-01	3	1.7	1.5	1.2
LEON-02	7.2	1.2	1.9	1.1
NBOS-01	2	3.1	1.3	1.5
NBOS-05	2.6	1.7	1.4	1.2
NOLC-01	1.7	2	1.2	1.3
NOLR-01	6.1	1.6	1.8	1.2

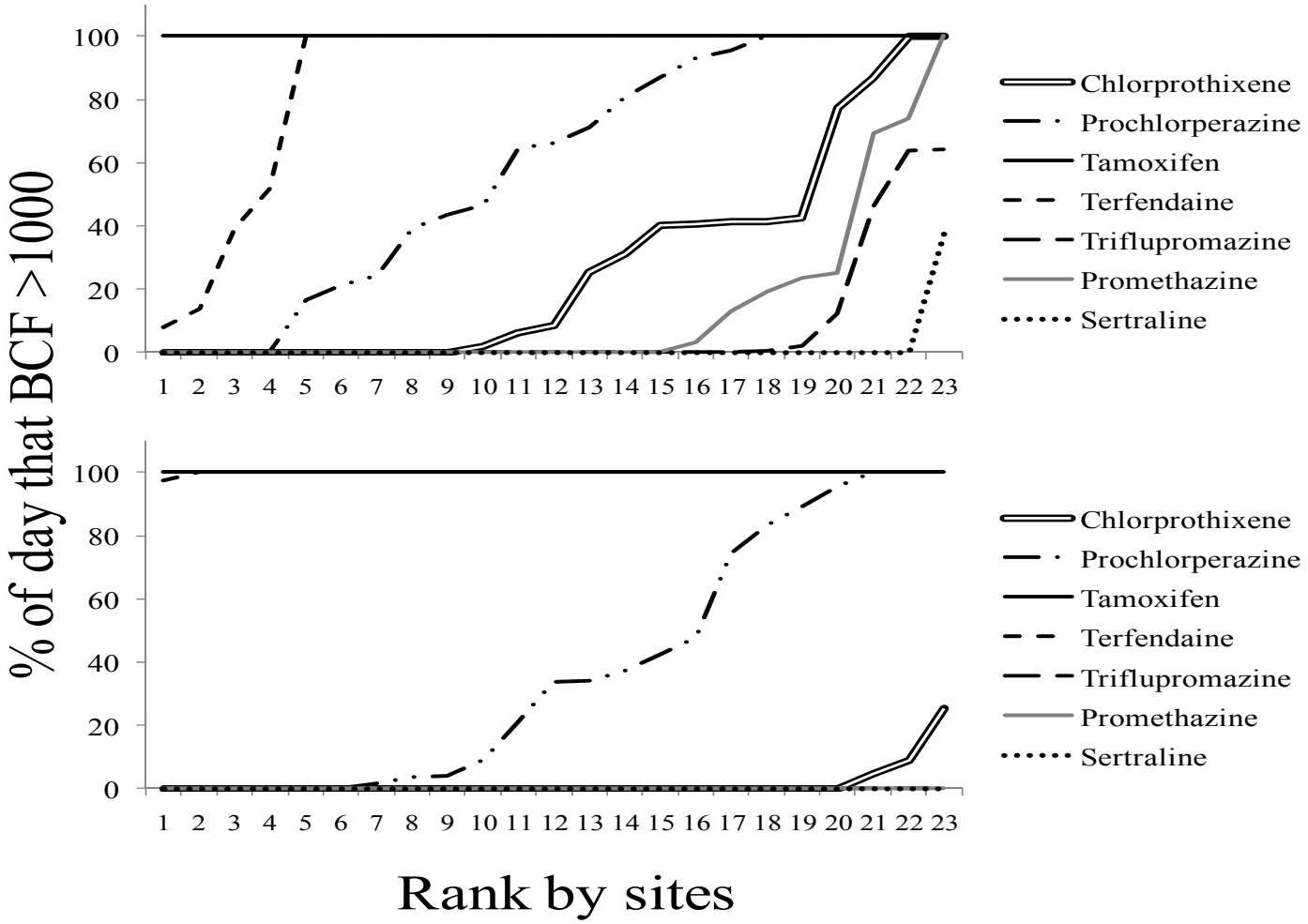


Figure 24. The percent of the day that predicted bioconcentration factors will be greater than 1000 for several pharmaceuticals that are weak bases at stream sites during low (2006) and high (2007) hydrology. Daily oscillations in pH at stream sites may influence the ionization state of these compounds. Differences in the patterns culminate from measured instream pH and the specific dissociation constant (pKa) for the weak base.

Table 16. The weighted mean K_{lipw} for seven weak base pharmaceuticals at 23 stream sites the Brazos River Watershed, TX during low flow (2006) and high flow (2007) conditions

	Chlorprothixene		Prochlorperazine		Tamoxifen		Terfendaine		Triflupromazine		Promethazine		Sertraline	
	2006	2007	2006	2007	2006	2007	2006	2007	2006	2007	2006	2007	2006	2007
	17000	21000	5600	7200	710000	910000	74000	94420	9200	12000	13000	16000	4199	5200
	18000	25000	6100	8700	770000	1100000	80000	113851	9900	14000	14000	19000	4400	6300
	31000	16000	11000	5500	1400000	710000	140000	73112	17000	9100	23000	13000	7900	4100
	27000	13000	9300	4200	1200000	550000	120000	56677	15000	7100	20000	9900	6700	3100
	29000	20000	10000	6900	1300000	870000	130000	89946	16000	11000	22000	15000	7400	5000
	13000	14000	4300	4600	560000	590000	58000	60897	7200	7600	10000	11000	3200	3400
	58000	18000	20000	6300	2800000	800000	290000	82464	34000	10000	40000	14000	16000	4600
	61000	22000	22000	7500	3000000	940000	310000	97941	36000	12000	42000	17000	17000	5400
	29000	11000	10000	3600	1300000	480000	130000	49079	16000	6100	21000	8600	7300	2700
	26000	27000	8900	9100	1100000	1100000	120000	119685	14000	15000	19000	20000	6500	6600
	77000	22000	27000	7300	4900000	924527	490000	96028	53000	12000	49000	16000	26000	5300
	19000	18000	6300	6200	800000	780000	83000	81143	10000	10000	14000	14000	4600	4497
	32000	18000	11000	3900	1400000	510000	150000	52445	18000	6500	24000	9200	8200	2900
	6400	12000	2100	3900	290000	510000	29000	52234	3600	6500	5000	9100	1600	2900
	7200	14000	2300	4700	320000	610000	32000	62562	4000	7800	5600	11000	1800	3500
	11000	18000	3700	6100	490000	770000	50000	79771	6200	9900	8600	14000	2800	4400
	6600	13000	2100	4500	290000	580000	30000	59496	3700	7400	5200	10000	1700	3300
	14000	8900	4700	2900	610000	390000	63000	39821	7800	5000	11000	7000	3500	2200
	32000	21000	11000	7100	1500000	900000	150000	93697	18000	12000	23000	16000	8400	5200
	2299	21000	7500	7100	950000	900000	98000	93587	12000	12000	17000	16000	5400	5200
	41000	18000	14000	6100	1800000	770000	190000	80236	23000	10000	30000	14000	11000	4400
	26000	14000	9100	4600	1100000	600000	120000	61552	15000	7700	20000	11000	6600	3400
	24000	17000	8200	5700	1100000	720000	110000	74819	13000	9300	18000	13000	6000	4100
Mean	26000	17000	9400	5800	1300000	740000	130000	77000	16000	9600	20000	13000	7300	4200
SE	3900	950	1300	350	220000	41000	22000	4400	2400	550	2400	720	1200	240
p		0.03		0.012		0.017		0.017		0.018		0.013		0.015

assessment of ionizable contaminants was demonstrated by the marked differences in allowable instream concentrations of traditional contaminants (e.g. ammonia, Figure 22), predicted toxicological responses of emerging ionizable contaminants (.e.g. sertraline, Figure 23), the likelihoods that other pharmaceuticals would exceed previously described regulatory guidelines for defining potentially bioaccumulative contaminants over the course of the day (Figure 24), as well the predicted weighted mean $LogD_{lipwater}$ for several pharmaceuticals (Table 16). To quantify the magnitude of difference of pH-dependent toxicological responses associated with daily fluctuations in pH at stream sites, we developed the Daily Oscillation Risk Ratio (DORR). We noted significant differences in the metric at the sites between low and high flow conditions for both ammonia and sertraline (Table 15).

While our study clearly showed that patterns of daily cycles in DO and pH were related to instream flows, relationships between concentrations of nutrients in the water column and diel oscillations were less apparent. Other researchers have suggested that such relationships are likely nonlinear and influences by several factors that may vary on a site by site basis (Heathwaite 2010, Moss 2010). Therefore additional factors besides flow and nutrients alone that influences photosynthetic activity, such as light availability, limitations of other nutrients, or possible toxicological effects of xenobiotics, could also confound relationships. In addition, differences in standing biomass and detritus material at the stream sites may have also affected P:R dynamics and thus influenced diel cycles in DO and pH. Furthermore, relying on water column concentrations of nutrients alone may be a poor indication of the actual eutrophic state at sites due to transient and episodic changes of nutrient storage, transport, and inputs (Winter and Duthie 2000, Dodds 2003).

For instance, concentrations of nutrients in the water column at even heavily impacted sites could be low if they are be rapidly sequestered by biota (consider the fact demand at such sites is likely greater due to increased biomass). Alternatively, water column nutrient concentrations may also be low at impacted sites if site-specific conditions facilitate binding to sediments.

Although our study did not definitively identify the specific causal factors contributing to these trends, it highlights the potential importance that these scenarios may have on aquatic risk characterization of ionizable contaminants. The similarity between diel patterns of DO and pH indicates that primary production (P) and respiration (R) dynamics can substantially influence site-specific conditions. Aquatic ecologists have long been aware of the interplay of P:R relationships in surface waters (Odum 1956); however, there has been a growing resurgence of this topic as scientists have become more aware of how changes to essential ecosystem functions due to climatological or anthropogenic influences may affect environmental contaminants in aquatic systems (Wenning et al 2010). Hydrologic changes due to interannual variability in precipitation rates, and anthropogenic influences, such as the construction of impoundments, inter-boundary water exchange, and modified land use, affects the biogeochemical properties and residency times of surface waters (Vorosmarty and Sahagian 2000, Sun et al 2008, Boxall et al 2010). Further, changes in flow regimes and transient pathways of water, along with both point and non-point source pollution, may also effect P:R dynamics in surface waters by influencing allochthonous nutrient inputs to wadeable streams (Heathwaite 2010).

A number of these ecosystem alterations are already evident in arid regions of the southwest and south central United States (Sun et al 2008). Predictions of climate change, population growth, and altered land use suggest potentially even greater effects on surface waters in the future (Boxall et al 2009). Texas may represent one of the States at greatest risk to climate change because of its high projected rates of population growth (doubling before 2050), declines in annual precipitation, increases in average air temperatures, and river basins that traverse arid and semiarid ecoregions. For these reasons, Sun et al (2008) scored several areas of Texas with high water supply stress index values. In fact, Austin, Texas represented the State capital facing the most severe water shortages in the future due to limited water supplies (Sun et al 2008). Ensuring a sustainable water supply to meet growing agriculture and populations needs is made more challenging by the necessity of maintaining adequate assimilation capacity for pollution dilution and flows in surface water to maintain the integrity of aquatic ecosystems (Moss 2010). It is therefore imperative that researchers develop a more comprehensive understanding of how site-specific water chemistry may be affected by changing hydrologic regimes and eutrophication, and in turn how these may influence anthropogenic risks to aquatic communities.

Ecological risk assessors have long recognized that the potential for contaminants to cause adverse effects in aquatic ecosystems is often mitigated by site-specific factors (Van Wezel 1998, Suter et al 2000, Van Wezel 1998, Niyogi and Wook 2004). One of the most important variables to consider when making inferences regarding ecological hazard in aqueous environments is pH because of its pivotal role in controlling

geochemical processes and reactions (USGS 2008). From a risk assessment perspective, pH is crucial because it can directly affect bioavailability and fate of some contaminants by altering their physicochemical properties (Van Wezel 1998, Valenti et al 2010a,b, Kim et al 2010) or indirectly by changing their interactions with other constituents in the water column (Paquin et al 2002, Niyogi and Wood 2004). The actual risk that contaminants pose to aquatic organisms may more closely be linked to the proportion of a contaminant that exists in the free form, rather than the total concentration measured in the water column (Escher and Hermens 2004). Because exposure is inevitably associated with the contaminant's physicochemical properties, the frequency and duration that a specific pH is measured at a site may play an important role in spatiotemporal risk characterization, which is typically poorly examined during risk assessments. However, estimating the temporal exposure profile of an ionizable contaminant at a specific site is challenged by the very dynamic nature of various intertwined factors controlling pH.

