ABSTRACT

Individual and Interactive Influences of Low Dissolved Oxygen and Calcium Channel Blockers in Inland Aquatic Systems

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Understanding and managing influences of multiple stressors represents a major water quality challenge, particularly in urbanizing regions. Because aquatic hazard assessments with chemical and nonchemical stressors can identify the global trends in occurrence and hazards of stressors for the protection of aquatic life, probabilistic aquatic hazard assessments were performed to examine whether water quality guidelines for dissolved oxygen (DO) are protective of aquatic life in inland waters. My analyses indicate that adverse effects of low DO to freshwater invertebrates and fish have been underestimated in inland waters. Additional low DO threshold information, including sublethal toxicity, for additional species such as warm water fish and mollusks across multiple life history stages is necessary to support environmental assessment and management of ecosystem protection goals. Similar techniques were used to examine the occurrence of calcium channel blockers (CCBs), a common class of vasodilators and cardio suppressants, in environmental matrices, and to predict hazards to non-target aquatic organisms in multiple environmental matrices and geographic regions. Whereas

environmental occurrence of CCBs in freshwater and effluent have predominantly been reported from North America and Europe, data is lacking from many developing regions around the world and hazards and risks of CCBs to non-target biota remains poorly understood. Therapeutic hazard values (THVs), a comparative pharmacology and toxicology approach, employed during probabilistic hazard assessments with environmental exposure distributions revealed that amlodipine and verapamil in effluent and freshwater exceeded THVs 28% of the time. Diltiazem exceeded minimum human therapeutic thresholds based on observations in fish plasma from the field ~18% of the time in surface waters. This approach demonstrated the utility of global assessments to identify specific CCBs and geographic regions where environmental assessments appear necessary. Subsequently, to understand adverse effects of individual and multiple stressors influencing cardiac function (DO, diltiazem, or DO x diltiazem), toxicity studies were performed using a comparative toxicology and pharmacology approach in fathead minnows (*Pimephales promelas*) across larval and adult life stages. DO x diltiazem toxicity studies with larval fish revealed acute lethality increased with decreasing DO levels and altered burst swimming behavior at DO water quality criteria levels deemed protective of aquatic life. In adult fathead minnows, low DO (3.0 mg DO/L) increased uptake of diltiazem and altered physiological responses (e.g., hematocrit, plasma lactate) at and above human therapeutic plasma levels. Failing to consider low DO influences with chemical exposure during toxicological studies of cardioactive medications and potentially other cardiotoxicants underestimates adverse outcomes in fish.

Individual and Interactive Influences of Low Dissolved Oxygen and Calcium Channel Blockers in Inland Aquatic Systems

by

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A Dissertation

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DEDICATION

To friends, family, the state of Wisconsin, and the Lake Superior watershed which influenced my career path in some shape or form.

ATTRIBUTION

Co-Author Contributions

Dr. Zhen Wang, Samuel P. Haddad, Dr. Jone Corrales, W. Casan Scott, and Prof. C. Kevin Chambliss all contributed intellectually and/or financially to one or more of my published works. My advisor, Prof. Bryan W. Brooks, contributed intellectually and financially to all chapters. Our discussions of research questions, experimental design and results interpretation, along with provisions of laboratory supplies and living stipend, were necessary to complete this dissertation. Discussions with Dr. Zhen Wang improved my first journal publication and chapter two in this dissertation. Intellectual assistance from W. Casan Scott resulted in the publication of chapter three. Finally, discussions and laboratory assistance from Dr. Jone Corrales, Prof. C. Kevin Chambliss and Samuel P. Haddad resulted in data presented in chapter four.

CHAPTER ONE

Introduction

Background and Significance

Continued global growth of the human population and its concentration to cities has created a new urban water cycle (Brooks, 2014; Postel, 2010). Effective water management is essential to maintain sufficient quality and quantities of water for its designated uses, especially as our access to consumer goods and medicines are increasing faster than our waste infrastructure. Consumption of water and consumer products including pharmaceuticals varies worldwide, while the number of persons above age 60 is expected to double by 2050 (Gaw and Brooks, 2016; Kookana et al., 2014). Coincidentally, 70% of the human population reside in coastal cities where local water resources are stressed from climate change, nutrient enrichment, and contaminant loadings (Brooks et al., 2006; Heathwaite, 2010; Hooper et al, 2013). Therefore, potential risks to aquatic organisms from urban water stressors such as contaminants of emerging concern and nutrients, leading to the increased occurrence of hypoxia (< 2 mg/L DO) worldwide, are of concern (Diaz, 2001; Kookana et al., 2014).

Occurrence, frequency, and duration of hypoxia in freshwater and marine systems throughout the world has been well documented (Cooper and Brush, 1991; Delorme, 1982; Diaz, 2001; Diaz and Rosenberg, 2008; Thornton et al., 1990). As such, with a majority of the human population residing in coastal cities, local water resources are stressed from climate change and nonpoint and point sources of contaminants (Brooks et al., 2006; Heathwaite, 2010; Hooper, 2013). Deleterious effects of hypoxia to aquatic

organisms has been observed in multiple species and even at moderate dissolved oxygen (DO) levels (McKim and Erickson, 1991; Thomas et al., 2006; Vaquer-Sunyer and Duarte, 2008; Wu, 2002). An empirical hypoxia assessment by Vaquer-Sunyer and Duarte (2008) demonstrated approximately 4.60 mg DO/L is necessary to prevent acute adverse effects to marine species (Vaquer-Sunyer and Duarte, 2008). Unfortunately, such an understanding of low DO hazards to freshwater species have received less study in recent years. Therefore, understanding the effects of hypoxia to aquatic organisms and the geographic regions where multiple stressors may be exacerbating chemical toxicity in non-target organisms is necessary to identify whether monitoring, assessment, and management efforts are adequate.

Occurrence of stressors other than hypoxia in aquatic systems have also been reported, especially in urban water cycles experiencing population growth and climate change (Brooks et al., 2006; Postel, 2010; Scott et al., 2016). Pharmaceuticals are continuously released from wastewater treatment plants (WWTP) resulting in life cycle exposures to aquatic organisms, particularly in effluent-dominated or dependent systems (Brooks et al., 2006). Around 98% of published literature on pharmaceuticals in the environment (PiE) has been published after 1995, and has increased by 5- and 10-fold in the past two decades (Daughton, 2016). This research growth has been spurred by an increasing ability to detect human and veterinary medicines in the environment, which has provided substantial evidence to determine exposure scenarios and consider their potential toxicological effects to non-target organisms (Halling-Sorensen et al., 1998; Monteiro and Boxall, 2010; Ternes, 1998). Unfortunately as attention to PiE continues to grow, our understanding of the environmental effects remains less defined (Brooks et al.,

2012). Most of these compounds are not acutely toxic at environmentally relevant concentrations and therefore significant challenges exist to characterize the sublethal effects of pharmaceuticals to non-target organisms (Brooks et al., 2009). Considering mechanism of action (MOA) of a chemical a priori and leveraging pharmacological safety data in a read-across approach has been purposed to anticipate or predict effects in ecotoxicological models (Ankley et al., 2007; Brooks et al. 2009; Rand-Weaver et al., 2013; Winter et al., 2010). Despite this concept of "intelligent testing" for human and ecological risk assessment of pharmaceuticals, data to support this approach is still limited (Winter et al., 2010, Brooks 2014, 2018).

In addition to a need for sufficient toxicity data for chemical stressors like pharmaceuticals, ecological risks of these compounds relative to and in combination with nonchemical stressors (e.g., pH, temperature, DO) has been emphasized (Boxall et al., 2012). Pharmaceuticals are released to surface waters with other contaminants (e.g., nutrients, pesticides) common to effluent dominated or dependent systems (Boxall et al., 2012; Brooks et al., 2006). Effects of pharmaceuticals to aquatic organisms relative to nonchemical stressors is far more complex (Boxall et al., 2012). The relative impact of pharmaceuticals compared to other stressors in the natural environment are unknown but necessary to make knowledgeable management decisions (Boxall et al., 2012).

Toxicological and biochemical responses to contaminants, such as ammonia (Lyu et al., 2013), crude oil (Dasgupta et al., 2016; Dasgupta et al., 2015), heavy metals (Fitzgerald et al., 2016; Hattlink et al., 2005; Malekpouri et al., 2016), and contaminants of emerging concern (Cypher et al., 2015; Hu et al., 2015; Prokkola et al., 2015) by fish have been shown to be DO-dependent. Recently, human therapeutic levels of the calcium

channel blocker diltiazem has been reported in fish plasma from estuaries along the Texas Gulf of Mexico (Scott et al., 2016). These urbanized watersheds, which are additionally impaired waterbodies on the Texas 303(d) list due to nonattainment of DO water quality standards (WQS), represent pronounced estuarine exposure scenarios for multiple stressors (Brooks et al., 2008; Du et al., 2016; Scott et al., 2016). Similar plasma diltiazem observations have occurred in fish exposed to WWTP effluent in Sweden and Japan, which lead to concerns regarding the worldwide occurrence of diltiazem and other calcium channel blockers in environmental matrices. Unfortunately, the ecological effects of diltiazem in fish are poorly understood and deserve future research to understand their potential pharmacological activity in fish.

Fish uptake modeling of pharmaceuticals has been described previously (Brooks, 2014; Du et al., 2014) and is based on physiological pharmacokinetic modeling accompanied with the conservation of drug targets in vertebrates, particularly in mammals and fish (Fitzsimmons et al., 2001; Gunnarsson et al., 2008; Huggett et al., 2003; Verbruggen et al., 2017). Our research group recently explored the utility of using therapeutic hazard values (THV) to identify pharmaceutical water concentrations predicted to bioconcentrate in fish plasma at human therapeutic levels (C_{min}- C_{max}). Read-across represents an approach using mammalian pharmacological data to predict and empirically measure the toxicological effects of drugs in non-target organism. Unfortunately, a minimal number of reports using fish plasma modeling and read-across exist (Rand-Weaver et al., 2013). The above approaches are necessary to effectively inform the applicability of mammalian to fish read-across and further broaden our understanding of pharmaceutical mixtures and multiple stressor (e.g., hypoxia,

temperature) impacts to aquatic organisms in urbanized ecosystems (Brooks, 2018; Brooks et al., 2006; Scott et al., 2016).