Data requirements for site-specific pH characterization seldom mandate the collection of continuous monitoring data and are generally focused on only capturing variability associated with spatial or coarse temporal scales (e.g., seasonal, annual fluctuations). For example, the Texas Commission of Environmental Quality (TCEQ) suggests that the 15^{th} percentile of a minimum of 30 values for pH at a site can be used to determine a site specific pH-based criterion (TCEQ 2007). The use of discrete sampling events to characterize site-specific pH inherently introduces uncertainty into ecological risk assessments; the magnitude of such uncertainty is related to site-specific conditions and depends on the time at which samples are collected. These potential data gaps are

likely to become more prominent in river basins of Texas, and other semi-arid regions, if predictions of population growth, climate change, and future water scarcity are realized.

It is highly probable that ignoring diel cycles in surface water pH could result in over or under estimates of site-specific risk for select ionizable contaminants. The implications of this potential shortcoming were evidenced by the significant differences between ammonia CMC at various times of the day. We did not attempt to quantify the occurrence of exceedences at the stream sites because current AWQC for ammonia recommends that TN concentrations should be monitored simultaneously with each pH measurements (US EPA 2009). Rather, our intent was to use recommended AWQC for ammonia as a model to examine how diel cycles in pH may influence risk because it accounts for additional factors besides concentration alone. Consistent with the observations in the present study, Crumpton and Isenhart (1988) noted pronounced diurnal patterns of total ammonium-ammonia concentrations at sites below a WWTP, and predicted the lowest total concentrations of unionized ammonia in the early morning. However, regardless of total ammonia concentrations, the researchers consistently predicted the highest unionized ammonia concentrations in the mid-afternoon, which they attributed to daytime increases of 5-10° C and 0.5-1.5 pH units. It is therefore likely that similar patterns exist for other ionizable organic contaminants (e.g., pharmaceuticals), as we have demonstrated here. Further, it appears necessary that such pH patterns should be considered for the speciation and bioavailability of inorganic contaminants (e.g., metals, nutrients), which are known to be influenced by pH (Crumpton and Isenhart 1988, Garban et al 1999, Jones et al 2004, Morris et al 2005, Nimick et al 2007).

During the development of requirements for characterization of site-specific pH for regulatory purposes(TCEQ 2007), it would be ideal to capture both the lowest and highest observed daily pH at a site so that data could be applicable for refining risk characterization of both weak acids and bases. Typically, the lowest observed pH would be measured at or just before dusk when little photosynthetic activity has yet occurred, while the highest observed pH would be measure in the early or mid-afternoon after photosynthetic activity has peaked. These values could then be integrated into equations and fit using sine and cosine functions to extrapolate daily patterns in pH oscillations. Furthermore, the collection of such data would allow for the calculations of other risk metrics, such as DORR.

Though the present study focused on weak bases, it is important to also consider the implications of climatological variability and nutrient enrichment on the aquatic toxicity of weak acids. For example, triclosan, a weak acid and common antimicrobial agent employed in personal care products, was demonstrated to be more toxic to aquatic life at lower pH (Orvos et al 2002). In addition, Kim et al. (2010) noted that lower water pH caused markedly greater toxicity for acetaminophen, enrofloxacin, and sulfathiazole to *D. magna*. Thus, based on our observations the lower pH conditions of early morning would be predicted to increase the hazard of these weak acids to aquatic organisms.. As demonstrated in the present study, the magnitude of pH variability was more pronounced during drought conditions. Ultimately, incorporating an approach that considers short term temporal variability is surface water pH appears important for reducing uncertainty during ecological risk assessment of select ionizable contaminants (Robinson and Roby 2006; Valenti et al 2010a).

Analysis of effects during ecological risk assessment is often completed by collecting data for laboratory toxicity tests in which individuals of a single species are exposed to a known concentration of contaminants (Kim et al 2010). Other studies have examined the influence of site-specific pH of toxicity of traditional ionizable contaminants, such as ammonia and pentachlorophenol. More recently several studies have quantified the magnitude of difference in biological responses over environmentally relevant pH gradients for some pharmaceuticals (Nakamura et al 2008, Valenti et al 2010, Kim et al 2010) and antimicrobials (Orvos et al 2002). Recently, Kim et al (2010). Other studies have shown that changes in ionizations state due to differences in exposure pH can affect BCFs for some organic compounds (Endo and Onozawa 1987, Fisher et al 1999) and others have demonstrated that increased BCFs correspond to heightened toxicological responses (Fent and Looser 1995, Kishino and Kobayashi 1995, Nakamura et al 2008). If observations from these previous studies are compared to our findings of pH variability in wadeable streams located in the Brazos River watershed, the accumulation and toxicity of some ionizable contaminants is predicted to vary with interannual stream flows as suggested by data shown in Table 5 and Figure 7 Thus, examining whole body burdens or critical tissue residues, rather than ambient water concentrations, may prove a more valuable tool for inferring actual exposure scenarios (Escher and Hermens 2004, Brooks et al 2009).

Other studies have also demonstrated that patterns of diel pH oscillation at sites have potential ecotoxicological implications because actual exposure scenarios for individuals may deviate over the course of the day. In a study focused not on steady state concentrations, but rather fluctuating internal concentrations due to changes in exposure

pH, Hargreaves and Kucuk (2001) demonstrated that total ammonia-nitrogen concentrations in the plasma of juvenile hybrid striped bass, channel catfish, and blue tilapia varied as a result of environmentally relevant daily oscillations in exposure pH. Similar trends in changing internal concentrations could occur for other ionizable contaminants, but it challenging to understand how the magnitude, frequency, and duration of sporadic fluctuations in concentrations at specific target sites within the body will affect pharmacological or toxicological responses in aquatic organisms. Though the present study demonstrates the importance of considering nutrient enrichment and interannual climate variability on pH dynamics of wadeable streams, future work is warranted to understand the consequences of such site-specific pH variability on the environmental impacts of ionizable contaminants.

APPENDICES

APPENDIX A:

Influence of pH on Amine Toxicology and Implications for Harmful Algal Bloom Ecology

Note: This publication was submitted as a response to other authors' comments concerning chapter two. It also appears in a special issue on Harmful Algal Bloom Species in Toxicon (2010) 55:1038-1043. Please refer to appendix B for the licensing agreement of this publication.

Abstract

In marine and estuarine conditions, pH-dependent aquatic toxicity associated with *Prymnesium parvum* has been previously demonstrated with greater toxicity observed at higher pH. Recent research by our group extended such observations to inland waters in Texas, USA. Because prymnesins (prymnesin-1, prymnesin-2) represent the only *P. parvum* toxins reported in the peer-reviewed literature, we hypothesized that *P. parvum* toxins may behave like weak bases in surface waters due to the presence of a primary amine. Employing a widely accepted computational model (ACD/Labs), we predicted physicochemical properties to support interpretation of experimental findings, specifically focusing on the amine-containing hydrophobic portion of these molecules. Using different computational models, alternative pKa values for prymnesins have been proposed by others. Herein, additional examination of our proposed hypothesis identifies the importance of considering subtle changes in ionization state on the lipophilicity, bioavailability (e.g., log D, log $D_{lip\text{-}water}$, Bioconcentration factor), and aquatic toxicity of ionizable chemicals over an environmentally-relevant pH gradient. If prymnesins represent causative toxins released by *P. parvum* as previously reported, a closer

evaluation of our findings further highlights the importance of considering site-specific pH during environmental assessment and management efforts for *P. parvum* in inland waters.

Response letter

Several previous studies demonstrated pH-dependent toxicity associated with the toxic alga *P. parvum* (Shilo and Aschner 1953, McLaughlin 1958, Ulitzer and Shilo 1966, Padilla 1970, Shilo and Sarig 1989). These studies focused on highly saline marine or brackish waters. Valenti et al. (2010, this issue of *Toxicon*) investigated whether pH influences the aquatic toxicity of *P. parvum* in less saline inland waters that are representative of reservoirs in Texas and the southwestern US where toxic blooms of this species have recently occurred (Roelke et al., 2007, 2010; Errera et al., 2008; Grover et al., 2010; Schwierzke et al., 2010). High aquatic toxicity was consistently observed near ambient pH (e.g., 8.5) in samples from two different reservoirs experiencing fish kills attributed to *P. parvum* blooms; however, toxicity was markedly reduced when pH was lowered. Such pH-dependent toxicity was further verified in laboratory experiments with cell-free filtrates from *P. parvum* monocultures (Valenti et al., 2010). Experimental approaches followed rigorous standardized methods for aquatic Toxicity Identification Evaluations (TIE) developed by the U. S. Environmental Protection Agency (1991). Together with results from prior studies in more saline waters, Valenti et al. (2010) concluded that increasing pH increases the potency of excreted toxins by *P. parvum* to model aquatic organisms over a wide range of salinities. During more recent studies that examined the aquatic toxicity of partially purified extracts, which were previously reported to be acutely toxic to fish (Schug et al. 2010), we observed acute mortality to

fish when bioassays were performed at pH 9 in laboratory reconstituted soft and hard waters (APHA et al. 1999), whereas toxicity was completely ameliorated when pH was lowered to 6 in both water types (Schug et al., unpublished data). Valenti et al. (2010) proposed that ionization of a primary amine on the compounds prymnesin-1 and -2 at low pH explained reduced aquatic toxicity based on a predicted pKa of 8.9 for the prymnesins. In a letter to the Editor (Chichewicz and Hambright, 2010, this issue of *Toxicon*), this proposition was sharply criticized. We thank *Toxicon* for the opportunity to respond to these criticisms here.

Three objections were raised to the mechanism we hypothesized in Valenti et al. (2010): (1) Presence of prymnesins in field samples and cultures was not analytically confirmed; (2) the predicted pKa of 8.9 was low relative to other compounds that contain primary amines; and (3) the calculation of ionization state distribution was incorrect, and that a recalculated distribution, using alternatively proposed pKa values, suggests that changes in ionization state will not be biologically important over the pH range previously examined by our research team. We initially reply to the first objection, and respond to the second and third together, because they involve several related physicochemical properties of the prymnesins that are relevant to their potential ambient aquatic toxicity.

To the first objection, we agree that measuring concentrations of prymnesins in our study would have strengthened our proposed hypothesis. However, we do not agree that a simple method is available for this purpose. For example, the use of chromatography – mass spectrometry, especially electrospray ionization – mass spectrometry (ESI-MS) methods for quantitative analysis of prymnesins, or any other

toxins produced by *P. parvum*, has not been conclusively demonstrated. The development of a straightforward method would require a pure analytical standard that could be applied for external standard or standard addition calibration. Of course, the ability to incorporate an internal standard would be ideal for highest precision. However, the strict need for coherence between the structure of the analyte of interest to be quantified and an internal standard to have identical ionization response dictates the need for a stable-isotopically-labeled form of the analyte. To date, only Yasumoto and coworkers have reported isolating prymnesin molecules (Igarashi et al. 1996, 1998, 1999; Morohashi et a. 2001), and replication of this work has yet to be reported in the peer-reviewed literature. Thus, analytical standards for the prymnesins, either normal or deuterated, or any structurally similar molecule are not currently available.

To address this question, our research team has worked towards developing general bioassay-guided fractionation procedures based on erythrocyte lysis and fish mortality in order to better elucidate the profile of toxic constituents produced by *P. parvum* (Schug et al., 2010). Laboratory cultures have been taken through multiple steps of fractionation, toxicity of fractions have been assessed, and active fractions have been analyzed by high performance liquid chromatography (HPLC)-ESI-MS. We have described the observation of a signal, previously attributed to a multiply-charged response for prymnesin-2 (Barkoh et al., 2008; Hamlett, 2008); however, inspection using a high resolution ion trap – time-of-flight mass spectrometer (Schug et al., 2010) revealed that this signal did not possess the expected isotope distribution pattern for a chlorine-containing molecule, and that the error in measured m/z ratios for the assigned sodiated ions was too high (> 120 ppm mass error on an instrument that reliably provides

< 5 ppm error). Prior analyses using a low resolution mass spectrometer may not have had sufficient resolution to observe and properly assign the expected signals for chlorine isotopes. Whereas Schug et al. (2010) were disappointed to report that the supposed ESI-MS response could not be attributed to prymnesins, this study emphasized the fact that there is currently no published validated method for quantitative analysis of prymnesins, or of any other potential toxins, produced by *P. parvum* (Schug et al., 2010).

The significance of the first objection to our research presented in Valenti et al. (2010) is that without quantitation of prymnesins in toxicity studies, it remains possible that other compounds explain the surface water impacts by *P. parvum* populations. We agree, and said as much (Valenti et al., 2010): "Alternatively, prymnesins might not be the only, or even the most important toxins produced by *P. parvum*." Because it remained infeasible for Valenti et al. (2010) to evaluate this possibility or make defensible inferences about other compounds not described and reported by others, we followed sound scientific principles and relied on the available peer-reviewed literature. To date, prymnesin-1 and -2 are the only identified compounds released by *P. parvum* that are responsible for aquatic toxicity (Igarashi et al., 1996, 1998, 1999; Murata and Yasumoto, 2000; Morohashi et al., 2001; Sasaki et al., 2001). To our knowledge, no published studies associate ambient toxicity to any other identified toxins produced by *P. parvum*. The pH-dependent toxicological relationships observed by Valenti et al. (2010) and others are consistent with numerous aquatic toxicity dose-response relationships of weak bases. Hence, Valenti et al. (2010) "hypothesized that toxins released by *P. parvum* are ionizable weak bases," and noted that prymnesin-1 and -2 are reported to possess a primary amine on their hydrophobic tail (Igarashi et al., 1996, 1998, 1999). We further

used ACD/Labs (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada) Chemsketch, pKa calculator, LogD calculator, and LogP calculator (Version 9) to estimate physicochemical parameters for prymnesin-1 and -2 (Table 4 in Valenti et al., 2010). ACD/Labs software is widely employed for such computational activities, is a trusted source of predicted properties for organic compounds, and each software module makes use of an extensive database of empirical data to estimate physicochemical properties. In fact, ACD/Labs is included in the American Chemical Society's SciFinder Scholar software for chemical property estimations (ACS, 2007) and was identified for physicochemical property estimation of environmental contaminants by the European Commission following a review of other property estimation software programs (ECA, 2008). The second and third objections (Chichewicz and Hambright, 2010) to our research presented in Valenti et al. (2010) take issue with the properties of prymnesins thus predicted, and the resulting ionization state distribution that we proposed as an explanation for the pH-dependent toxicity associated with *P. parvum* blooms. One of the major contentions is that the pKa of 8.9 ± 0.1 modeled by ACD/Labs pKa calculator for the primary amine on the hydrophobic portion of prymnesin-1 and prymnesin-2 is unexpectedly low. The prediction is based on an initial value for alkylated primary amines (10.7) found in the Internal Reaction Centers Database, which is then modulated in the calculation due to inductive effects imparted to the ionization center based on the functional nature of the remainder of the molecule. The pKa calculation package provides a detailed rationale for the predicted value; however, the manufacturer's website acknowledges the possibility for error in the accuracy of predicted values.

The accuracy of this value can be further explored. For example, different software packages (SPARC, ChemAxon MARVIN) used to predict the pKa for a surrogate compound, 4-amino-1,6-heptadiene, which mimics the amine-containing C-12 to C-16 portion of prymnesins, and whole prymnesins, showed pKa values of 9.8 and 10.4, respectively, which are appreciably higher than the value reported by our group for the prymnesin fragment (although it is unclear in Cichewicz and Hambright (2010) whether these pKa values from either SPARC or ChemAxonMARVIN relate to the surrogate or to a prymnesin molecule, preventing rigorous examination of the proposed pKa values; in addition, errors associated with pKa predictions by SPARC or ChemAxonMARVIN were not provided). Interestingly, our analysis of the same surrogate compound using the ACD/Labs pKa calculator yielded a value of 9.3 ± 0.1 units. Clearly, some discrepancies can arise from predictions of pKa values using different software packages, which each possess inherent advantages and disadvantages (Kah and Brown, 2008; ECA, 2008). In the end, it is difficult, if not impossible, to ascertain which value is most accurate without future experimental studies of purified prymnesins. In our work presented in Valenti et al. (2010), a computationally-predicted model was sought to compare properties with experimental data. More recent ACD/Labs modeling of pKa values for the entire prymnesin structures are consistent with those presented in Valenti et al. (2010) for the hydrophobic portion of the molecules (Schug et al., unpublished data). As noted above, experimental data from laboratory and field samples clearly showed a pH-dependent toxicity relationship; predictions from the ACD/Labs calculator correlate well with the experimental findings of Valenti et al. (2010).

Table 4 in Valenti et al. (2010) presents values for percent ionization of the primary amine, based on calculated Log D parameters (ACD/Labs Log D calculator). These results can be countered by presenting a classical calculation of degree of protonation/deprotonation in aqueous media. We certainly acknowledge that a compound with a pKa of 8.9 would be expected to have a higher degree of ionization in aqueous media than presented in Table 4, and perhaps our previous representation of such values could have been more clearly described. The important caveat of our results (Table 4 of Valenti et al., 2010), however, is that the log D calculator accounts not only for the ionizability of the compound in aqueous media, but also in organic media. Thus, a more complex and comprehensive equilibrium model is considered by the log D calculator of ACD/Labs. Because the hydrophobic portion of the compound has a high log D (and log P) value, it would be expected to partition significantly to, and be present primarily in, hydrophobic media. The lower dielectric constant of this matrix reduces the ionizability of the compound. As a result, the predicted degree of ionization, which is weighted by taking into account the fraction of the molecule present in different solvent environments, is much lower than what would be expected by simply considering acid/base equilibria in aqueous media. Table 1 in the present paper provides predicted partitioning parameters based on ionization in aqueous media based on various proposed pKa values.

If the prymnesins are produced by *P. parvum* and result in toxicity to aquatic organisms as previously reported (Igarashi et al., 1996, 1998, 1999; Murata and Yasumoto, 2000; Morohashi et al., 2001; Sasaki et al., 2001), then the approach employed by us in Valenti et al. (2010) is appropriate. Both fundamental principles and

considerable evidence from the aquatic toxicology and mammalian pharmacology and toxicology literature for ionizable compounds show that it is crucial to examine the implications of various pKa values for amine lipophilicity and, thus, bioavailability (e.g., interactions with biological membranes), bioaccumulation, and toxicity to aquatic organisms over environmentally-relevant pH ranges. Such considerations are especially important for toxins produced by *P. parvum*. Well documented hemolytic effects point to interactions with cell membranes as a major component of exerted toxicity (Meldahl et al., 1994; Mariussen et al., 2005).

For nonionizable organic chemicals, log P (or, log K_{ow}) is a universally accepted parameter for predicting water to lipid partitioning properties, bioaccumulation and toxicokinetics of contaminants in aquatic systems (Schwarzenbach et al., 1993; Rand, 1995; Boethling and MacKay, 2000), and toxico(pharmaco)kinetics of chemicals related to human health (Boethling and MacKay, 2000; Hardman and Limbird, 2001; Klaussen, 2008). For ionizable compounds, log D represents a pH-dependent corollary for log P in such efforts (Scherrer and Howard, 1977; Schwarzenbach et al., 1993; Rand, 1995; Boethling and MacKay, 2000; Hardman and Limbird, 2001; Erickson et al., 2006a, 2006b; Klaussen, 2008; Kah and Brown, 2008; Fu et al., 2009), where (for bases):

$$\text{Log D}_{(\text{Aqueous})} = \log P + \log [1/(1+10^{(pKa-pH)})]$$

Eq. 1.

However, liposome-water systems (log $D_{lip\text{-}water}$) are considered more accurate surrogates for biological systems than octanol-water partitioning predictions, particularly for

membrane-toxic agents and hydrophobic ionogenic organic compounds (Escher and Schwarzenbach, 2000), where:

$$\text{Log } D_{\text{lip-water}} = 0.78 \text{ x log D} + 1.12 \qquad \text{Eq. 2.}$$

Estimates of bioconcentration factors (BCF) are also possible when employing such partition coefficients, as provided by ACD/Labs and other software packages (Kah and Brown, 2008; Fu et al., 2009).

It is instructive to appreciate that the various pKa values proposed for prymnesins (8.9, 9.8, 10.4) lead to strong predictions of pH-dependence of log D and log $D_{\text{lip-water}}$ values over an environmentally relevant pH gradient (Figure 1). Lipophilicity of the hydrophobic portion of prymnesins is predicted to increase with pH, and log D exceeds 3.5, a value identified as a threshold for triggering aquatic bioaccumulation concerns (US EPA, 2000), regardless of the value of pKa predicted for prymnesins across the pH gradient examined by Valenti et al. (2010). BCF values are also predicted to increase over two orders of magnitude between pH 6.5 and 8.5, which exceed regulatory bioaccumulation thresholds (US EPA, 2000; Fu et al., 2009; Table 1). Though it may not be possible for prymnesins to appreciably bioaccumulate in aquatic organisms following waterborne exposure due to their large molecular size, based on information presented in Table 1 and Figure 1, there is a markedly greater potential for these molecules to "interact with target sites (e.g., gill membranes)" at higher environmentally relevant pH (Valenti et al., 2010), even if a pKa value of 10.4 of prymnesins is considered.

Experimental evidence from the peer-reviewed literature clearly provides support for predicted pH-dependent lipophilicity and toxicity of ionizable contaminants in aquatic

organisms (Figure 1), as suggested by findings in Valenti et al. (2010). For example, fluoxetine, a secondary amine with a pKa value of 10.1, was reported at pH 7, 8, and 9 to have respective % unionized species of 0.079, 0.79, and 7.4 %, and corresponding LC_{50} values of 5.5, 1.3, and 0.20 mg L^{-1}, demonstrating greater aquatic toxicity with higher pH (Nakamura et al., 2008). Further, there was greater than a 4-fold difference in aquatic toxicity between pH 7 and 8 despite a change in ionization state of less than 1% , and greater than a 27-fold difference in toxicity with an ~8% change in ionization (Nakamura et al., 2008). Predicted and experimental log $D_{lip\text{-}water}$ values and BCFs for fluoxetine were also markedly greater at higher pH levels, with BCFs ranging from 8.8 to 260 between pH 7 and 9 (Nakamura et al., 2008). But such partitioning behavior and bioaccumulation in a fish model would be expected for fluoxetine and other weak bases, based on pharmaco(toxico)kinetic information from the aquatic toxicology (Erickson et al., 2006a, 2006b; Valenti et al., 2009; Berninger and Brooks, 2010) and mammalian pharmacology (Hardman and Limbird, 2001) and toxicology (Klaussen, 2008) literature. As another example, pH-dependent increases in aquatic toxicity to the model crustacean *Artemia salinia* occurred for the primary amines octylamine (pKa = 10.75), decylamine (pKa = 10.7), dodecylamine (pKa = 10.67), tetradecylamine (pKa = 10.67), pentadecylamine (pKa = 10.67) and hexadecylamine (pKa = 10.67) (Finlay and Callow, 1997), over an environmentally-relevant pH gradient similar to those examined by Valenti et al. (2010). Thus, it appears highly probable that small changes in ionization state can influence lipophilicity, bioavailability, bioaccumulation and aquatic toxicity of amines. Even if the pKa of prymnesins is as high as proposed in the objections to our

research (Chichewicz and Hambright, 2010), our hypothesized mechanism for pH-dependent toxicity associated with *P. parvum* populations remains quite reasonable.

There is clearly a need for an advanced understanding of *P. parvum* bloom dynamics and the fate and function of its toxins in inland waters of the United States as their frequency and severity have increased after first being documented in Texas in the 1980s (James and De la Cruz, 1989; Watson, 2001). Numerous published reports have attributed fish mortality to *P. parvum* in laboratory and field studies and identified such toxicological effects to result from the release of toxins by *P. parvum* (for a review see Brooks et al., 2010). Further, site-specific differences in chemical, physical and biological parameters appear critical for influencing the magnitude of toxins produced by *P. parvum* (Granéli and Johansson, 2003; Baker et al., 2007, 2009). Previous studies with *P. parvum* have demonstrated a pH-dependent toxicological relationship under marine and brackish conditions (Shilo and Aschner, 1953; McLaughlin, 1958; Ulitzer and Shilo, 1966; Padilla, 1970; Shilo and Sarig, 1989). In our research presented in Valenti et al. (2010), we extended this relationship to lower salinity waters and proposed a chemical mechanism based on the only identified and characterized toxins associated with this species. Further consideration of the principles involved only strengthens this suggestion.