Scope of dissertation

In this dissertation, I aimed to better understand the environmental hazards of multiple stressors by examining a model weak base pharmaceutical (e.g., diltiazem) and a common nonchemical stressor (e.g., DO) in fish. Both of these stressors co-occur in aquatic systems and have the potential to adversely affect cardiac function. In the second chapter, an examination of the national guidelines and regional water quality criteria for the nonchemical stressor DO was performed and identified inadequate environmental assessment and management strategies for DO in inland waters. In chapter three, a novel probabilistic hazard analysis was performed with individual and mixtures of CCBs by leveraging existing chemical environmental occurrence data and mammalian pharmacological information to identify global hazards to non-target organisms. Based on my observations in chapters two and three, these predictive tools can identify regions where environmental assessment and management efforts appear inadequate. In chapters four and five, I advanced toxicokinetic and comparative pharmacology efforts using a model calcium channel blocker (diltiazem) and model fish species across an environmentally relevant DO gradient. Such basic and applied studies have the capacity, when paired with fish plasma modeling and read-across approaches, to predict the effects of human therapeutic plasma levels in non-target aquatic vertebrates. Such approaches are necessary and essential for robust ecological risk assessment and management of cardiotoxicants.

CHAPTER TWO

Revisiting Inland Hypoxia: Diverse Exceedances of Dissolved Oxygen Thresholds for Freshwater Aquatic Life

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Abstract

Water resources in many regions are stressed by impairments resulting from climate change, population growth and urbanization. In the United States (US), water quality criteria (WQC) and standards (WQS) were established to protect surface waters and associated designated uses, including aquatic life. In inland waters of the south central US, for example, depressed dissolved oxygen (DO) consistently results in impaired aquatic systems due to noncompliance with DO WQC and WQS. In the present study, we systematically examined currently available DO threshold data for freshwater fish and invertebrates and performed probabilistic aquatic hazard assessments with low DO toxicity data that were used to derive the US Environmental Protection Agency's (EPA) Ambient Water Quality Criteria (AWQC) for DO and newly published information. Aquatic hazard assessments predicted acute invertebrate DO thresholds for Ephemeroptera, Plecoptera, or Trichoptera (EPT) taxa and species inhabiting lotic systems to be more sensitive than fish. For example, these organisms were predicted to have acute low DO toxicity thresholds exceeding the US EPA guidelines 17, 26, 31 and 38% and 13, 24, 30 and 39% of the time at 8.0, 5.0, 4.0 and 3.0 mg DO/L, respectively. Based on our analysis, it appears possible that low DO effects to freshwater organisms

have been underestimated. We also identified influences of temperature on low DO thresholds and pronounced differences in implementation and assessment of the US EPA AWQC among habitats, seasons, and geographic regions. These results suggest some implemented DO guidelines may adversely affect the survival, growth, and reproduction of freshwater aquatic organisms in a region susceptible to climate change and rapid population growth. Given the global decline of species, particularly invertebrates, low DO threshold information, including sublethal (e.g., reproduction, behavior) responses, for additional species (e.g., mollusks, other invertebrates, warm water fish) across seasons, habitats, and life history stages using consistent experimental designs is needed to support more sustainable environmental assessment efforts and management of biodiversity protection goals in inland waters.

Introduction

Freshwater systems can experience significant modification in response to climate change, population growth, and other anthropogenic stressors such as nutrient enrichment, contaminants of emerging concern, pH, and dissolved oxygen (DO). These alterations are particularly observed in arid to semi-arid regions (Brooks et al. 2006; Delorme 1982; Heathwaite 2010). Nutrient enrichment of freshwater systems due to anthropogenic point and nonpoint sources can indirectly result in depressed DO and, in extreme scenarios, hypoxic or anoxic conditions leading to poor water quality (Brooks et al. 2006; Delorme 1982; Heathwaite 2010; Valenti et al. 2011). In aquatic systems, an increase in temperature co-occurring with carbon dioxide accumulation can also exaggerate hypoxia due to elevated oxygen demand and lower oxygen solubility at high

temperature (Brewer and Peltzer 2009; Pörtner 2010). Hypoxia in aquatic ecosystems is typically defined as low levels of DO from near maximum solubility to below 2 mg DO/L (Committee on Environment and Natural Resources 2003). Hypoxic conditions have occasionally occurred naturally in some systems, such as Lake Erie (Delorme 1982; Zhou et al. 2013) and the Chesapeake Bay (Cooper and Brush 1991; Committee on Environment and Natural Resources 2003). However, nutrient enrichment and increased organic matter due to anthropogenic activities has resulted in intensified magnitude, frequency, and duration of hypoxia and anoxia in freshwater and marine systems (Diaz and Breitburg 2009; Committee on Environment and Natural Resources 2003). Low DO concentrations typically occur in hypolimnetic waters with high organic matter, poor circulation, defined stratification, or seasonal ice cover (Chambers et al. 1997; Diaz and Breitburg 2009). Depressed DO levels produce adverse effects on metabolic and behavioral processes in aquatic organisms. For example, moderate hypoxia (2 to 5 mg DO/L) can cause physiological or biochemical stress (e.g., hormonal responses, oxidative stress) in fish and invertebrates, while severe hypoxia can impact survival (mortality), growth, reproduction, and population trajectories of aquatic life (Brett and Blackburn 1981; Doudoroff and Shumway 1970). Unfortunately, though hypoxia has received much study in marine and coastal systems, depressed DO has received relatively limited attention in freshwater ecosystems over the past few decades (Pollock et al. 2007). In the US, the 303(d) list (Section 303(d)) of the Clean Water Act (CWA) includes impaired surface waters that do not attain water quality standards (WQS). In states experiencing dramatic population growth and climate change, such as Texas, freshwater impoundments and tidally influenced rivers have been consistently listed on 303(d) lists

due to noncompliance with DO water quality criteria (WQC) and standards (Brooks et al. 2008; Brooks et al. 2011). Reservoirs located in these arid to semi-arid regions are particularly prone to hypolimnetic and even metalimnetic hypoxia due to high loads of organic matter, droughts, withdrawal rates, and spatial variability (Brooks et al. 2011; Diaz and Breitburg 2009; Thornton et al. 1990). Though reservoir zones (e.g., riverine, transition, lacustrine) represent different aquatic habitats that should be considered during surface water quality assessment and management (Lind et al. 1993), various reservoir habitats are not routinely considered during surface water quality assessments of DO and other contaminants (Brooks et al. 2008; Brooks et al. 2011). Whether habitat-specific implementation and assessment of AWQC, including DO, differs among states and other geographic regions remains poorly described, but differing implementation practices can introduce uncertainty during surface water quality assessments and management activities. The US CWA mandates states and authorized tribes to develop, implement, enforce, and periodically update WQC to protect designated uses of aquatic ecosystems. Based on the 1986 US EPA AWQC for DO, these WQC were intended to protect aquatic life uses and were predominantly dependent on available low DO toxicity data for growth impairment in cold and warm water fish (U.S. Environmental Protection Agency 1986; U.S. Environmental Protection Agency 2012). In 1986, the recommended freshwater DO AWQC were derived for the protection of no to slight (10%) growth/production impairment to fish populations because these DO concentrations were also expected to provide adequate protection for other aquatic organisms (i.e., invertebrates; U.S. Environmental Protection Agency 1986). Canada and UK published DO water quality guidelines after the US EPA in 1987 and 1992, respectively, with the UK specifically

referencing both fresh and marine waters (Canadian Council of Ministers of the Environment 2001; Stiff et al. 1992). Similar to the US AWQC (U.S. Environmental Protection Agency 1986). Canada recommended DO criteria across different developmental stages, while the UK aquatic life criteria were categorized based on the fishery (e.g., salmonid, cyprinid, less sensitive cyprinid). No revisions have occurred to the EPA AWQC since its initial publication 30 years ago; whether such criteria are protective of threatened and endangered species is largely understudied (Woods et al. 2010). However, DO is of particular importance because of the increased frequency of hypoxic events worldwide over the past few decades (Diaz 2001; Committee on Environment and Natural Resources 2003) and future projections of population growth, landscape modification, and climate change. Whether more recently published low DO toxicity data could improve our understanding of the adverse effects of hypoxia in inland waters, and thus reduce uncertainty during surface water quality assessment and management efforts, is not understood. Thus, in the present study, we (1) examined the current status of historical (pre-1986) and more recent low DO toxicity data (post-1986) for freshwater fish and invertebrates, hypothesizing more recent data would differ from historical information; (2) employed probabilistic aquatic hazard assessments to determine the percent of species affected by low DO relative to WQC; and (3) identified whether implementation and assessment of DO WQC differs among freshwater habitats, seasons, and the south central geographic area of the US, a region susceptible to climate change and population growth. We further examined the relationship between temperature and low DO thresholds because increasing temperature decreases oxygen water solubility under conditions when metabolic demands increase with less oxygen

availability and the US AWQC are based on water temperature (cold water vs. warm water) and fish (salmonid vs. nonsalmonid species).

Methods

Data collection

Acute and chronic toxicity data (lethal or effect concentrations, LC50s or EC50s) for low DO and corresponding experimental conditions (e.g., DO, pH, temperature) of freshwater fishes and invertebrates were collected from the peer-reviewed literatures and the US EPA AQWQC document (U.S. Environmental Protection Agency 1986). Acute toxicity endpoints included individual species' LC50 (≤96 h, ≥96 h) values, while chronic endpoints included EC10 and EC50s for the effects of DO on growth (>96 h). For data quality consistency, toxicity data were selected using the following approach. Only published DO experiments that documented experimental designs and study procedures were used for further analyses. These study procedures included sufficient water renewals, clearly identified DO control methods (constant or declining DO), organismal conditions (species, size, weight, life stage, source, diet, acclimation period), daily water chemistry observations (DO, pH, temperature), adequate controls, at least initial and final mortality observations (with sufficient control survival), and statistically calculated standard toxicity values (LC50 or ECx) (Sprague 1973). DO treatment levels reported simply as values greater or less than a concentration were excluded from probabilistic analyses. In the present study, low DO toxicity refers to a calculated lower DO threshold for either decreased survival or growth of an organism. Toxicity data used for species

sensitivity distributions (SSDs) are listed in supplementary information (Supplementary: Table: 16). Fish growth data calculated from both laboratory and mesocosm studies were used in our analyses because they were explicitly included in the derivation of the 1986 AWQC for DO.