References

American Chemical Society, 2007. Sci-Finder Scholar, American Chemical Society, Washington, DC.

Baker, J.W., Grover, J.P., Brooks, B.W., Urena-Boeck, F., Roelke, D.L., Errera, R.M., Kiesling, R.L., 2007. Growth and toxicity of *Prymnesium parvum* (Haptophyta) as a function of salinity, light, and temperature. J. Phycol. 43, 219–227.

Baker, J.W., Grover, J.P., Ramachandrannair, R., Black, C., Valenti, T.W., Brooks, B.W., Roelke, D.L., 2009. Growth at the edge of the niche: an experimental study of the harmful alga *Prymnesium parvum*. Limnol. Oceanogr. 54, 1679–1687.

Barkoh, A., Paret, J.M., Lyon, D.D., Begley, D.C., Smith, D.G., Schlechte, J.W., 2008. Evaluation of barley straw and a commercial probiotic for controlling *Prymnesium parvum* in fish production ponds. N. Am. J. Aquacult. 70, 80-91.

Berninger, J.P., Brooks, B.W. 2010 in press. Leveraging mammalian pharmaceutical toxicity and pharmacology data to predict chronic fish responses to pharmaceuticals. Toxicol. Lett

Boethling, R.S., MacKay, D., 2000. Handbook of Property Estimation Methods for Chemicals: Environmental and Health Sciences, CRC Press, Boca Raton, FL. 481 p.

Brooks, B.W., James, S.V., Valenti, T.W., Urena-Boeck, F., Serrano, C., Schwierzke, L., Mydlarz, L.D., Grover, J.P., Roelke, D.L., 2010. Comparative toxicity of *Prymnesium parvum* in inland waters. J. Am. Water Resour. Assoc. in press

Cichewicz, R.H., Hambright, K.D., 2010. Letter to the Editor: A revised amino group pKa for prymnesins does not provide decisive evidence for a pH-dependent mechanism of *Prymnesium parvum*'s toxicity. Toxicon in press.

Erickson, R.J., McKim, J.M., Lien, G.J., Hoffman, A.D., Batterman, S.L., 2006a. Uptake and elimination of ionizable organic chemicals at fish gills: I. Model formulation, parameterization, and behavior. Environ. Toxicol. Chem. 25, 1512–1521.

Erickson, R.J., McKim, J.M., Lien, G.J., Hoffman, A.D., Batterman, S.L., 2006b. Uptake and elimination of ionizable organic chemicals at fish gills: II. Observed and predicted effects of pH, alkalinity, and chemical properties. Environ. Toxicol. Chem. 25, 1522–1532.

Errera, R.M., Roelke, D.L., Kiesling, R., Brooks, B.W., Grover, J.P., Ureña-Boeck, F., Baker, J.W., Schwierzke, L., Pinckney, J.L., 2008. The effect of nitrogen and phosphorus availability, barley straw extract, and immigration on *Prymnesium parvum* community dominance and toxicity: Results from in-lake microcosm experiments, Texas, USA. Aquat. Microb. Ecol. 52, 33-44.

Escher, B.I., Schwarzenbach, R.P., 2000. Evaluation of liposome-water partitioning of organic acids and bases. 1. Development of a sorption model. Environ. Sci. Technol. 32, 3954-3961.

European Chemicals Agency, 2008. Guidance on information requirements and chemicals safety assessment. Endpoint specific guidance (Chapters R.7a and R.7c). European Chemicals Agency, Helsinki, Finland.

Finley, J.A., Callow, M.E., 1997. The toxicity of alkyl amines: the effects of pH. Biofouling 11, 19-30.

Fu, W., Franco, A., Trapp, S., 2009. Methods for estimating the bioconcentration factor of ionizable organic chemicals. Environ. Toxicol. Chem. 28, 1372-1379.

Graneli, E., Johansson, N., 2003. Effects of the toxic haptophyte *Prymnesium parvum* on the
survival and feeding of a ciliate: the influence of different nutrient conditions. Mar. Ecol. Prog. Ser. 254, 49-56.

Grover, J.P., Baker, J.W., Roelke, D.L., Brooks, B.W., 2010. Mathematical models of population dynamics of *Prymnesium parvum* in inland waters. J. Am. Water Resour. Assoc. in press.

Hamlett, P., 2008. LC-MS-ESI characterization of golden alga (*Prymnesium parvum)* toxin in lake water. Proceedings of the 56th ASMS Conference on Mass Spectrometry and Allied Topics, June 1-5, 2008, Denver, Colorado.

Hardman , J.G., Limbird, L.E. (Eds), 2001. Goodman & Gillman's: The Pharmacological Basis of Therapeutics, 10th Ed. McGraw-Hill, New York. 2148 p.

Igarashi, T., Satake, M., Yasumoto, T., 1996. Prymnesin-2: a potent ichthyotoxic and hemolytic glycoside isolated from the red tide alga *Prymnesium parvum*. J. Am. Chem. Soc. 118, 479–480.

Igarashi, T., Aritake, S., Yasumoto, T., 1998. Biological activities of pyrmnesin-2 isolated from a red tie alga *Prymnesium parvum*. Nat. Toxins 6, 35-41.

Igarashi, T., Satake, M., Yasumoto, T., 1999. Structural and partial stereochemical assignments from prymnesin-1 and prymnesin-2: Potent hemolytic and ichthyotoxic glycosides isolated from the red tide alga *Prymnesium parvum*. J. Am. Chem. Soc. 121, 8499-8511.

James, T.L., De La Cruz, A., 1989. *Prymnesium parvum* Carter (Chrysophyceae) as a suspect of mass mortalities of fish and shellfish communities in western Texas. Texas J. Sci. 41, 429–430.

Kah, M. Brown, C.D., 2008. LogD: Lipophilicity for ionisable compounds. Chemosphere 72:1401-1408.

Klaussen, C.D. (Ed), 2008. Casarett and Doull's Toxicology: The Basic Science of Poisons, 7th Ed. McGraw-Hill, New York. 1280 p.

Mariussen, E., Nelson, G., Fonnum, F., 2005. A toxic extract of the marine phytoflagellate *Prymnesium parvum* induces calcium-dependent release of glutamate from rat brain synaptosomes. J. Toxicol. Env. Heal. A. 68, 67-79.

McLaughlin, J. 1958. Euryhaline chrysomonads: nutrition and toxigensis in *Prymnesium parvum*, with notes on *Isochrysis galbana* and *Monochrysis lutheri*. J.Protozool. 5, 75-81.

Meldahl, A.S., Edvardsen, B., Fonnum, F., 1994. Toxicity of four potentially ichhyotoxic marine phytoflagellates determined by four different test methods. J. Toxicol. Env. Health. 42, 289-301.

Morohashi A., Satake M., Oshima Y., Igarashi T., Yasumoto, T., 2001. Absolute configuration at C14 and C85 in Prymnesin-2, a potent hemolytic and ichthyotoxic glycoside isolated from the red tide alga *Prymnesium parvum*. Chirality 13, 601-605.

Murata, M., Yasumoto, T., 2000. The structure elucidation and biological activities of high molecular weight algal toxins: maitotoxin, prymnesins and zooxanthellatoxins. Nat. Prod. Rep. 17, 293-314.

Nakamura, Y., Yamamoto, H., Sekizawa, J., Kondo, T, Hirai, N, Tatarako, 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.\

Padilla, G.M., 1970. Growth and toxigenesis of the chrysomonad *Prymnesium parvum* as a function of salinity. J. Protozool. 17, 546-462.

Rand, G.M. (Ed), 1995. Fundamentals of Aquatic Toxicology: Effects, Environmental fate and Risk Assessment. CRC Press, Boca Raton, FL. 1148 p.

Roelke, D.L., Errera, R., Kiesling, R., Brooks, B.W., Grover, J.P., Schwierzke, L., Ureña-Boeck, F., Baker, J., Pinckney, J.L., 2007. Effects of nutrient enrichment on *Prymnesium parvum* population dynamics and toxicity: Results from field experiments, Lake Possum Kingdom, USA. Aquat. Microb. Ecol. 46, 125-140.

Roelke, D.L., Schwierzke, L., Brooks, B.W., Grover, J.P., Errera, R.M., Valenti, T.W., Pinckney, J.L., 2010. Factors influencing *Prymnesium parvum* population dynamics during bloom initiation: Results from in-lake mesocosm experiments. J. Am. Water Resour. Assoc. in press.

Roelke, D.L., Gable, G.M., Valenti, T.W., Grover, J.P., Brooks, B.W., Pinckney, J.L., 2010. Hydraulic flushing as a *Prymnesium parvum* bloom-terminating mechanism in a subtropical lake. Harmful Algae in press.

Sasaki M., Shida T., Tachibana K., 2001. Synthesis and stereochemical confirmation of the HI/JK ring system of prymnesins, potent hemolytic and ichthyotoxic glycoside toxins isolated from the red tide alga. Tetrahedron Letters 42, 5725-5728.

Scherrer, R.A., Howard, S.M., 1977. Use of distribution coefficients in quantitative structure-activity relationships. J. Med. Chem. 20, 53-58.

Schug, K.A., Skingel, T.S., Spencer. S.E., Serrano, C., Le, C.Q., Schug, C.A., Valenti Jr., T.W., Brooks, B.W., Mydlarz, L.D., Grover, J.P., 2010. Hemolysis, fish mortality and LC-ESI-MS of cultured crude and fractionated golden alga (*Prymnesium parvum*). J. Am. Water Resour. Assoc. in press.

Schwarzenbach, R.P., Gschwend, P.M., Imboden, D.M., 1993. Environmental Organic Chemistry. John Wiley and Sons, New York. 681 p.

Schwierzke, L., Roelke, D.L., Brooks, B.W., Grover, J.P., Valenti, T.W., Lahousse, M., Pinckney, J.L., 2010. *Prymnesium parvum* population dynamics during bloom development: an assessment of the roles of grazers and virus. J. Am. Water Resour. Assoc. in press.

Shilo, M., Aschner, M., 1953. Factors governing the toxicity of cultures containing the phytoflagellate *Prymnesium parvum* Carter. J. Gen. Microbiol. 8, 333-343.

Shilo, M., Sarig S., 1989. Fish Culture in Warm Water Systems: Problems and Trends. CRC Press, Boca Raton, Florida. 259 p.

Ulitzur S., Shilo, M., 1966. Mode of action of *Prymnesium parvum* ichthyotoxin. J. Protozool. 13, 332-336.

United States Environmental Protection Agency, 1991. Methods for aquatic toxicity identification evaluation: Phase 1 toxicity characterization procedures, 2nd Ed. EPA-600-6-91-003. Office of Research and Development, Washington, DC.

United States Environmental Protection Agency, 2000. Bioaccumulation testing and interpretation for the purpose of sediment quality assessment: Status and needs. EPA-823-R-00-001. Office of Water and Office of Solid Wastes, Washington, DC.

Valenti. T.W., Jr., Perez Hurtado, P., Chambliss. C.K., Brooks, B.W., 2009. Aquatic toxicity of sertraline to *Pimephales promelas* at environmentally relevant surface water pH. Environ. Toxicol. Chem. 28, 2685-2694.

Valenti, T. W., Jr, James, S.V., Lahousse, M.J., Schug, K.A., Roelke, D.L., Grover, J.P., Brooks,B.W., 2010. A mechanistic explanation for pH-dependent ambient aquatic toxicity of *Prymnesium parvum* Carter. Toxicon in press.

Veith, G.D., Kosian, P., 1983. Estimating bioconcentration potential from octanol/water partition coefficients. In: Mackay, D., Paterson, S., Eisenreich, S.J., Simons, M.S. (Eds). Physical Behavior of PCBs in the Great Lakes. Ann Arbor Sciences Publishers, Ann Arbor, MI. pp. 269-282.

Watson, S., 2001. Literature Review of the Microalga *Prymnesium parvum* and Its Associated Toxicity. Texas Parks and Wildlife Department, Austin TX. (http://www.tpwd.state.tx.us/landwater/water/environconcerns/hab/ga/literature/index.phtml; accessed December 17,2009).