Aquatic hazard assessments

Geometric means were calculated for species LC50 or ECx values when study conditions within 1 °C, the same life stage, and multiple toxicity values were reported. When multiple LC50 or ECx values were available for the same species from studies at temperatures varying by greater than 1 °C or by life stage, these data values were separately included in taxa SSD development. Low DO toxicity values were selected to be inclusive of all available temperature conditions, life stages, and study designs. Toxicity data were first ranked in ascending order and assigned percentiles using the Weibull equation:

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

where j is the percent rank, i is the rank assigned to an acute (LC50) or chronic concentration (EC10 or EC50), n is the number of species examined, and $n + 1$ accounts for the assumption that there is always one less than all species tested (Posthuma et al. 2002). SSDs were then constructed following the procedures described in Wheeler et al. (2002), having log concentrations of toxicity values (LC or EC) as x-axis and the proportion of species being affected as y-axis (SigmaPlot Version 11.0 Systat Software, Inc., San Jose, CA, USA). Analyses of covariance (ANCOVA, SPSS, Chicago, IL, USA) were conducted to compare the slopes and intercepts of Weibull ranked probit normalized

regression models of specific classified datasets (e.g., Ephemeroptera, Plecoptera, or Trichoptera (EPT) taxa vs. non-EPT taxa). Due to a variety of low DO toxicity data, which spanned five decades across multiple species (e.g., *Hyalella azteca*, *Hexagenia limbata*, *Onchorynchus mykiss*), different SSDs were generated (i.e., EPT, lotic habitat, pre-1986) for fish and invertebrates. Probabilistic aquatic hazard assessments using developed SSDs were then performed to determine the percentage of toxicity thresholds (e.g., LC50, EC50) likely to be exceeded at the existing US EPA AWQC. Slopes and y-intercepts were extracted from SSD regression models and centile values were calculated (Microsoft Excel 2016 Microsoft Corp, Richmond, WA, USA) using the equation:

$$Centile\ value = NORMDIST((b \times \log 10(x)) + a))$$

where the *NORMSDIST* returns the standard normal cumulative distribution function of a selected value, and *b* and *a* represent the slope and intercept, respectively, from the linear regression.

To quantify differences in SSDs, hazard concentrations (HC) at the 80th percentile (i.e., HC20 or 20% protection level) were calculated from each SSD. More common HC95 or HC90 (i.e., 95 or 90% protection level for DO, respectively) values were not compared in this study because over half of the SSDs contained less than 20 data values (minimum was 5) and would introduce higher uncertainty in such predictions (Grist et al. 2002; Wheeler et al. 2002). HC values derived from each dataset were calculated and compared to compute an HC ratio. When a ratio was greater than one, the dataset/ species were considered sensitive to DO. HCs and their corresponding 95% confidence interval were computed by Monte Carlo simulation, following the log-normal procedure available in the SAS package (SAS 9.4, Cary, NC, USA), and were determined

at 10th, 50th, 80th, 90th, 95th, and 99th centiles.

Temperature-dependent DO thresholds

To investigate potential temperature effects on DO thresholds of freshwater species, a comprehensive meta-analysis of various acute toxicity endpoints (LC50s andEC50s) for DO across multiple temperatures ($n \geq 3$) was conducted for the data generated from an individual study. Linear regression was applied to fit relationships between temperature and acute toxicity endpoints (SigmaPlot 13.0, San Jose, CA, USA). To define the inherent effect of temperature on freshwater communities and populations, temperature-dependent SSDs (i.e., 15, 20, and 25 °C) were constructed for examining the effects of temperature on SSDs. To further quantitatively compare the differences among temperature-dependent SSDs for DO, the HC value and 95% CIs were computed for each SSD by Monte Carlo simulation, following the log-normal procedure available in SAS (SAS 9.4, Cary, NC, USA). To minimize the uncertainty caused by data quantity ($n = 5$), relative species sensitivities among temperatures for DO were compared on the basis of HC20 values. A linear regression function ($y = a + b\ x$) was also applied to fit these data (SigmaPlot 13.0, San Jose, CA, USA).

Geographic- and habitat-specific DO water quality criteria and standards

The south central region of the US is characterized by diverse watersheds, urbanization, population growth, and appreciable annual rainfall gradients. For example, annual rainfall in Texas spans over 114 cm per year from west to east, and contains three of the top ten

largest and fastest growing metropolitan areas in the US (Dallas/Ft. Worth, Houston, San Antonio) and are thus potentially representative of other regions experiencing climate change and population growth. Subsequently, WQC for DO in the south central US, which corresponded to states in US EPA Region 6 (Arkansas, Louisiana, New Mexico, Oklahoma, Texas), were examined to determine whether habitat and geographic differences in implementation and assessment of WQC and WQS exist (Texas Commission on Environmental Quality 2010; Arkansas Pollution Control and Ecology Commission 2011; Louisiana Department of Environmental Quality 2012; New Mexico Environment Department 2000; Oklahoma Water Resources Board 2007).

Results

Freshwater invertebrate and fish thresholds to low DO

The majority of studies reporting standard lethality thresholds were conducted with invertebrates prior to the US EPA AWQC (pre-1986). Additionally, a large amount of low DO toxicity data published pre-1986 included chronic data for growth studies of multiple cold water (e.g., chinook salmon: *Oncorhynchus kisutch*) and warm water (e.g., largemouth bass: *Micropterus salmoides*) fish. Acute and chronic DO toxicity data for fishes and invertebrates published over the last five plus decades with over 70 different fish and invertebrate species are provided in supplementary information (Supplementary: Table: 16). Prior to publication of the AWQC in 1986, no standard calculated fish toxicity values (e.g., LC50) were found (Supplementary: Table: 16) because the majority of these historical studies reported the percent mortality at some DO treatment level(s).

However, recent publications have derived DO LC50 values for a variety of fish species including rainbow trout (*Oncorhynchus mykiss*), suckers (*Deltistes luxatus* and *Chasmistes brevirostris*), common smelt (*Retropinna retropinna*), inanga (*Galaxias maculatus*), common bully (*Gobiomorphus cotidianus*), short-finned eel (*Anguilla australis*), shiner (*Notropis topeka*), and catfish (*Rhamdia quelen*) (Supplementary: Table: 16). Acute and chronic invertebrate LC50 values published pre- 1986 ranged from 0.03 (Jacob et al. 1984; Sprague et al., 1963) to 8.75 (Jacob et al. 1984) mg DO/L and 4.50 (Nebeker et al. 1992) to 5.00 (Nebeker et al. 1992) mg DO/ L, respectively, while more recent (post-1986) acute and chronic invertebrate LC50 values ranged from 0.51 (Nebeker et al. 1992) to 1.95 (Nebeker et al. 1996) mg DO/L and 0.49 (Nebeker et al. 1992) to 2.00 (Nebeker et al. 1996) mg DO/L, respectively (Supplementary: Table: 16). Only 8 and 13 acute DO toxicity values were published since 1986 for freshwater invertebrates and fish, respectively, and one chronic invertebrate DO toxicity value has been published since 1986. Such studies of invertebrate species largely focused on organisms from lentic and lotic habitats. Specifically, those species in both lotic and lentic habitats comprised ~58% of the available low DO toxicity data for invertebrates and represented the most robust invertebrate data set we examined. Similarly, fish DO toxicity data, which included mainly growth studies, were mainly comprised by species inhabiting cold waters (optimal temperature ~11 °C).

Aquatic hazard assessments

Nine invertebrate and five fish SSDs were generated using acute and chronic DO toxicity data. The dataset with the largest and smallest range of LC50 values were 8.72

and 1.44 mg DO/L (Table 1). Because pre-1986 data on acute DO toxicity only was identified for invertebrates, this dataset was compared to all available acute invertebrate and fish DO toxicity data published pre- and post-1986 and was significantly different from each other with the pre-1986 dataset more sensitive (Fig. 1a; ANCOVA, slope $p > 0.217$; y-int $p < 0.001$). These distributions differed at in the middle of the SSD and converged at the lower and upper end. Both datasets were dominated by invertebrates, with the latter dataset encompassing acute fish DO toxicity data and invertebrate data. This acute dataset including both invertebrate and fish DO toxicity data is comprised of 86.5 and 13.5% invertebrate and fish values, respectively. The datasets containing acute invertebrate DO toxicity data include some taxonomic diversity (Table 1) but those SSDs including all available invertebrate data were dominated by EPT taxa compared to non-EPT taxa (~3-fold difference in n and no. of species).

When invertebrates were classified based on lentic or lotic habitats (Merritt and Cummins 1996), our results indicated all three SSDs were significantly different from each other (ANCOVA; lentic and lotic vs lentic $p < 0.014$; lotic vs lentic $p < 0.008$; both vs lotic, $p < 0.001$). Lentic and lotic datasets contained 71.4 and 92.6% EPT taxa. When we then classified acute invertebrate datasets by EPT and non-EPT taxa, our results indicated that the EPT and non-EPT taxa SSDs were also significantly different. Additionally, acute and chronic lethality (LC50) SSDs were significantly different (ANCOVA, $p < 0.001$), though the slopes were not ($p > 0.762$). As mentioned above, the pre-1986 dataset, which included only acute invertebrate DO toxicity data, was significantly different from both invertebrate and fish DO toxicity data; our results revealed acute invertebrate and acute fish SSDs were also significantly different

(ANCOVA, $p < 0.001$). Acute fish DO toxicity data were dominated by warm water species with one acute cold water LC50 value identified for rainbow trout.

When HCs were computed from each dataset and HC80s compared based on the minimum data values in several SSDs, the HC80 ratios for acute DO toxicity involving invertebrate from pre-1986 were consistently greater than one, suggesting that invertebrate mortality thresholds were more sensitive than fish. Specifically, the acute invertebrate pre-1986 DO toxicity SSD was more sensitive than the SSD including the most recent acute invertebrate data and the predicted HC80 was 10.6% lower following the addition of newly published data because these toxicity values fell within the bottom half of the distribution (Supplementary: Figure 20). This is reflected by the acute invertebrate to fish HC ratio of 2.3, again suggesting that invertebrates are twice as sensitive to decreases in DO as fish. Increasing the exposure duration to low DO increased sensitivity, which was reflected in the chronic to acute invertebrate lethality HC ratio of 1.9. Further, EPT taxa were ~2.5× more sensitive than non-EPT taxa. However, ratios comparing fish growth (EC10) to acute invertebrate (LC50) were consistently greater than one regardless of habitat (cold or warm water, lotic or lentic), suggesting fish growth responses are more sensitive to DO than invertebrate mortality.