Table 1. Predicted physicochemical properties for the hydrophobic portion of prymnesin-1 (reported to contain a primary amine) over a range of environmentally relevant pH using several proposed pKa values, based on Log P = 7.5 (Valenti et al., 2010).

		pKa	
Physicochemical Property	8.9	9.8	10.4
Log D (pH 6.5)	5.1	4.2	3.6
Log D (pH 7.5)	6.1	5.2	4.6
Log D (pH 8.5)	7.0	6.2	5.6
Log Dlip-water (pH 6.5)	5.1	4.4	3.9
Log Dlip-water (pH 7.5)	5.9	5.2	4.7
Log Dlip-water (pH 8.5)	6.5	5.9	5.5
Bioconcentration factor (pH 6.5)	4200	830	280
Bioconcentration factor (pH 7.5)	25000	5100	1700
Bioconcentration factor (pH 8.5)	120000	30000	10000

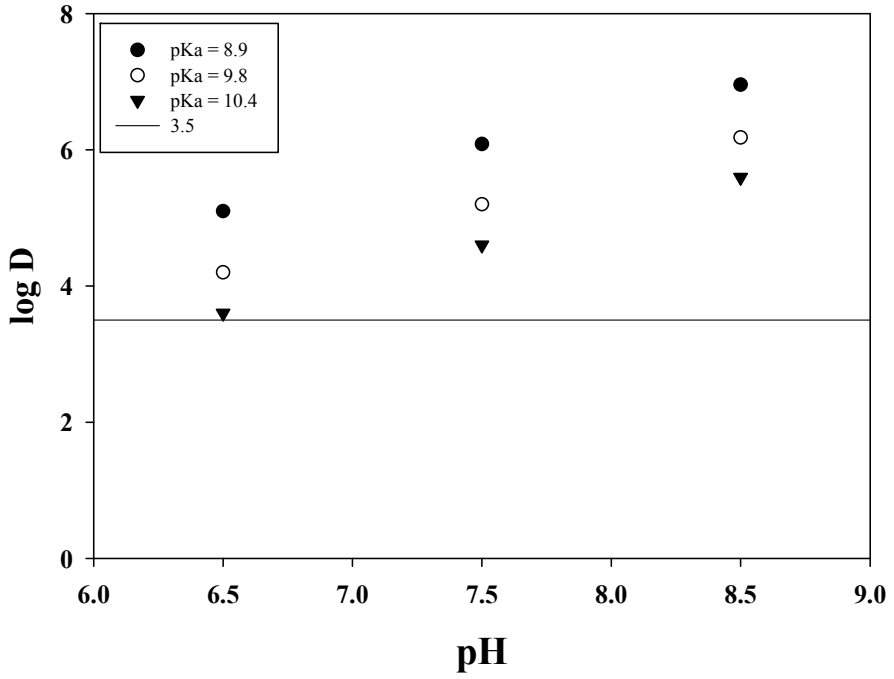
Log D = Log P + Log $[1/(1+10^{pka-pH})]$ (Scherrer and Howard, 1977)

Log $D_{lip-water}$ = (0.78 x Log D) + 1.12 (Escher and Schwarzenbach, 2000)

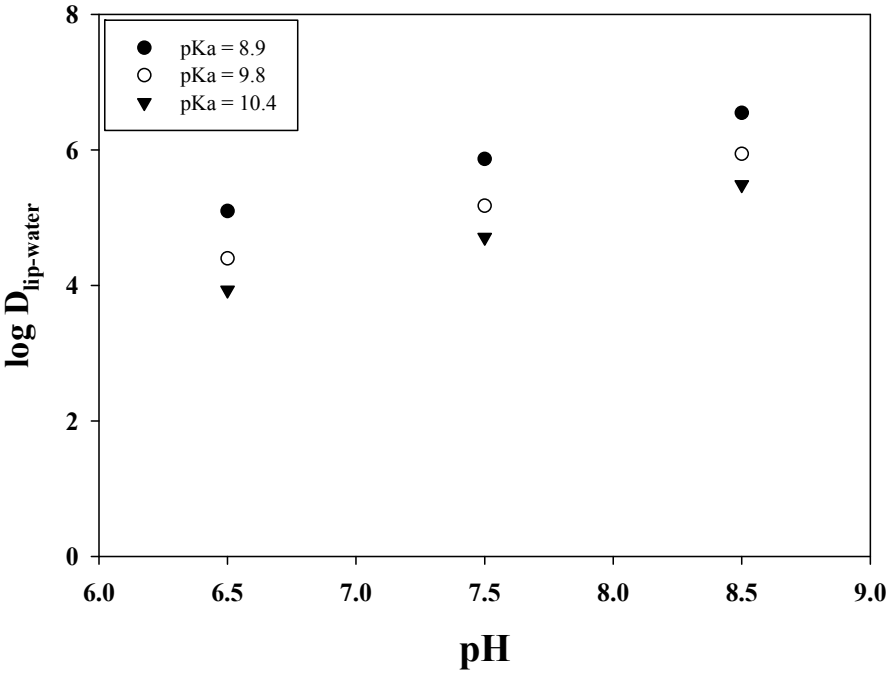
Bioconcentration factor = (0.79 x Log D) - 0.4 (Veith and Kosian, 1983)

Figure 1. (A) The log D and (B) log $D_{lip\text{-}water}$ values for the hydrophobic portion of prymnesin-1 across an environmentally-relevant pH gradient at various pKa values.

A.



B.



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REFERENCES

1) Allan CJ, Roulet NT, Hill AR. 1993. The biogeochemistry of pristine, headwater Precambrian shield watersheds: an analysis of material transport within a heterogeneous landscape. Biogeochemistry 22: 37-79.

2) Allan DJ. 1995. Stream ecology: Structure and function of running waters. Chapman & Hall. London, UK.

3) American Public Health Association, American Water Works Association, Water Environment Foundation. 1998. Standard Methods for the Examination of Water and Wastewater, 20th ed. American Public Health Association, Washington, DC.

4) Anderson DM, Gilbert PM, Burkholder JM. 2002. Harmful algae blooms and eutrophication: nutrient sources, composition, and consequences. Estuaries 25, 704-726.

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

6) Arnot JA, Gobas FAPC. 2006. A review of bioconcentration factor (BCF) and bioaccumulation factor (BAF) assessments for organic chemicals in aquatic organisms. Environ Rev 14: 257-297.

7) Atchison GJ, Henry MG, Sandheinrich, MB. 1987. Effects of metals on fish behavior: a review. Environ Biol Fish 18: 11-25

8) Bailes C, Hudson DL. 1982. A Guide to Texas Lakes, Including the Brazos, Colorado, Frio and Guadalupe Rivers. Houston, TX, Pacesetter Press.

9) Baker JW, Grover JP, Brooks BW, Ureña-Boeck F, Roelke DL, Errera RM, Kiesling RL. 2007. Growth and toxicity of *Prymnesium parvum* (Haptophyta) as a function of salinity, light, and temperature. J Phycol 43, 219-227.

10) Baker JW, Grover JP, Ramachandrannair R, Black C, Valenti TW, Brooks BW, Roelke DL. 2009. Growth at the edge of the niche: an experimental study of the harmful alga *Prymnesium parvum*. Limnol Oceanogr 54, 1679-1687.

11) Baraban SC, Taylor MR, Castro PA, Baier H. 2005. Pentylenetetrazole induced changes in zebrafish behavior, neural activity, and c-fos expression. Neuroscience 131: 759-768.

12) Bass SL, Gerlai R. 2008. Zebrafish (*Danio rerio*) responds differentially to stimulus fish: The effects of sympatric and allopartic predators and harmless fish. Behav Brain Res 186: 107-117.

13) Beitinger TL. 1990. Behavioral reactions for the assessment of stress in fishes. J Great Lakes Res 16: 495-528.

14) Benmansour S, Cecchi M, Morilak DA, Gerhardt GA, Javors MA, Gould GG, Frazer A. 1999. Effects of chronic antidepressant treatments on serotonin transporter function, density, and mRNA level. J Neurosci 19: 10494-14501.

15) Berges JA, Franklin DJ, Harrison PJ. 2001. Evolution of an artificial seawater medium: improvements in enriched seawater, artificial water over the last two decades. J Phycol 37, 1138-1145.

16) Blackman GE, Robertson-Cuninghame RC. 1953. The influence of pH on the phytotoxicity of 2:4-dichlorophenxyacetic acid to *Lemna minor*. New Phytol 52: 71-75.

17) Blaser RE, Chadwick L, McGinnis GC. 2010. Behavior measures of anxiety in zebrafish (*Danio rerio*). Behav Brain Res 208:56-62.

18) Blaxter JHS, Hallers-Tjabbes CCT. 1992. The effect of pollutants on sensory systems and behaviour of aquatic animals. Neth J Aquat Ecol 26: 43-58.

19) Bound JP, Voulvoulis N. 2004. Pharmaceuticals in the aquatic environment- a comparison of risk assessment strategies. Chemosphere 56: 1143-1155.

20) Bowling DR, Pataki DE, Randerson JT. 2008. Carbon isotopes in terrestrial ecosystems and CO_2 fluxes. New Phytologist. 178: 24-40.

21) Boxall ABA, Hardy A, Beulke S, Boucard T, Burgin L, Falloon PD, Haygarth PM, Hutchinson T, Kovats RS, Leonardi G, Levy LS, Nichols G, Parsons SA, Potts L, Stone D, Topp E, Turley DB, Walsh K, Wellington EMH, Williams RJ. 2009. Impacts of climate change on indirect human exposure to pathogens and chemicals form agriculture. Environ Health Persp 117: 508-514.

22) Brain RA, Wilson CJ, Johnson DJ, Sanderson H, Bestari BJ, Hanson ML, Sibley PK, Solomon KR. 2005. Toxicity of a mixture of tetracyclines to *Lemna gibba* and *Myriophyllum sibiricum* evaluated in aquatic microcosms. Environ Pollut 138 426-443.

23) Brooks BW, Ankley GT, Hobson JF, Lazorchak JM, Meyerhoff RD, 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/Taylor and Francis, pp. 97-128.

24) Brooks BW, Chambliss CK, Stanley JK, Ramirez A, Banks KE, Johnson RD, Lewis RJ. 2005. Determination of select antidepressants in fish from an effluent-dominated stream. Environ Toxicol Chem 24: 464-469.

25) Brooks BW, Foran CM, Richards S, Weston JJ, Turner PK, Stanley JK, Solomon K, Slattery M, La Point TW. 2003. Aquatic ecotoxicology of fluoxetine. Toxicol Lett 142: 169-83.

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

27) Brooks BW, Huggett DB, Brain RA, Ankley GT. 2009. 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 (in press). Accepted 12/12/2008.

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

29) Brooks BW, James SV, Valenti TW, Urena-Boeck F, Serrano C, Schwierzke L, Mydlarz LD, Grover JP, Roelke DL. 2010. Comparative Toxicity of *Prymnesium parvum* in Inland Waters. J Am Water Resour Assoc 46: 45-62.

30) Brown JN, Paxéusb N, Förlinc L, Joakim Larsson DG. 2007. Variations in bioconcentration of human pharmaceuticals from sewage effluents into fish blood plasma. Environ Toxicol Pharm 24: 267-274.

31) Burcher CL, Benfield EF. 2006. Physical and biological responses of streams to suburbanization of historically agricultural watersheds. J N Am Benthol Soc 25: 356-369.

32) Carpenter SR, Fisher SG, Grimm NB, Kitchell JF. 1992. Global change and freshwater ecosystems. Annu Rev Ecol Syst 23: 119-139.

33) Christensen AM, Faaborg-Anderson S, Ingerslev F, Baun A. 2007. Mixture and single-substance toxicity of selective serotonin reuptake inhibitors toward algae and crustaceans. Environ Toxicol Chem 26: 85-91.

34) Cleuvers M. 2003. Aquatic ecotoxicity of pharmaceuticals including the assessment of combination effects. Toxicology letters 142: 185-194.

35) Connell DW, Miller GJ. 1984. Chemistry and ecotoxicology of pollution. John Wily & Sons. New York. p. 443.

36) Crumpton WG, Isenhart TM. 1988. Diurnal patterns of ammonium and un-ionized ammonia in streams receiving secondary treatment effluent. B Environ Contam Tox 40: 539-544.