Aquatic hazard assessments predicted 14, 23, 28, and 35% of acute low DO toxicity values pre-1986 exceed the existing US EPA AWQC at 8.0, 5.0, 4.0, and 3.0 mg DO/L. When this dataset included the newly published invertebrate and fish DO toxicity values, the results predicted 7, 15, 20, and 28% of species to be adversely affected at same DO concentrations, respectively. In comparison, including acute fish DO toxicity data with the acute invertebrate data decreased the percent exceeded by 4–5% and again

indicated, based on the data available, that freshwater invertebrates are more susceptible to DO than fish. When the acute invertebrate datasets were then classified based on habitat types, those species inhabiting lotic environments were predicted to be more adversely affected at 8.0, 5.0, 4.0, and 3.0 mg DO/L (13, 24, 30, and 39%) than those species inhabiting lentic or both lentic and lotic habitats (1–29 to 13–30%, respectively; Fig. 1b; Table 1). Interestingly, a similar percentages of species affected were predicted for those invertebrates divided to EPT (17, 26, 31, and 38%) and non-EPT taxa (5, 10, 14, and 19%) at the same DO concentrations (Fig. 1c; Table 1).

Though there were only five chronic invertebrate DO toxicity values, encompassing 7–30-day exposure durations, 22, 33, 39, and 45% of species were predicted to be affected at 8.0, 5.0, 4.0, and 3.0 mg DO/L (Fig. 1d, Table 1). Compared to predicted acute DO thresholds for aquatic invertebrates, there was an increase of ~10% in the number of affected invertebrates when exposure duration was greater than 7 days. The percent of acute fish DO toxicity values exceeding the US EPA AWQC at 8.0, 5.0, 4.0, and 3.0mg/L (~0, 2, 5, and 11%, respectively; Fig. 1e, Table 1) were low compared to invertebrates. Again, such predictions were based on post-1986 acute fish toxicity values (LC50s) and could not be compared to DO toxicity values available pre-1986 because standard toxicity values were not identified. Using the cold water fish growth EC10 values, results indicated 23, 48, 61, and 76%of fish to be adversely affected at 8.0, 5.0, 4.0, and 3.0 mg DO/L, respectively (Fig. 1e, Table 1). Similarly, 2, 48, 83, and 99% of fishes are predicted to be adversely affected at the same DO levels, using the warm water fish growth EC_{10} values, although only five values were available from four different species.

Table 1. Summary of species sensitivity distributions associated with aquatic hazard assessments for freshwater organisms. The number of the data points (*n*) and number of species (*Sp*) in each dataset; the *Median*, *Variance* and *Range* for each dataset are provided, and the correlation coefficient (r^2), slope (*b*) and intercept (*a*) for each of the log-normal fitting model are listed.

									Hazard Concentration (mg DO/L (95% CI))					
Distributions	***n***	***Sp***	***Median Toxicity Value***	***Var***	***Range***	***r2***	***a***	***b***	***10%***	***50%***	***80%***	***90%***	***95%***	***99%***
Lotic invertebrates	27	17	2.40	4.62	7.85	0.86	-0.66	1.97	0.49 (0.32-0.61)	2.17 (1.80-2.49)	5.78 (4.63-7.31)	9.65 (7.34-13.3)	14.7 (10.7-22.2)	32.6 (21.5-58.1)
Lentic invertebrates	8	4	2.06	1.20	3.28	0.97	-1.34	4.00	1.04 (0.93-1.15)	2.16 (2.06-2.30)	3.51 (3.28-3.87)	4.52 (4.14-5.14)	5.58 (5.00-6.50)	8.25 (7.10-10.2)
Lotic and lentic invertebrates	48	31	1.53	5.62	8.72	0.90	-0.15	1.44	0.16 (0.12-0.20)	1.28 (1.09-1.46)	4.91 (4.02-6.01)	9.93 (7.78-12.9)	17.8 (13.3-24.3)	52.9 (36.2-81.8)
All acute invertebrates	83	52	1.99	4.87	8.72	0.88	-0.33	1.64	0.30 (0.25-0.34)	1.57 (1.43-1.71)	4.68 (4.13-5.27)	8.27 (7.08-9.65)	13.2 (11.0-16.0)	32.0 (25.3-41.2)
Post-1986 invertebrates	8	6	0.93	0.28	1.44	0.97	-0.003	3.83	0.46 (0.41-0.52)	1.00 (0.94-1.06)	1.66 (1.54-1.84)	2.16 (1.95-2.49)	2.68 (2.38-3.20)	4.04 (3.43-5.13)
Pre-1986 invertebrates	75	47	2.20	5.11	8.72	0.86	-0.35	1.56	0.27 (0.21-0.32)	1.73 (1.51-1.95)	5.86 (4.94-7.00)	11.1 (9.00-14.0)	18.8 (14.7-24.9)	50.6 (36.6-73.6)
Chronic invertebrate LC_{50}	5	5	4.5	3.97	4.51	0.78	-0.62	1.53	0.37 (0.028-0.73)	2.53 (1.22-4.26)	8.96 (4.40-58.5)	17.4 (7.12-240)	30.0 (10.2-810)	83.4 (20.0-8589)
Warm water fish EC10	5	4	5.00	0.66	2.02	0.80	-7.14	10.3	3.58 (2.94-3.98)	4.79 (4.52-5.17)	5.80 (5.42-6.67)	6.40 (5.87-7.75)	6.95 (6.25-8.81)	8.11 (7.01-11.3)
Cold water fish EC10	16	7	5.20	2.53	6.82	0.64	-2.33	3.40	2.03 (0.31-2.65)	4.83 (3.25-5.80)	8.53 (6.18-19.2)	11.5 (7.73-43.0)	14.7 (9.33-87.6)	23.3 (13.0-336)
Warm water fish EC50	5	4	1.38	0.51	1.75	0.94	-0.24	2.82	0.48 (0.28-0.62)	1.35 (1.12-1.58)	2.68 (2.12-3.66)	3.83 (2.85-5.94)	5.15 (3.59-9.05)	8.95 (5.56-19.9)
Cold water fish EC50	16	7	2.26	0.81	3.72	0.89	-1.47	4.56	1.10 (0.90-1.24)	2.10 (1.92-2.26)	3.21 (2.87-3.65)	4.01 (3.48-4.77)	4.82 (4.08-5.97)	6.80 (5.46-9.15)
Warm and cold water fish LC50	13	9	1.59	0.45	2.13	0.90	-0.33	3.28	0.52 (0.39-0.61)	1.26 (1.12-1.40)	2.28 (1.95-2.75)	3.11 (2.55-4.02)	4.01 (3.16-5.54)	6.46 (4.74-10.1)

EPT taxa	62	38	2.66	5.51	8.72	0.82	-0.39	1.49	0.28 (0.21-0.35)	1.93 (1.63-2.23)	6.86 (5.51-8.67)	13.3 (10.2-18.2)	23.1 (16.8-33.5	64.5 (42.8-107)
None EPT taxa	21	14	0.96	0.94	4.27	0.72	-0.006	1.81	0.19 (0.04-0.30)	0.99 (0.64-1.29)	2.89 (1.82-5.58)	5.06 (2.89-14.1)	8.03 (4.18-32.2)	19.1 (8.17-149)
All fish and invertebrate LC50	96	61	1.82	4.42	8.72	0.89	-0.33	1.75	0.28 (0.23-0.32)	1.64 (1.46-1.80)	5.22 (4.51-6.02)	9.59 (8.00-11.5)	15.8 (12.8-19.8)	40.6 (30.8-54.9)

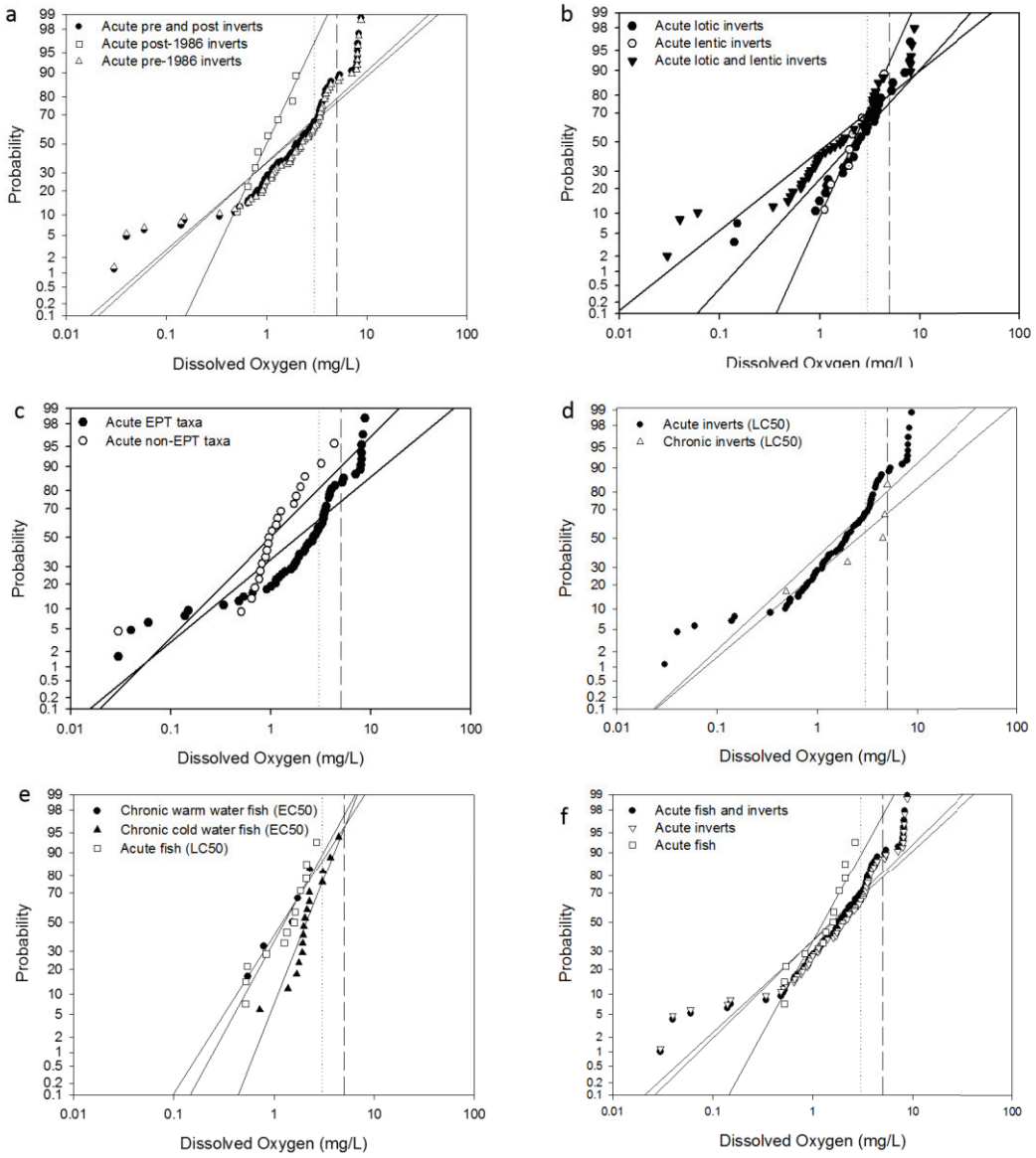

Figure 1: Aquatic hazard assessments of invertebrate and fish dissolved oxygen thresholds from both acute and chronic lethality (LC50) and chronic fish growth (EC) toxicity studies. (A) All acute (2-96 h) invertebrate toxicity data (LC50s) relative to those classified as published pre and post the United States Environmental Protection Agency's Ambient Water Quality Criteria (1986); (B) Acute Lotic, Lentic, and Lotic and Lentic invertebrate (LC50s); (C) Acute invertebrate data (LC50s) divided into the orders (taxa) Ephemeroptera, Plecoptera, and Trichoptera (EPT) or non-EPT taxa; (D) acute and chronic invertebrate toxicity data (LC50s); (E) Chronic warm and cold water fish growth effect concentrations (EC50) relative to acute fish acute toxicity data (LC50); (F) Acute fish and invertebrate toxicity data (LC50) relative to acute invertebrate or acute fish toxicity data (LC50). Vertical lines (left to right) represent water quality criteria for DO subcategory high aquatic life use, commonly assigned to water bodies in Texas, where 24-hour DO minimum are not to extend beyond 8 hours (3 mg/L, dotted) and the 24-hour mean minimum (5 mg/L, long dash) that cannot be exceeded over 24 hours (TCEQ, 2010).

Temperature-dependent DO thresholds

Temperature is an important factor in chemical-induced toxicity, which typically increases with increasing temperature. To examine potential errors and variability associated with different experimental conditions, we systematically examined low DO toxicity data from individual studies at multiple temperatures. There were 3 of 9 invertebrate (LC50) and 1 of 2 fish (EC90, EC50 growth) cases following a positive linear relationship, indicating low DO toxicity increased with elevating temperatures (Supplementary: Figure: 21 and Supplementary: Figure: 22). Three acute temperature-dependent SSDs (15, 20, 25 °C) were also constructed using five similarly available low DO toxicity values for species within the order Ephemeroptera (Supplementary: Figure: 21). When temperature-dependent HC80 values were considered, an insignificant yet positive relationship between temperature and HC80 values was observed, which suggests low DO toxicity may be expected to increase with increasing surface water temperatures (Supplementary: Figure: 21).

Geographic- and habitat-specific DO water quality criteria and standards

States in the south central US were found to have diverging surface water quality assessment approaches that varied by habitat, time of the year, and aquatic life use designations relative to AWQC (Table 2). In the state of Texas, for example, historical high and limited aquatic life use WQC consisted of a 24-h mean and absolute minimum DO WQC at 5 and 3 mg DO/L, respectively, for reservoir systems, and were dependent on the type of waterbody (stream, tidally influenced river; Texas Commission on

Environmental Quality 2003). These WQS were revised in 2010 to implement DO concentrations based on aquatic life use (Texas Commission on Environmental Quality 2010). To compare different implementation practices within the south central US, we found the number of different WQC and standards per state ranged between 1 and 41, not including site-specific criteria within states of Arkansas, Louisiana, New Mexico, Oklahoma, and Texas. Subsequently, we compared the number of early life stage (ELS) and other life stage (OLS) WQC or WQS recommended for cold and warm water fish species within this region and found the number of states with these distinct criteria ranged from 0 to 4 and 0 to 36, respectively (Table 3). All six states have derived DO criteria for warm water species but only three of the six states (Arkansas, New Mexico, Oklahoma) appear to have derived criteria for cold water fishes due to inherent habitat differences. Arkansas has the most distinctly different DO WQC (41) accounting for ELS and OLS, while Louisiana has apparently derived the fewest criterion values (Table 3).

Table 2. Specific dissolved oxygen (DO) water quality criteria in the south central United States of America. The Aquatic Life Category indicates a State DO criteria specified for cold and/or warm water aquatic life. The Habitat Specific Category describes whether states contain specified DO criteria for lakes, streams, and/or impoundments, not including site-specific criteria. Seasonal DO Categories indicate whether State specific DO criteria were derived based on seasons, and the Mean and/or Minimum DO Criteria demonstrates the range of criteria values and whether such DO criteria exist as mean and/or minimum values in each State (APCEC 2011; LDEQ 2012; NMED 2000; OWRB 2007; TCEQ 2010). *Other – In Texas, two different freshwater seasonal criteria are derived and include one specific for 'freshwater in spring' and 'other' (interpreted as the criteria implemented for the other seasons).

EPA Region 6 States	Aquatic Life Categories	Habitat Specific Category	Seasonal DO Categories	Mean and/or Minimum DO Criteria
Texas	Yes	No	Spring and Other*	6.0-2.0/4.0-1.5 (mean/minimum)
New Mexico	Yes	No	None	6.0-5.0 (minimum)
Oklahoma	Yes	No	April-June, Summer, and Winter	7.0-3.0 (minimum)
Arkansas	Yes	Yes	March-May, Critical, and Primary	6.0-2.0 (minimum)
Louisiana	Yes	No	None	5.0 (minimum)

Table 3. Individual US EPA Region 6 state water quality criteria (WQC) that were derived for the protection of early life stages (ELS) and other life stages (OLS). The total number of DO WQC for each state were separated into those providing protection for cold and warm water fisheries. Based on the actual DO concentration, each individual state WQC is placed into a corresponding predicted degree of fisheries production impairment (APCEC 2011; LDEQ 2012; NMED 2000; OWRB 2007; TCEQ 2010; USEPA 1986). Note, AR = Arkansas, LA = Louisiana, NM = New Mexico, OK = Oklahoma, TX = Texas. Cold water DO concentrations for early life stages (the first value is the recommended water column concentration and the value in parentheses represents intergravel DO concentrations in mg DO/L): no production impairment = 11 (8); slight production impairment = 9 (6); moderate production impairment = 8 (5); severe production impairment = 7 (4); limit to avoid acute mortality = 6 (3). Cold water DO concentrations for other life stages (water column concentrations in mg DO/L): no production impairment = 8; slight production impairment = 6; moderate production impairment = 5; severe production impairment = 4; limit to avoid acute mortality = 3. Warm water DO concentrations for early life stages (water column concentrations in mg DO/L): no production impairment = 6.5; slight production impairment = 5.5; moderate production impairment = 5; severe production impairment = 4.5; limit to avoid acute mortality = 4. Warm water DO concentrations for other life stages (water column concentrations in mg DO/L): no production impairment = 6; slight production impairment = 5; moderate production impairment = 4; severe production impairment = 3.5; limit to avoid acute mortality = 3 (modified from the U.S. EPA Ambient Water Quality Criteria for Dissolved Oxygen, 1986. (*) = Three WQC values exist in New Mexico and they apply to both ELS and OLS

Water Classification	State	Criteria Value	Total Number of Dissolved Oxygen WQC		Degree of Production/Growth Impairment									
					No (0%)		Slight (10%)		Moderate (20%)		Severe (40%)		Acute (>50%)	
			ELS	OLS	ELS	OLS	ELS	OLS	ELS	OLS	ELS	OLS	ELS	OLS
Cold Waters	AR	Minimum	1	2	-	-	-	2	-	-	-	-	1	-
Cold Waters	LA	Minimum	-	-	-	-	-	-	-	-	-	-	-	-
Cold Waters	NM	Minimum	*3		-	-	-	3	-	-	-	-	3	-
Cold Waters	OK	Minimum	1	2	-	-	-	2	-	-	1	-	-	-
Cold Waters	TX	Minimum	-	-	-	-	-	-	-	-	-	-	-	-
Cold Waters	TX	Mean	-	-	-	-	-	-	-	-	-	-	-	-
Warm Waters	AR	Minimum	2	36	1	11	-	15	1	1	-	-	-	9
Warm Waters	LA	Minimum	1		-	-	-	1	1		-		-	

Warm Waters	NM	Minimum	3		-	-	-	3	3	-	-	-	-	-	
Warm Waters	OK	Minimum	2	4	-	-	1	2	-	-	-	-	1	2	
Warm Waters	TX	Minimum	4	5	-	1	-	1	1	1	1	-	2	2	
Warm Waters	TX	Mean	4	5	-	-	2	-	1	1	-	-	1	3	

Discussion

Because the occurrence of worldwide hypoxia may increase in both freshwater (Watson et al. 2016) and marine environments (Brewer and Peltzer 2009; Pörtner 2010), the present study examined variability in acute and chronic DO thresholds among freshwater species. We found experimental designs and study protocols (e.g., experimental exposure temperatures, species life stages) for low DO toxicity studies to vary considerably among fish and invertebrates. For a number of studies, potential confounding factors could not be resolved in large part due to data paucity and experimental conditions. For example, Elshout et al. (2013) previously identified juvenile fish to have higher DO tolerances compared to adults based on LOEC values yet we could not identify adequate data to further explore such relationships in the present study. In addition, we were unable to conclude whether the DO toxicity data that formed the basis for derivation of the 1986 AWQC (U.S. Environmental Protection Agency 1986) were confounded by differences among wild and hatchery fish, seasonal conditions, acclimation to laboratory conditions, or by an exceptionally wide range of experimental conditions including varied feeding regimes and age of organisms. For example, time of the year is known to play an important role controlling food consumption and growth rate of both chinook and coho salmon were higher in June than July (and October for coho salmon) at temperatures near 18 °C (Warren et al. 1973).