37) Dafni, Z., Shilo, M., 1966. The cytotoxic principle of the phytoflagellate *Prymnesium parvum*. J Cell Biol 28, 461-471.

38) Dietl M, Palacios JM. 1988. Autoradiographic studies of serotonin receptors. In The Serotonin Receptors (ed. E. Sanders-Bush), pp. 89-138. Clifton, NJ: The Humana Press.

39) Dodds WK. 2003. Misuse of inorganic N and soluble reactive P concentrations to indicate nutrient status of surface waters. J N Am Benthol Soc 22: 171-181.

40) Doney SC, Mahowald N, Lima I, Freely RA, Mackenzie FT, Lamarque JF, Rasch PJ. 2007. Impact of anthropogenic atmospheric nitrogen and sulfur deposition on ocean acidification and the inorganic carbon system. Proceeding of the National Academy of Science. 104: 14580-14585.

41) Døving KB. 1991. Assessment of animal behaviour as a method to indicate environmental toxicity. Comp Biochem Physiol C 100: 247-252.

42) Driscoll CT, Yatsko CP, Unangst FJ. 1987. Longitudinal and temporal trends in the water chemistry of the north branch of the Moose River. Biogeochemistry. 3: 37-61.

43) Dzialowski EM, Turner PK, Brooks BW. 2006. Physiological and reproductive effects of Beta adrenergic receptor antagonists on *Daphnia magna*. Arch Environ Contamin Toxicol 50, 503-510.

44) EMA. European Agency for the Evaluation of Medical Products 2001. Draft CPMP discussion paper on environmental risk assessment of non-genetically modified organism (NON-GMO) containing medicinal products for human use. CPMP/SWP/4447/00.

45) Endo T, Onozawa M. 1987. Effects of pH and temperature on the uptake of oxolinic acid in goldfish. Nippon Suisan Gakkaishi 53: 551-555.

46) Erickson RJ, McKim JM, Lien GJ, Hoffman AD, Batterman SL. 2006. Uptake and elimination of ionizable organic chemicals at fish gills: I. Model formulation, parameterization, and behavior. Environ Toxicol Chem 25: 1512-1521.

47) Escher BI, Hermens JLM. 2004. Internal exposure: Linking bioavailability to effects. Environ Sci Technol 38 455-462.

48) Escher, B.I., Schwarzenbach, R.P., 2000. Evaluation of liposome-water partitioning of organic acids and bases. 1. Development of a sorption model. Environ Sci Technol 32 3954-3961.

49) Farrington JW. 1991. Biogeochemical processes governing exposure and uptake of organic pollutant compounds in aquatic organisms. Environ Health Persp 90: 75-84.

50) FDA (Food and Drug Administration). 1998. Guidance for industry environmental assessment of human drugs and biologics applications. Center for Drug Evaluation and Research and Center for Biologics Evaluation and Research. www.fda.gov/cder/guidance/index.htm

51) Fent K, Looser PW. 1995. Bioaccumulation and bioavailability of tributyltin chloride: Influence of pH and humic acid. Wat Res 29 1631-1637.

52) Findlay S. 1995. Importance of surface-subsurface exchange in stream ecosystems: The hyporheic zone. Limnol Oceanogr 40: 159-164.

53) Fisher SW, Hwang H, Atanasoff M, Landru PF. 1999. Lethal body residues for pentachlorophenol in zebra mussels (*Dreissena polymorpha*) under varying conditions of temperature and pH. Ecotoxl Environ Saf 43: 274-283.

54) Fistarol, G.O., Legrand, C., Graneli, E., 2003. Allelopathic effect of *Prymnesium parvum* on a natural plankton community. Mar Ecol Prog Ser 255: 115-125.

55) Fitzhugh RD, Furman T, Webb JR, Cosby BJ, Driscoll CT. 1999. Longitudinal and seasonal patterns of stream acidity in a headwater catchment on the Appalachian Plateau, West Virginia, USA. Biogeochemistry 47: 39-62.

56) Fitzsimmons PN, Fernadez JD, Hoffman AD, Butterworth BC, Nichols JW.. 2001. Branchial elimination of superhydrophobic organic compounds by rainbow trout (*Oncorhynchus mykiss*). Aquatic Toxicol 55: 23-34.

57) Frazer A. Serotonergic and noradrenergic reuptake inhibitors: predictions of clinical effects form in vitro potencies. J Clin Psychiatry 62: 16-23.

58) Fu, W., Franco, A., Trapp, S., 2009. Methods for estimating the bioconcentration factor of ionizable organic chemicals. Environ Toxicol Chem 28, 1372-1379.

59) Fulton BA, Brain RA, Usenko S, Back JA, King RS, Brooks BW. 2009. Influence of N and P concentrations and ratios on Lemna gibba growth responses to triclosan in laboratory and stream mesocosm experiments. Environ Toxicol Chem 28: 2610-2621.

60) Fung IY, Doney SC, Lindsay K, John J. 2005. Evolution of carbon sinks in a changing climate. Proceeding of the National Academy of Science. 102: 11201-11206.

61) Garban B, Ollivon D, Jairy A, Carru AM, Chesterikoff A. 1999. The role of phytoplankton in pollutant transfer processes in rivers. Example of River Marne (France). Biogeochemistry. 44: 1-27.

62) Gerlai R, Lahav M, Guo S, Rosenthal A. 2000. Drinks like a fish: zebra fish (*Danio rerio*) as a behavior genetic model to study alcohol effects. Pharmacology Biochemistry and Behavior 67: 773-782.

63) Gernhardt A. 2007. Aquatic behavioral ecotoxicology- Prospects and limitations. Hum Ecol Risk Assess 13: 481-491.

64) Gleick P (Ed.) 2003 a. The World's Water- The biennial report of freshwater resources 2002-2003. Island Press, Washington DC. 334p.

65) Gleick PH. 2003 b. Global freshwater resources: Soft-path solutions for the 21st century. Science 302: 1524-1528.

66) Gould GG, Brooks BW, Frazer A. 2007. [3H] citaplopram binding to serotonin transporter sites in minnow brains. Basic Clin Pharmacol 101: 203-210.

67) Granéli E, Hansen PJ. 2006. Allelopathy in harmful algae: a mechanism to compete for resources? In: Granéli, E., Turner, J.T. (Eds.), Ecology of Harmful Algae, Ecological Studies, Springer-Verlag, Berlin Heidelberg, Germany, 189-201.

68) Graneli E, Johansson N. 2003. Effects of the toxic haptophyte *Prymnesium parvum* on the survival and feeding of a ciliate: the influence of different nutrient conditions. Mar Ecol Prog Ser 254: 49-56.

69) Green JM, Hale T. 2005. Increasing and decreasing pH to enhance the biological activity of nicosulfuron. Weed technology 19: 468-475.

70) Grimm NB, Grove JM, Pickett ST, Redman CL. 2004. Integrated approaches to long-term studies of urban ecological systems. Bioscience 50: 571-584.

71) Grippo MA, Heath AG. 2003. The effect of mercury on the feeding behavior of fathead minnows (*Pimephales promelas*). Ecotoxicology and Environmental Safety 55: 187-198.

72) Grover, J.P., Baker, J.W., Urena-Boeck, F., Brooks B.W., Errera, R.M., Roelke, D.L., Kiesling, R.L., 2007. Laboratory tests of ammonium and barley straw extract as agents to suppress abundance of the harmful alga *Prymnesium parvum* and its toxicity to fish. Wat Res 41: 2503-2512.

73) Guasch H, Armengol J, Marti E, Sabater S. 1998. Diurnal variation in dissolved oxygen and carbon dioxide in two low-order streams. Wat Res 32: 1067-1074.

74) Guillard, R.L., 1975. Culture of phytoplankton for feeding marine invertebrates. In: Smith, W.L., Charley, M.H., (Eds.), Culture of marine invertebrate animals. Proceedings - 1st Conference on Culture of Marine Invertebrate Animals Greenport, 29-60.

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

76) Hallegraeff, G.M. 2003. Harmful algal blooms: a global overview. In: Hallegraeff, G.M., Anderson, D.M., Cembella, A.D, (Eds.). Manual on harmful marine microalgae. Monographs on oceanographic methodology, 11. 25-49.

77) Halstead BG, Tash JC. 1982. Unusual diel pHs in water as related to aquatic vegetation. Hydrobiologia 96: 217-224.

78) Heathwaite AI. 2010. Multiple stressors on water availability at global and catchment scales: understanding human impact on nutrient cycles to protect water quality and water availability in the long term. Freshwater Biol 55: 241-257.

79) Herberer T. 2002. Occurrence, fate, and removal of pharmaceutical residuals in the aquatic environment: A review of recent research data. Toxicol Lett 131: 5-17.

80) Hernandez MA, Rathinavelu A. 2006. Basic pharmacology-Understanding drug actions and reactions. Boca Raton, FL. Taylor &Francis Group. 392 pp.

81) Hill T, Neal C. 1997. Spatial and temporal variation in pH, alkalinity and conductivity in surface runoff and groundwater from the Upper River Severn catchment. Hydrol Earth Syst Sc 1: 697-715.

82) Holmes A,Yang RJ, MurphyDL, Crawley JN. 2003. Evaluation of antidepressant-related behavioral responses in mice lacking the serotonin Transporter. Neuropsychopharmacol 27: 914-923.

83) Howland RJM, Tappin AD, Uncles RJ, Plummer DH, Bloomer NJ. 2000. Distribution and seasonal variability of pH and alkalinity in the Tweed Estuary, UK. Sci Total Environ 251: 125-138.

84) Huggett DB, Cook JC, Ericson JF, Williams RT. 2003. A theoretical model for utilizing mammalian pharmacology and safety data to prioritize potential impacts of human pharmaceuticals to fish. HERA 9: 1789-1799.

85) Hunt RJ, Krabbenhoft DP, Anderson MP. 1997. Hydrogeochemical heterogeneity in natural and constructed wetlands. Biogeochemistry. 39: 271-293.

86) Hurd B, Leary N, Jones R, Smith J. 1999. Relative regional vulnerability of water resources to climate change. J Am Water Resour As 35: 1399-1409.

87) Igarashi T, Aritake S, Yasumoto T. 1998. Biological activities of pyrmnesin-2 isolated from a red tie alga *Prymnesium parvum*. Nat Toxins 6, 35-41.

88) Igarashi T, Satake M, Yasumoto T. 1996. Prymnesin-2: a potent ichthyotoxic and hemolytic glycoside isolated from the red tide alga *Prymnesium parvum*. J Am Chem Soc 118, 479-480.

89) Igarashi T, Satake M, Yasumoto T. 1999. Structural and partial stereochemical assignments from prymnesin-1 and prymnesin-2: Potent hemolytic and ichthyotoxic glycosides isolated from the red tide alga *Prymnesium parvum*. J Am Chem Soc 121, 8499-8511.

90) James, T.L., De La Cruz, A., 1989. *Prymnesium parvum* Carter (Chrysophyceae) as a suspect of mass mortalities of fish and shellfish communities in western Texas. Texas J Sci 41: 429-430.

91) Jefferies RL. 2000. Allochthonous inputs: integrating population changes and food-web dynamics. TREE. 15: 19-22.

92) Johansson N, Granéli E. 1999. Influence of different nutrient conditions on cell density, chemical composition and toxicity of *Prymnesium parvum* (Haptophyta) in semi-continuous cultures. J Exp Mar Biol Ecol 239: 243-258.

93) Jollow DJ, Brodie BB. 1972. Mechanisms of drug absorption and drug solution. Pharmacology 8: 21-32.

94) Jones CA, Nimick DA, McCleskey RB. 2004. Relative effect of temperature and pH on diel cycling of dissolved trace elements in Prickly Pear Creek, Montana. Water Air Soil Poll 153: 95-113.

95) Kasumyan AO. 2001. Effects of chemical pollutants on foraging behavior and sensitivity of fish to food stimuli. J Ichthyol 41: 76-87.

96) Kent R, Belitz K, Burton CA. 2005. Algal productivity and nitrate assimilation in an effluent dominated concrete lined stream. J Am Water Resour As 41: 1109-1128.

97) Kim J, Park J, Kin PG, Lee C, Choi K, Choi K. 2010. Implications of global environmental changes on chemical toxicity-effect of water temperature, pH, and ultraviolet B irradiation on acute toxicity of several pharmaceuticals in *Daphnia magna*. Ecotoxicology. 19: 662-669.