As noted above, when additional acute toxicity data for invertebrates were incorporated in our analyses, the likelihood of encountering species threshold to DO above the recommended US EPA AWQC increased. Such observations are likely explained by the selection of species and experimental endpoints studied since 1986. As

indicated by our DO sensitivity metrics (i.e., SSDs, HC ratios), invertebrates, particularly EPT and lotic taxa compared to non-EPT and lentic species, were more sensitive to acute DO than fish. EPT taxa are commonly used as sensitive bioindicators of environmental quality in aquatic ecosystems (Cairns and Pratt 1993) because these organisms are quite sensitive to reduced DO and other types of pollution, which tends to be correlated with specific habitat types (Jacobsen et al. 2003). Elevated DO sensitivity of invertebrates was also observed in a study of marine benthic organisms, especially crustaceans and mollusks (Vaquer-Sunyer and Duarte 2008). Based on the data availability examined in the present study, we observed lotic taxa to be more sensitive than lentic species, largely because EPT taxa primarily contributed to lotic distributions. Robust DO thresholds were not available for numerous benthic invertebrates and fish species, including threatened and endangered organisms. Thus, whether existing DO WQC and WQS are protective of most of these imperiled species has not been examined (Woods et al. 2010). Additional high-quality low DO toxicity data is needed for freshwater fish and invertebrates from lotic and lentic habitats to more effectively understand differences in DO sensitivity among freshwater organisms and support more sustainable environmental quality assessment and management.

When chronic data is not available for contaminants, acute-to-chronic (ACR) ratios have been used to predict sublethal responses from acute toxicity data. For hypoxia in freshwater systems, the DO concentration where ≥50% growth impairments occur has historically been reported to accompany the onset of fish mortality (Doudoroff and Shumway 1970; U.S. Environmental Protection Agency 1986). In the present study, we derived a novel ACR of 2.63 from the 80th percentile of warm water fish LC50 and

warm water fish growth EC10 distributions (Supplementary: Figure: 23). This ratio supports the hypothesis that the sensitivity of fish growth and potentially other chronic responses occur at DO concentrations almost three times the LC50 value (Vaquer-Sunyer and Duarte 2008). An SSD derived ACR from cold water fish or freshwater invertebrates could not be calculated due to a lack of available data. However, we derived an ACR of 3.8 for rainbow trout using a geometric LC50 and available EC10 data for growth. Prior to 1986, DO chronic toxicity data were mostly available for fish, especially those in the family Salmonidae based on economical and sociological reasons (U.S. Environmental Protection Agency 1986). Most of the studies used to derive the AWQC predominantly investigated growth along with some studies of embryonic development and swimming behavior. Most chronic DO toxicity studies prior to 1986 failed to include a full life cycle, examine both embryo and larval stages, or encompass an adequate period of post-larval feeding and growth (U.S. Environmental Protection Agency 1986). Further, studies of the effects of DO to cold water fish reproduction, fecundity, or fertility, which are important endpoints relevant to ecological risk assessment and management (Ankley et al. 2010; U.S. Environmental Protection Agency 1986), were also lacking. Prior to 1986, two studies were conducted with warm water fish that investigated the effects of DO on reproduction with fathead minnows and black crappie, but the quality of a life cycle experiment with fathead minnows is uncertain due to 50% mean larval survival in some experimental controls (U.S. Environmental Protection Agency 1986; Brungs 1971). Clearly, future studies are necessary to understand reproductive thresholds of DO to freshwater fish, amphibians, and invertebrates.

To assess the likelihood of acute DO hazards to freshwater communities, we performed aquatic hazard assessments, which indicated 7, 15, 20, and 28% of invertebrates, and fish are expected to be adversely affected at 8.0, 5.0, 4.0, and 3.0 mg DO/L, respectively (Fig. 1f). A similar assessment was conducted by Vaquer-Sunyer and Duarte (2008) with marine benthic organisms in which cumulative distributions were created utilizing median lethal concentrations, sublethal thresholds, and median lethal times that were classified by organism types ranging from echinoderms to fish. Vaquer-Sunyer and Duarte (2008) identified that the most sensitive groups of organisms exhibiting the highest LC50 and lowest LT50 90th percentiles were the crustaceans and mollusks, respectively. In the present study, including the acute warm and cold water fish LC50 values with the acute invertebrate LC50 values decreased the predicted affects by 4–5% and illustrated the invertebrate community was more sensitive to DO than fish. Conversely, fish exhibited the highest sublethal response to DO of the compiled marine benthic organisms, which included endpoints such as avoidance of hypoxic waters, behavior, and increased ventilation, which differs from the type of fish chronic toxicity data used in the current study. However, in both assessments, fish chronic responses were the most sensitive to DO. In the present study, the 50th and 90th percentile warm and cold water fish LC50 values were 1.26 and 4.01 mg DO/L, respectively, which are similar concentrations from two different water types. Freshwater to saltwater and vice versa toxicity extrapolations were previously investigated by Wheeler et al. (2002) who indicated differences in toxicity sensitivity depending on the chemical (e.g., ammonia, metals, pesticides, narcotics) that could be accounted for with an appropriate adjustment factor. Regardless, DO sensitivities across saltwater and freshwater organisms require

further research to develop a comparative understanding of DO thresholds among fish and invertebrates, and support ecosystem protection goals related to biodiversity.

It appears possible that DO effects to marine and freshwater organisms have been underestimated. For example, Vaquer-Sunyer and Duarte (2008) illustrated the effects of DO to marine benthic organisms were above the conventional 2 mg DO/L definition of hypoxia; such predictions for marine invertebrates are consistent with fish and invertebrate SSDs and corresponding 80th percentile values in the present study (Table 1). Further, Vaquer-Sunyer and Duarte (2008) predicted a 90th percentile median LC50 value for marine organisms of 4.59 mg DO/L, which is similar to the 80th percentile concentration of 4.64 mg DO/L predicted to adversely affect 20% of freshwater species from a community SSD for acute DO toxicity data to invertebrates and fish (Fig. 1). Therefore, DO concentrations approximately twice the 2 mg DO/L hypoxia threshold are expected to cause significant mortality in marine and freshwater organisms. These DO thresholds are in direct contrast to the 2.3 mg DO/L ASWQC derived limit to avoid juvenile and adult mortality. Such a difference may be due to the small range of DO LC50 values available (~1.29) for both juvenile and adults used in the EPA saltwater criteria recommendations, while the LC50 range in our present study was 8.72. The conventional 2 mg DO/L threshold is commonly used to indicate the potential risk to fisheries, but again, to conserve diversity and avoid mortality events, higher DO levels are predicted necessary to maintain most aquatic life populations (Vaquer-Sunyer and Duarte 2008). In fact, the ASWQC recommends a general 4.8 mg DO/L level to prevent no more than a 25% chronic growth reduction in species (U.S. Environmental Protection Agency 2000). In comparison, this value is twice as high as our predicted 75th percentile

values of 2.41 and 3.21 mg DO/L from warm and cold water fish growth (25% growth reduction) EC50 SSDs, respectively. Given the global decline of species, particularly invertebrates, during the Anthropocene (Dirzoet al. 2014), future studies are necessary to experimentally examine such predictions of DO thresholds for inland waters.

We further examined temperature influences on DO thresholds to freshwater organisms. Oxygen plays a critical role influencing acute temperature limits of organisms and relationships among temperature limits of physiological and biochemical pathways associated with the oxygen supply cascade. For example, the oxygen-limited thermal tolerance (OLTT) model describes physiological activities of ectotherms when exposed to various temperatures (Frederich and Pörtner 2000; Pörtner 2001). This model suggests that aquatic ectotherms, like fishes, generally live within a confined range of temperatures where they function aerobically without displaying any sign of stress (e.g., behavioral disorders). Beyond the optimum temperature range, however, ectotherms encounter a mismatch of energy demand and supply and eventually shift to anaerobic respiration at extreme high or low temperatures to increase energy supply for sustaining essential cellular and physiological functions (Pörtner 2010). When such changes in temperature and oxygen concentration are introduced, total metabolism, basal metabolism, and scope of activity of aquatic organisms' decreases, while the frequency of locomotory acts and mechanical power decline (Svetlichny et al. 2000). Therefore, oxygen deficiency (e.g., hypoxia) within body tissues results in changes in growth, survival, reproduction and even population distribution and abundance under thermal stress (Perry et al. 2005; Pörtner 2010).

Previous studies have compared temperature-dependent chemical toxicity alone (Zhou et al. 2014) and between geological regions (e.g., temperate, tropical) to the same chemicals (Wang et al. 2014), but fewer studies have compared stressor-dependent toxicity to other abiotic factors such as pH (Wang et al. 2016). For the majority of ectotherms, their physiological performances (e.g., metabolism, appetite, behavior) follow a thermal curve and experience increased mortality when temperature deviates from optimum (Bao et al. 2008; Pörtner 2002; Schulte et al. 2011). While the derived US EPA AWQC were designed to be protective of high seasonal surface water temperatures, most organisms used to derive the criteria were studied at optimal thermal conditions during acute and chronic exposures. To examine temperature-dependent DO thresholds of freshwater organisms, a total of nine invertebrate and two fish species were found to be studied across at least three different temperatures within the same study (Supplementary: Figure: 21 and Supplementary: Figure: 22). For invertebrates, only five species emphemeropterans were studied across the same three temperatures (15, 20, and 25 °C). HC80 values for this temperature-dependent SSD were 3.94, 6.36, and 12.5 mg DO/L at 15, 20, and 25 °C, respectively. Clearly, the HC80 values increased with increasing temperature but, again, are only representative of five species within the order. Coho and Chinook salmon were two additional species studied across more than three different temperatures (Supplementary: Figure: 22). A temperature-dependent DO toxicity relationship with growth was clearly observed in Chinook salmon while the relationship was less clear for cohos. However, these studies were conducted with juveniles at different developmental stages, weights, diets, and months of the year that could confound the results. In fact, as mentioned above, time of year was observed to

play an important role in controlling the food consumption and growth rate of both chinook and coho salmon (Warren et al. 1973). Additional research is clearly needed to understand the influences of temperature on DO thresholds of freshwater organisms, particularly when considering predictions of climate change.

Divergent implementation practice efforts in surface water quality assessment and management of DO was observed in a region characterized by diverse watersheds, experiencing population growth, and susceptible to climate change. We specifically observed surface water quality practices to differ across habitats, seasons, and aquatic life relative to the US EPA AWQC in the south central US (Table 2). For example, New Mexico and Oklahoma have derived criteria based on cold and warm water aquatic life/communities and both states even derive specific aquatic life/community values for cool water organisms (New Mexico Environment Department 2000; Oklahoma Water Resources Board 2007). Similarly, Arkansas derives DO WQS based on the presence of trout and by habitat categories specific to different stream watershed sizes throughout the state, and has specific DO standards derived for lakes and reservoirs (Arkansas Pollution Control and Ecology Commission 2011). Some states (Texas, Oklahoma, Arkansas) have specific seasonal DO criteria for at least the spring season (March to June depending on the state), yet routine monitoring for surface water quality parameters, including DO, largely occurs in summer months. Further, Louisiana does not have specific DO criteria for habitats or seasons (Louisiana Department of Environmental Quality 2012). Though the US EPA AWQC recommended instantaneous minimum DO concentrations to be achieved at all times (U.S. Environmental Protection Agency 1986), our analysis reveals some DO criteria within the south central US appear inadequate to prevent species from

adverse mortality (Table 3). These observations are salient given challenges to develop, implement and enforce criteria and standards elsewhere in developed and developing countries. For example, as of 2014, 27 states within the US did not have numeric criteria for total nitrogen or phosphorus (Manuel 2014), despite influences of nutrient enrichment on surface water quality and the development of harmful algal blooms (Watson et al. 2015; Brooks et al. 2016). Given such nutrient enrichment, population growth, rising surface water temperatures, and potential climate-induced sensitivity of organisms (Heathwaite 2010; Hooper et al. 2013; Solomon et al. 2007), additional studies examining whether current DO criteria and standards are adequate to protect freshwater organisms across seasons, habitats, and life history stages are warranted.

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CHAPTER THREE

Global Scanning Assessment of Calcium Channel Blockers in the Environment: Review and Analysis of Occurrence, Ecotoxicology and Hazards in Aquatic Systems

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Abstract

As an urban water cycle is increasingly realized, aquatic systems are influenced by sewage and wastewater effluent discharges of variable quality. Such urbanization results in exposures of non-target aquatic organisms to medicines and other contaminants. In the present study, we performed a unique global hazard assessment of calcium channel blockers (CCB) in multiple environmental matrices. Effluent and freshwater observations were primarily from North America (62% and 76%, respectively) and Europe (21% and 10%, respectively) with limited-to-no information from rapidly urbanizing regions of developing countries in Asia-Pacific, South America, and Africa. Only 9% and 18% of occurrence data were from influent sewage and marine systems, though developing countries routinely discharge poorly treated wastewater to heavily populated coastal regions. Probabilistic environmental exposure distribution (EED) 5th and 95th percentiles for all CCBs were 1.5 and 309.1 ng/L in influent, 5.0 and 448.7 ng/L for effluent, 1.3 and 202.3 ng/L in freshwater, and 0.17 and 12.9 ng/L in saltwater, respectively. Unfortunately, global hazards and risks of CCBs to non-target organisms remain poorly understood, particularly for sublethal exposures. Thus, therapeutic hazard values (THV) were calculated and employed during probabilistic hazard assessments with EEDs when

sufficient data was available. Amlodipine and verapamil in effluents and freshwater systems exceeded THVs 28% of the time, highlighting the need to understand ecological consequences of these CCBs. This global scanning approach demonstrated the utility of global assessments to identify specific CCBs, chemical mixtures with common mechanisms of action, and geographic locations for which environmental assessment efforts appear warranted.

1. Introduction

Whereas unprecedented growth and concentration of human populations is occurring in urban areas, resource consumption, including chemical use, is also concentrating (Brooks, 2014; Postel, 2010). Advancing sustainable water management is increasingly important as global access to chemical products is increasing faster than wastewater management systems and infrastructure are being implemented. For example, 80% of global sewage production remains untreated. Consumption of consumer goods, including human pharmaceuticals, varies worldwide, while the number of persons above age 60 is expected to double by 2050 (Gaw and Brooks, 2016; Kookana et al., 2014). Coincidentally, 70% of the human population reside in coastal cities where local water resources are stressed from insufficient waste management, climate change, and contaminant loadings (Hooper, 2013; Vorosmarty et al., 2010; Water, 2009). Herein, potential risks of pharmaceuticals in the aquatic environment are of increasing concern to water resources, wildlife, and public health (Arnold et al., 2014; Ashbolt et al., 2013), particularly in developing countries (Kookana et al., 2014). Pharmaceuticals are often continuously released from wastewater treatment plants (WWTP) resulting in potential

life cycle exposures to non-target aquatic organisms, especially in arid to semi-arid geographic regions where effluent-dominated or dependent systems are common (Ankley et al., 2007; Brooks et al., 2006). Around 98% of published literature on pharmaceuticals in the environment (PiE) has been published since 1995 and has increased by 5- and 10-fold in the past two decades (Daughton, 2016). This research growth has been spurred by an increasing ability to detect human and veterinary medicines in the environment, which has provided information to support exposure assessments and to consider their potential toxicological effects to non-target organisms (Halling-Soensen et al., 1998; Monteiro and Boxall, 2010; Ternes, 1998). However, various classes of pharmaceuticals have received differential attention. For example, initial studies emphasized endocrine disrupting compounds while more recent assessments have focused on antibiotics, antidepressants, antihistamines, and others (Brooks, 2014; Gaw and Brooks, 2016; Kookana et al., 2014; Kristofco and Brooks, 2017). Unfortunately, environmental hazards and risks of calcium channel blockers (CCB) to non-target aquatic organisms remain poorly examined. CCBs represent a class of compounds previously identified to pose potential risks to ecosystems (Berninger and Brooks, 2010). These commonly prescribed substances are reported to accumulate in tissues of freshwater and terrestrial wildlife (Fick et al., 2010b; Lazarus et al., 2015; Scott et al., 2016). Calcium antagonists were discovered in the 1960s (Spedding and Paoletti, 1992) and then introduced to the market as medicines in the 1980s. These antagonists are intended to elicit therapeutic benefits through voltage dependent calcium channel inhibition for treatment of hypertension and angina (Law et al., 2013). Similar to other pharmaceuticals and down the drain compounds, CCBs are primarily introduced to the environment through reclaimed wastewater discharges following excretion as parent

compounds or metabolites from patients. For example, approximately 30% of verapamil is excreted as the parent compound without metabolism while other CCBs can be almost entirely excreted as inactive metabolites (Law et al., 2013). As urbanizing aquatic systems are increasingly influenced by WWTP effluent discharges and untreated sewage, understanding environmental hazards and risks of chronic low dose CCB exposures to non-target organisms is necessary for effective water management (Ankley et al., 2007; Brooks et al., 2006). For example, understanding differential hazards and risks of specific pharmaceuticals across geographic regions has recently been reported and emphasized as a critical research need (Boxall et al., 2012; Rudd et al., 2014). In the present study, we performed a novel global scanning assessment for CCBs in the environment. The objectives of this study were to critically review the current knowledge of CCB occurrence and to initially assess associated hazards in various environmental water matrices. We specifically examined the refereed literature for CCB occurrence and ecotoxicology data. When data availability was sufficient, environmental exposure distributions for specific CCBs were developed. These distributions were then used to predict the probability of exceeding individual CCB therapeutic hazard values (THV) in surface waters and effluents among geographic regions.

2. Materials and methods

2.1 Literature review of calcium channel blockers

A list of CCBs was compiled from the Mammalian Pharmacokinetic Prioritization for Aquatic Species Targeting (MaPPFAST) database (Berninger et al., 2016). Literature searches through March 12, 2017 returned approximately 143 relevant publications from

almost 2800 hits. A similar search was conducted for CCB ecotoxicity data. In these publications, quantitative data on CCBs was collated based on standard study parameters, analytical instrumentation, and geographic region (e.g., Africa, Asia-Pacific, Europe, North America, and South America) as previously described (Corrales et al., 2015; Kristofco and Brooks, 2017).

2.2 Probabilistic environmental hazard assessments

2.2.1 Environmental exposure distributions

After CCB occurrence data was compiled and collated, probabilistic environmental exposure distributions (EEDs) were created using maximum measured environmental concentrations (MEC) for each water matrix when greater than occurrence observations were available (Wheeler et al., 2002) for a matrix. MECs were used due to differential data reporting to represent conservative exposure conditions. All graphs were created in Sigmaplot 11.0 (Systat Software, Inc.). Distributions were then used to perform probabilistic environmental hazard assessments (PEHAs) to estimate probabilities of encountering environmental occurrence of each CCB at or above a threshold concentration. This approach generally followed those methods previously described (Corrales et al., 2015; Kristofco and Brooks, 2017; Solomon and Takacs, 2001). MECs were ranked in ascending order and assigned percentiles using the Weibull formula (Eq. (1)):

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

where j is the percent rank, i is the rank assigned to a MEC, n is the number of chemicals examined, and $n+1$ accounts for the assumption that there is always one less than all

occurrences measured (Posthuma et al., 2010). EEDs were then constructed with CCB concentrations as the x-axis and percent rank as the y-axis (log common and probability transformed, respectively). Linear regression analyzes were performed and the slope and y-intercept were extracted to calculate centile values (Microsoft Excel 2016 Microsoft Corp, Richmond, WA, USA) using the equation:

$$Centile\ value = NORMDIST((b \times log\ 10(x)) + a) \quad (2)$$

where the *NORMSDIST* returns a standard normal cumulative distribution function of a selected value, and *b* and *a* represent the slope and intercept, respectively, from a linear regression. These exceedance values were derived from each EED for various water matrices and geographic regions.