98) Kim, Y.S., Padilla, G.M., 1977. Hemolytically active components from *P. parvum* and *G. breve* toxins. Life Sci. 21, 1287-1292.

99) King DL. 1970. The role of carbon in eutrophication. Journal water pollution control federation. 42: 2035-2051.

100) King RS, Taylor JT, Back JA, Fulton BA, Brooks BW. 2009. Linking observational and experimental approaches for the development of regional nutrient criteria for wadeable streams. Final Report. #CP-966137-01. United States Environmental Protection Agency Region 6.

101) Kishino T, Kobayashi K. 1995. Relation between toxicity and accumulation of chlorophenols at various pH, and their absorption mechanism in fish. Wat Res 29: 431-442.

102) Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A nation reconnaissance. Environ Sci Technol 36: 1202-1211.

103) Kreke N, Dietrich DR. 2008. Physiological endpoints for potential SSRI interactions in fish. Cri Rev Toxicol 37: 215-247.

104) Kummer K (Ed). 2004. Pharmaceuticals in the environment- Sources, fate, effects, and risks. Springer, Verlag, Berlin.

105) Kuwabara JS. 1992. Associations between benthic flora and diel changes in dissolved arsenic, phosphorus, and related physico-chemical parameters. J N Am Benthol Soc 11: 218-228.

106) Kwon Y. 2001. Handbook of essential pharmacokinetics, pharmacodynamics, and drug metabolism for industrial scientists. Kluwer Academic / Plenum Publishers, New York, NY, USA.

107) Landrum P, Harkey GA, Kukkonen. 1996. The significance of bioconcentration and bioaccumulation- Chapter 4 in Ecotoxicology-A Hierarchical Treatment. Editors Newman and Jagoe. Lewis Publishers, Boca Raton.

108) Lapiz-Bluhm MD, Bondi CO, Doyen J, Rodriguez GA, Bedard-Arana T, Morilak DA. 2008. Behavioural assays to model cognitive and affective dimensions of depression and anxiety in rats. Journal of Neuroendocrinology 20: 1115-1137.

109) Larrson DGJ, Adlofsson-Erici M, Parkkonen J, Pettersson M, Berg AH, Olsson PE Forlin L. 1999. Ethinyloestradiol- an undesired fish contraceptive? Aquat Toxicol 45: 91-97.

110) Larsen, A., Bryant, S., 1998. Growth rate and toxicity of *Prymnesium parvum* and *Prymnesium patelliferum* (Haptophyta) in response to changes in salinity, light and temperature. Sarsia 83:409-418.

111) Larsen, A., Eikrem, W., Paasche, E., 1993. Growth and toxicity in *Prymnesium patelliferum* (Prymnesiophyceae) isolated from Norweigian Waters. Can. J. Botany 71:1357-1362.

112) Levin ED, Bencan Z, Cerutti DT. 2007. Anxiolytic effects of nicotine in zebrafish. Physiology and Behavior 90: 54-58.

113) Liebert, F., Deerns, W.M., 1920. Onderzoek naar de oorzak van een Vischsterfte in den Polder Workumer Nieuwland, nabij Workum. Verhandungen en Rapporten uitgegeven door Rijkinstituten voor Visscherijonderzoek 1, pp. 81-93.

114) Lindholm, T., Ohman, P., Kurki-Helasmo, K. Kincaid, B., Meriluoto, J., 1999. Toxic algae and fish mortality in a brackish-water lake in Aland, SW Finland. Hydrobiologia 397, 109-120.

115) Little EE, Finger S.E. 1990. Swimming behavior as an indicator of sublethal toxicity in fish. Environ Toxicol Chem 9: 13-19.

116) Little EE, Flerov BA, Ruzhinskaya NN. 1985. Behavioral approaches in aquatic toxicity investigations: a review. In: Mehrle, P.M., Gray, R.H., Kendall, R.L. (Eds.), Toxic Substances in the Aquatic Environment: An International Aspect. American Fisheries Society, Water Quality Section, Bethesda, MD, pp. 72-98.

117) Maberly SC. 1996. Diel, episodic and seasonal changes in pH and concentrations of inorganic carbon in a productive lake. Freshwater Biol 35: 579-598.

118) MacQueen G, Born L, Steiner M. 2001. The selective serotonin reuptake inhibitor sertraline: Its profile and use in psychiatric disorders. CNS Drug Rev7: 1-24.

119) Maltby L. 1999. Studying stress: The importance of organism-level responses. Ecol Appl 9: 380-385.

120) Marcucella H., Abramson CI. 1978. Behavioral toxicology and teleost fish. In: Mostofsky, D.J. (Ed.), The Behavior of Fish and Other Aquatic Animals. Academic Press, London, UK, pp. 33-77.

121) Marti E, Aumatell J, Gode L, Poch M, Sabater F. 2004. Nutrient retention efficiency in streams receiving inputs from wastewater treatment plants. J Environ Qual 33: 285-293.

122) Maximino C, de Brito TM, Colmanetti R, Pontes AAA, de Castro HM, De Lacerda RIT, Gouveai A. 2010. Parametric analyses of anxiety in zebrafish scototaxis. Behavioral Brain Research 210: 1-7.

123) McLaughlin, J. 1958. Euryhaline chrysomonads: nutrition and toxigensis in *Prymnesium parvum,* with notes on *Isochrysis galbana* and *Monochrysis lutheri*. J.Protozool. 5, 75-81.

124) Meldahl, A.S., Edvardsen, B., Fonnum, F., 1994. Toxicity of four potentially ichhyotoxic marine phytoflagellates determined by four different test methods. J. Toxicol. Env. Health. 42, 289-301.

125) Minagh E, Heman R, O'Rourke K, Lyng FM, Davoren, M. 2009. Aquatic ecotoxicity of the selective serotonin reuptake inhibitor sertraline hydrochloride n a battery of freshwater test species. Ecotox Environ Safe 72: 434-440.

126) Morris JM, Nimick DA, Farag AM, Meyer JS. 2005. Does biofilm contribute to diel cycling of Zn in High Ore Creek, Montana? Biogeochemistry. 76: 233-259.

127) Moss B. 2010. Climate change, nutrient pollution and the bargain of Dr Faustus. Freshwater Biol 55: 175-187.

128) Murata, M., Yasumoto, T., 2000. The structure elucidation and biological activities of high molecular weight algal toxins: maitotoxin, prymnesins, and zooxanthellatoxins. Nat. Prod. Rep. 17, 293-314.

129) Murdock SH, Hoque N, Michael M, While S, Pecotte B. 1997. The Texas challenge: Population change and the future of Texas. Texas A&M Press, College Station, TX. 233 pp.

130) Nakamura Y, Yamamoto H, Sekizawa J, Kondo T, Hirai N, Tatarako 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.

131) Nimick DA, Harper DD, Farag AM, Cleasby TE. 2007. Influence of in-stream diel concentration cycles of dissolved trace metals on acute toxicity to one-year old cutthroat trout (*Oncorhynchus clarki lewisi*). Environ Toxicol Chem 26: 2667-2678.

132) Odum HT. 1956. Primary production in flowing waters. Limnol Oceanog 1, 102-117.

133) Oki T, Kanae S. 2006. Global hydrological cycles and world water resources. Science 313: 1068-1072.

134) Olli, K., Trunov, K., 2007. Self-toxicity of *Prymnesium parvum* (Prymnesiophyceae). Phycologia 46, 109-112.

135) Omernik JM, Bailey RG. 1997. Distinguishing between watersheds and ecoregions. J Am Water Resour As 33: 935-949.

136) Omernik JM, Griffith GE. 1991. Ecological regions versus hydrologic units: Framework for managing water quality. J Soil Water Conserv 46: 334-340.

137) Orvos DR, Versteeg DJ, Inauen J, Capdevielle M, Rothenstein A, Cunningham V. 2002. Aquatic toxicity of triclosan. Environ Toxicol Chem 21: 1338-1349.

138) Owen MJ, Morgan WN, Plott SJ, Nemeroff CB. Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. J Pharmacol Exp Ther 283: 1305-1322.

139) Padilla, G.M., 1970. Growth and toxigenesis of the chrysomonad *Prymnesium parvum* as a function of salinity. J. Protozool. 17, 546-462.

140) Pearl, H.W., 1988. Nuisance phytoplankton blooms in coastal, estuarine, and inland waters. Limnol. Oceanogr. 33, 823-847.

141) Perreault HAN, Semsar K, Godwin J. 2003. Fluoxetine treatment decreases territorial aggression in coral reef fish. Physiology and Behavior 79: 719-724.

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

143) Pratt CW, Voet D, Voet JG. 2008. Fundamentals of Biochemistry: Life at the molecular level. John Wiley & Sons, New York. pp. 1098.

144) Ramirez AJ, Brain RA, O'Donnel J, Usenko S, Mottaleb MA, Perez-Hurtado P, Dobbins LL, Pitts J, Snyder B, Wathen J, Stahl L, Brooks BW, Chambliss CK. 2009. Occurrence of pharmaceuticals and personal care products in fish: Results of a national pilot study in the U.S. Environ Toxicol Chem 28: 2587-2597.

145) Rand GM, Petrocelli SR. 1985. Introduction. In: Rand, G.M., Petrocelli, S.R. (Eds.), Fundamentals of Aquatic Toxicology: Methods and Applications. Hemisphere, London, UK, pp. 1-28.

146) Rascher CM, Driscoll CT, Peters NE. 1987. Concentration and flux of solutes from snow and forest floor during snowmelt in the West-Central Adirondack region of New York. Biogeochemistry 3: 209-224.

147) Raven, J.A., 1970. Exogenous inorganic carbon sources in plant photosynthesis. Biol. Rev. 45, 167-221.

148) Rebsdorf A, Thyssen N, Erlandsen M. 1991. Regional and temporal variation in pH, alkalinity, and carbon dioxide in Danish streams, related to soil type and land use. Freshwater Ecology 25: 419-435.

149) Reich, K., Aschner, M., 1947. Mass development and control of the phytoflagellate *Prymnesium parvum* in fish ponds in Palestine. J. Bot. 4, 14-23.

150) Robinson RB, Roby JC. 2006. Concentration-duration-frequency curves for pH in a stream in the Great Smoky Mountains. J Envir Engrg 132: 1600-1605.

151) Roelke, D.L., Errera, R., Kiesling, R., Brooks, B.W., Grover, J.P., Schwierzke, L., Ureña-Boeck, Baker F, Pinckney J, J.L., 2007. Effects of nutrient enrichment on *Prymnesium parvum* population dynamics and toxicity: Results from field experiments, Lake Possum Kingdom, USA. Aquat. Microb. Ecol. 46, 125-140.

152) Roelke DL, Schwierzke L, Brooks BW, Grover JP, Errera RM, Valenti TW, Pinckney JL. 2010. Factors influencing Prymnesium parvum population dynamics during bloom initiation: Results from In-lake mesocosm experiments. J Am Water Resour Assoc 46: 76-91.

153) Rosetta, C.H., McManus, G.B., 2003. Feeding by ciliates on two harmful algal bloom species, *Prymnesium parvum* and *Prorocentrum minimum*. Harmful Algae 2, 109-126.

154) RxList. http://www.rxlist.com/script/main/hp.asp.

155) Sackerman J, Donegan JJ, Cunningham CS, Nguyen NN, Lawless K, Long A, Benno RH, Gould GG. 2010. Zebrafish behavior in novel environments: Effects of acute exposure to anxiolytic compounds and choice of Danio rerio Line. Int J Comp Psychol 23: 43-61.

156) Sand-Jensen K, Pedersen MF, Nielsen SL. 1992. Photosynthetic use of inorganic carbon among primary and secondary plants in streams. Freshwater biology. 27: 283-293.

157) Sanderson H, Johnson DJ, Reitsma R, Brain RA, Wilson CJ, Solomon KR. 2004. Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. Regulatory toxicology and pharmacology 39: 158-183.

158) Sandheinrich MB, Atchison GJ. 1990. Sublethal toxicant effects on fish foraging behavior: Empirical vs. mechanistic approaches. Environ Toxicol Chem 9: 107-119.

159) Santschi PH. 1988. Factors controlling the biogeochemical cycles of trace elements in fresh and coastal marine waters as revealed by artificial radioisotopes. Limnol Oceanogr 33: 848-866.

160) Sarrikoski J, Lindstrom R, Tyynela M, Viluksela M. 1986. Factors affecting the absorption of phenolics and carboxylic acid in the guppy (*Poecilia reticulate*). Ecotoxicol Environ Saf 11: 158-173.

161) Sarrikoski J, Vilukesela M. 1981. Influence of pH on the toxicity of substituted phenols in fish. Arch Environ Contam Toxicol 10: 747-753.

162) Scheffel U, Kim S, Cline EJ, Kuhar MJ. 1994. Occupancy of the serotonin transporter by fluoxetine, paroxetine, and sertraline: In vivo studies with [125I]RTI-55. Synapse 16: 263-268.

163) Scherer E. 1992. Behavioural responses as indicators of environmental alterations: approaches, results, developments. J Appl Ichthyol 8:122-131.

164) Schindler DW, Dillon PJ, Schreier H. 2006. A review of anthropogenic sources of nitrogen and their effects on Canadian aquatic ecosystems. Biogeochemistry. 79: 25-44.

165) Schug KA, Skingel TS, Spencer SE, Serrano C, Le CQ, Schug CA, Valenti TW, Brooks BW, Mydlarz LD, Grover JP. 2010. Hemolysis, fish mortality and LC-ESI-MS of cultured crude and fractionated golden alga (*Prymnesium parvum*). J Am Water Resour Assoc 46: 34-44.

166) Schultz M, Furlong ET. 2008. Trace analysis of antidepressant pharmaceuticals and their select degradates in aquatic matrixes by LC/ESI/MS/MS. Anal Chem 80: 1756-1762.

167) Schwierzke L, Roelke DL, Brooks BW, Grover JP, Valenti TW, Lahousse M, Pinckney JL. 2010. *Prymnesium parvum* Population Dynamics During Bloom Development: A Role Assessment of Grazers and Virus. J Am Water Resour Assoc 46: 63-75.

168) SciFinder Scholar [Columbus, Ohio : American Chemical Society, Chemical Abstracts Service]

169) Scott GR, Sloman KA. 2004. The effects of environmental pollutants on complex fish behavior: integrating behavioral and physiological indicators of toxicity. Aquatic Toxicology 68: 369-392.

170) Serra EL, Medalha CC, Mattioli R. 1999. Natural preference of zebrafish (*Danio rerio*) for a dark environment. Braz J Med Biol Res 32: 1551-1553.

171) Shilo, M., Aschner, M., 1953. Factors governing the toxicity of cultures containing the phytoflagellate *Prymnesium parvum* Carter. J. Gen. Microbiol. 8, 333-343.

172) Simon EW, Beevers H. 1951. The quantitative relationship between pH and the activity of weak acids and bases in biological experiments. Science 114: 124-126.

173) Simon EW, Beevers H. 1952. The effect of pH on the biological activities of weak acids and base: II. Other relationships between pH and activity. New Phytolt 51: 191-197.

174) Skovgaard A., Hansen, P.J., 2003. Food uptake in the harmful alga *Prymnesium parvum* mediated by excreted toxins. Limnol. Oceanogr. 48, 1161-1166.

175) Smakhtin VU. 2001. Low flow hydrology: a review. J Hydrol 240:147-186.

176) Smith DF. 1999. Neuroimaging of serotonin uptake sites and antidepressant binding sites in the thalamus of humans and 'higher' animals. Eur Neuropsychpharmacol 9: 537-544.

177) Smith VH, Tilman GD, Nekola JC. 1999. Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. Environmental pollution. 100: 179-196.

178) Sprouse J, Clarke T, Reynolds L, Heym J, Rollema H. 1996. Comparison of the effects of sertraline and its metabolite desmethylsertraline on blockade of central 5-HT reuptake In Vivo. Neuropsychopharmacol 14: 225-231.

179) Stanley JK, Ramirez AJ, Chambliss CK, Brooks BW. 2007. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. Chemosphere 69: 6-16.

180) Stephenson GL, Koper N, Atkinson GF, Solomon KR, Scroggins RP. 2000. Use of nonlinear regression techniques for describing concentration-response relationships of plant species exposed to contaminated site soils. Environ Toxicol Chem 19: 2968-2981.

181) Stoecker, D., Tillmann, U., Granéli, E., 2006. Phagotrophy in harmful algae. In: Granéli, E., Turner, J.T. (Eds.), Ecology of Harmful Algae, Ecological Studies, Springer-Verlag, Berlin Heidelberg, Germany, 177-187.

182) Sun G, McNulty SG, Moore Myers HA, Cohen EC. 2008. Impacts of multiple stresses on water demand and supply across the southeastern United States. J Am Water Resour As 44: 1441-1457.

183) Suter GW, Barnthouse LW, Bartell SM, Cormier SM, Mackay D, Mackay N, Norton SB. 2007. Ecological risk assessment- Second edition. CRC Press. Taylor & Francis Group. Boca Raton, FL, USA.

184) Suter GW, Efroymson RA, Sample BE, Jones DS. 2000. Ecological risk assessment for contaminated sites. Lewis Publishers. Boca Raton, FL, USA.

185) Talling JF. 1976. The depletion of carbon dioxide from lake water by phytoplankton. Journal of ecology. 64: 79-121.

186) Tank SE, Lesack LFW, McQueen DJ. 2009. Elevated pH regulates bacterial carbon cycling in lakes with high photosynthetic activity. Ecology 90: 1910-1922.

187) Texas Commission of Environmental Quality. 2007. DRAFT: 2006 Guidance for Assessing and Reporting Surface Water Quality in Texas (June 27, 2007). Monitoring Operations, Surface Water Quality Monitoring Program, Austin, TX.

188) Texas Commission of Environmental Quality. 2003. Surface Water Quality Monitoring Procedures Volume 1: Physical and Chemical Monitoring Methods for Water, Sediment and Tissue. Publication NO. RG-415, December 2003, Austin, TX.

189) Texas Commission on Environmental Quality. 2008. Surface Water Quality Monitoring Information System. Austin, TX, USA.

190) Tillmann, U., 2003. Kill and eat your predator: a winning strategy of the planktonic flagellate *Prymnesium parvum*. Aquat. Microb. Ecol. 32, 73-84.

191) Toze S. 2006a. Water reuse and health risks- real vs. perceived. Desalination. 187: 41-51.

192) Toze S. 2006b. Reuse of effluent water- benefits and risks. Agricultural water management. 80: 147-159.

193) U.S. Environmental Protection Agency. 1985. Ambient water quality criteria for ammonia- 1984. EPA-440-5-85-001.Office of regulations and standards, Criteria and standards division, Washington, DC.

194) U.S. Environmental Protection Agency. 1986. Ambient water quality criteria for pentachlorophenol- 1986. EPA-440-5-86-009. Office of Regulations and Standards, Criteria and Standards Division, Washington, DC.

195) United States Environmental Protection Agency. 1991. Methods for aquatic toxicity identification evaluation: Phase 1 toxicity characterization procedures-Second edition. EPA-600-6-91-003. Office of Research and Development, Washington, DC.

196) U.S. Environmental Protection Agency. 1994. 10-day Chronic Toxicity Test using *Daphnia magna* or *Daphnia pulex*. SOP #2028. Environmental Response Team. Compendium of ERT Standard Operating Protocols. Office of Solid Waste and Emergency Response, Edison, NJ.

197) U.S. Environmental Protection Agency. 1996. 1995 Updates: Water quality criteria documents for the protection of aquatic life in ambient water. EPA-820-B-96-001. Office of Water, Washington, DC.

198) U.S. Environmental Protection Agency. 1999. 1999 update of ambient water quality criteria for ammonia. EPA-822-R-99-014. Office of Water, Washington, DC.

199) U.S. Environmental Protection Agency, 2000. Bioaccumulation testing and interpretation for the purpose of sediment quality assessment: Status and needs. EPA-823-R-00-001. Office of Water and Office of Solid Wastes, Washington, DC.

200) U.S. Environmental Protection Agency. 2002. Methods for measuring acute toxicity of effluents and receiving waters to freshwater and marine organisms EPA-821-R-02-012. Office of Research and Development, Washington, DC.

201) U.S. Environmental Protection Agency. 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.

202) U. S. Environmental Protection Agency. 2009. Draft 2009 Update aquatic life ambient water quality criteria for ammonia-Freshwater. EPA-822-D-09-001. Office of Water, Washington, DC.

203) U.S. Geological Survey, variously dated, National field manual for the collection of water-quality data: U.S. Geological Survey Techniques of Water-Resources Investigations, book 9, chaps. A1-A9, available online at http://pubs.water.usgs.gov/twri9A.

204) Ulitzur S, Shilo M. 1966. Mode of action of *Prymnesium parvum* ichthyotoxin. J. Protozool. 13: 332-336.

205) Uronen P, Kuuppo P, Legrand C, Tamminen T. 2007. Allelopathic effects of toxic haptophyte *Prymnesium parvum* lead to release of dissolved organic carbon and increase in bacterial biomass. Microbial Ecol 54: 183-193.

206) Valenti TW, Perez-Hurtado P, Chambliss CK, Brooks BW. 2010a. Aquatic toxicity of sertraline to *Pimephales promelas* at environmentally relevant surface water pH. Environ Toxicol Chem 28: 2685-2694.

207) Valenti TW, SV James, MJ Lahousse, KA Schug, DL Roelke, JP Grover, BW Brooks. 2010b. A mechanistic explanation for pH-dependent ambient aquatic toxicity of *Prymnesium parvum* carter. Toxicon 55:990-998.

208) Van der Oost R, Beyer J, Vermeulen NPE. 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. Environ Toxicol Phar 13: 57-149.

209) Van Wezel AP. 1998. Chemical and biological aspects of ecotoxicological risk assessment of ionizable and neutral organic compounds in fresh and marine waters: a review. Environ Rev 6: 123-137.

210) Veith, G.D., Kosian, P., 1983. Estimating bioconcentration potential from octanol/water partition coefficients. In: Mackay, D., Paterson, S., Eisenreich, S.J., Simons, M.S. (Eds). Physical behavior of PCBs in the Great Lakes, Ann Arbor Sciences Publishers, Ann Arbor, MI. pp. 269-282.

211) Vieno NM, Tuhkanen T, Kronberg L. 2005. Seasonal variation in the occurrence of pharmaceuticals in effluents from a sewage treatment plant and in the recipient water. Environ Sci Technol 39: 8220-8226.

212) Vorosmarty CJ, Sahagian D. 2000. Anthropogenic disturbance of the terrestrial water cycle. Bioscience. 50: 753-765.

213) Walsh CJ, Roy AH, Feminella JW, Cottingham PD, Groffman PM, Morgan RP. 2005. The urban stream syndrome: current knowledge and the search for a cure. J N Am Benthol Soc 24: 706-723.

214) Webb SF. 2001. A data-based perspective on the environment risk assessment of human pharmaceuticals I- collation of available ecotoxicity data. In Kummerer K (Ed.), Pharmaceuticals in the Environment. Springer, NY, NY, USA.

215) Weis JS, Samson J, Zhou T, Skurnick J, Weis P. 2003. Evaluating prey capture by larval mummichogs (*Fundulus heteroclitus*) as a potential biomarker for contaminants. Marine Environmental Research 55: 27-38.

216) Wenning RJ, Finger SE, Guilhermino L, Helm RC, Hooper MJ, Menzie CA, Munns WR, Rombke J, Stahl RG. 2010. Global climate change and environmental contaminants: A SETAC call for research. IEAM 6: 197-198.

217) Wheeler PA, Hellebust JA. 1981. Uptake and concentration of alkylamines by marine diatoms. Plant Physiol 67: 367-372.

218) Whitley LS. 1968. The resistance of tubicid worms to three common pollutants. Hydrobiologia 32: 193-205.

219) Williams RT, Cook JC. 2007. Exposure to pharmaceuticals present in the environment. Drug Inf J 41: 133-141.

220) Winters JG, Duthie HC. 2000. Epilithic diatoms as indicators of stream total N and total P concentrations. J N Am Benthol Soc 19: 32-49.

221) Yariv J, Hestrin S. 1961. Toxicity of the extracellular phase of *Prymnesium parvum* cultures. J Gen Microbiol 24: 165-175.

222) Zingone A, Enevoldsen H.O. 2000. The diversity of harmful algal blooms: a challenge for science and management. Ocean Coast Manage 43: 725-748.