2.2.2 Therapeutic hazard values

To identify whether CCB concentrations in water matrices may adversely affect fish, THVs were calculated for each compound to estimate therapeutic hazards relative to various EEDs. A THV is a predicted pharmaceutical water concentration expected to bioaccumulate in fish plasma to a human therapeutic level (C_{max} or C_{min}; Eq. (3); (Brooks, 2014)).

$$THV = C_{min}/P_{Blood:Water} \quad (3)$$

Despite some previously noted limitations (Brooks, 2014), plasma modeling approaches have been employed by our research group (Berninger et al., 2011; Valenti et al., 2012; Du et et al., 2014; Scott et al., 2016) and others (Fick et al., 2010a; Margiotta-Casaluci et al., 2014, 2016) to predict fish internal doses of pharmaceuticals. These concepts were

initially derived from fish uptake modeling performed by Fitzsimmons et al. (2001) (Eq. (4)) and incorporated by Huggett et al. (2003) to prioritize pharmaceuticals of

$$LogP_{Blood:Water} = log\ ((10^{0.73\ x\ logK_{OW}} x\ 0.16) + 0.84) \quad (4)$$

environmental concern by estimating fish plasma steady state drug concentrations (Eq. (5)).

$$Fish\ plasma\ concentration = [Aqueous]\ x\ logP_{Blood:Water} \quad (5)$$

2.3 Concentration addition modeling

To determine whether occurrence of individual components in CCB mixtures with a common mode of action may exceed a combined therapeutic hazard concentration, studies that quantitated multiple CCBs within the same study and location were collated. Concentration addition (CA) is generally considered an appropriate approach to approximate ecological effects when compounds exert toxicity through a common mode of action. Additionally, CA has been suggested as a 'worst case' assumption to conservatively overestimate mixture responses and has successfully been applied for prediction of mixture effects for estrogenic agents, pesticides, herbicides, and other pollutants (Kortenkamp et al., 2009). Herein, MECs were combined within the same dataset, ranked, and plotted as described above. Distributions and associated MECs were calculated for each compound at the 5th, 20th, 50th, 80th, and 95th centiles, which were then divided by THVs to create an additive Therapeutic Hazard Ratio (∑THR; Gaw and Brooks, 2016) similar to previous CA methods ((Faust et al., 2001; Kortenkamp et al., 2009); Eq. (6)).

$$Additive\ THR = \sum(MEC_n/THV_i) + (MEC_n/THV_i) + \ldots \quad (6)$$

where $\sum$ is the sum of each ratio at MEC_n, n is the percentile from each distribution (e.g., diltiazem, verapamil), and i is the compound specific THV for each CCB. Ratios per compound and MEC were summed. For example, if values exceeding 1 were observed, then CA modeling predicted an additive CCB THR exceedance from environmental occurrence studies examining multiple CCB in a specific matrix.

3. Results and discussion

3.1 Global occurrence of calcium channel blockers

Published articles detecting CCBs in environmental water matrices have steadily increased over the past 15 years (Figure 2). Ground water, influent sewage, effluent discharge, freshwater, and saltwater/estuarine surface waters represented the primary media studied though others investigations have included invertebrates, fish, birds, sludge, and sediments (Supplementary: Table 17). Similar CCBs were detected in influent, effluent, freshwater, and saltwater (Tables 4-7), which allowed for comparative study of these water matrices. When data availability was sufficient (e.g., >5 data values), distributions of CCBs across all global (Figure 3) and specific geographic regions by matrix identified diverse geographic hazard profiles (Figs. 3A-E & 4A-F). Unfortunately, the occurrence of CCBs were not available for multiple regions. In fact, the majority of occurrence data have been studied in North America, Europe and parts of Asia-Pacific. Limited data was available from South America. Further, the occurrence of CCBs in large geographic regions such as Africa and Antarctica, and specific regions (e.g., the Middle East), were not available, and thus remain poorly understood.

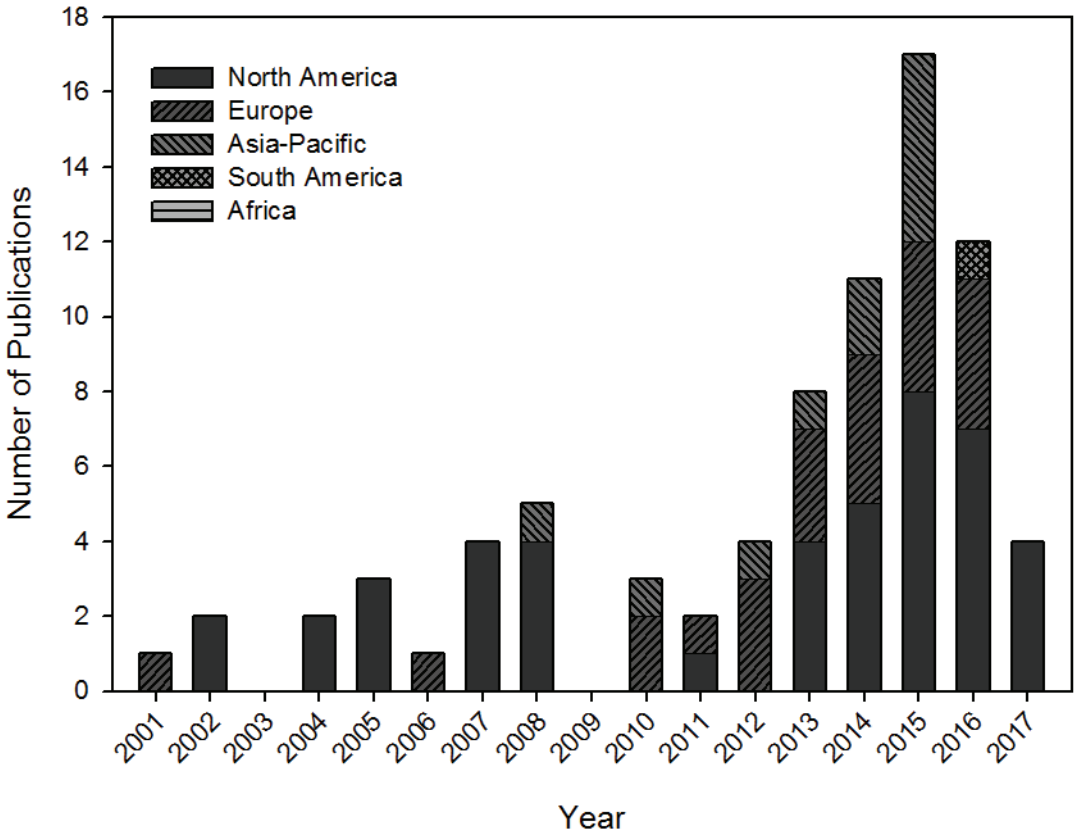

Figure 2: Peer-reviewed studies measuring the occurrence of calcium channel blockers in environmental matrices through time (until March 2017).

Table 4. Detection frequency and geographic distribution of calcium channel blockers in influent sewage.

	Influent Detections (ng/L)					Geographic Distribution				
Compound	**# Times Studied**	**# Times Detected**	**Ratio**	**Min**	**Max**	**Africa**	**Asia-Pacific**	**Europe**	**North America**	**South America**
*Amlodipine	4	2	2/4	ND	247	-	1	3	1	-
Clevidipine	0	0	0/0	ND	ND	-	-	-	-	-
Dehydronifedipine	2	1	1/2	ND	< 10.0	-	-	-	2	-
Desmethyldiltiazem	1	1	1/1	20.3	32.1	-	-	-	1	-
Diltiazem	15	15	15/15	ND	1800	-	2	5	8	-
Felodipine	0	0	0/0	ND	ND	-	-	-	-	-
Flunarizine	0	0	0/0	ND	ND	-	-	-	-	-
Isradipine	0	0	0/0	ND	ND	-	-	-	-	-
Mibefradil	0	0	0/0	ND	ND	-	-	-	-	-
Nicardipine	0	0	0/0	ND	ND	-	-	-	-	-
Nifedipine	7	3	3/7	ND	440		2	2	2	1
Nimodipine	0	0	0/0	ND	ND	-	-	-	-	-
Nisoldipine	0	0	0/0	ND	ND	-	-	-	-	-
Nitrendipine	0	0	0/0	ND	ND	-	-	-	-	-
Norverapamil	1	0	0/1	ND	ND	-	-	1	-	-
Verapamil	9	8	8/9	ND	72	-	2	6	1	-

* Huber et al (2016) measured amlodipine in effluent from two countries (Europe and North America); therefore, the total studies throughout the geographic regions (5) does not match the # times studied (4).

Table 5. Detection frequency and geographic distribution of calcium channel blockers in effluent wastewater.

	Effluent Detections (ng/L)					Geographic Distribution				
Compound	**# Times Studied**	**# Times Detected**	**Ratio**	**Min**	**Max**	**Africa**	**Asia-Pacific**	**Europe**	**North America**	**South America**
*Amlodipine	7	4	4/7	ND	448	-	2	4	2	-
Clevidipine	0	0	0/0	ND	ND	-	-	-	-	-
Dehydronifedipine	5	4	4/5	ND	15	-	-	-	5	-
Desmethyldiltiazem	2	2	2/2	14.1	110	-	-	-	2	-
Diltiazem	30	29	29/30	ND	425	-	2	10	18	-
Felodipine	0	0	0/0	ND	ND	-	-	-	-	-
Flunarizine	0	0	0/0	ND	ND	-	-	-	-	-
Isradipine	0	0	0/0	ND	ND	-	-	-	-	-
Mibefradil	0	0	0/0	ND	ND	-	-	-	-	-
Nicardipine	0	0	0/0	ND	ND	-	-	-	-	-
Nifedipine	9	5	5/9	ND	89		3	2	3	1
Nimodipine	0	0	0/0	ND	ND	-	-	-	-	-
Nisoldipine	0	0	0/0	ND	ND	-	-	-	-	-
Nitrendipine	0	0	0/0	ND	ND	-	-	-	-	-
Norverapamil	5	2	2/5	ND	71	-	-	3	2	-
Verapamil	14	12	12/14	ND	190	-	1	9	4	-

* Huber et al (2016) measured amlodipine in effluent from two countries (Europe and North America); therefore, the total studies throughout the geographic regions (8) does not match the # times studied (7).

Table 6. Detection frequency and geographic distribution of calcium channel blockers in freshwater.

	Freshwater Detections (ng/L)					Geographic Distribution				
Compound	**# Times Studied**	**# Times Detected**	**Ratio**	**Min**	**Max**	**Africa**	**Asia-Pacific**	**Europe**	**North America**	**South America**
*Amlodipine	8	6	6/8	ND	25	-	3	3	2	-
Clevidipine	0	0	0/0	ND	ND	-	-	-	-	-
Diltiazem	37	30	30/37	ND	173	-	2	7	28	-
Dehydronifedipine	11	6	6/11	ND	30	-	-	-	11	-
Desmethyldiltiazem	4	4	4/4	< 0.149	65	-	-	-	4	-
Felodipine	0	0	0/0	ND	ND	-	-	-	-	-
Flunarizine	0	0	0/0	ND	ND	-	-	-	-	-
Isradipine	0	0	0/0	ND	ND	-	-	-	-	-
Mibefradil	0	0	0/0	ND	ND	-	-	-	-	-
Nicardipine	0	0	0/0	ND	ND	-	-	-	-	-
Nifedipine	8	2	2/8	ND	50	-	3	3	2	-
Nimodipine	0	0	0/0	ND	ND	-	-	-	-	-
Nisoldipine	0	0	0/0	ND	ND	-	-	-	-	-
Nitrendipine	0	0	0/0	ND	ND	-	-	-	-	-
Norverapamil	7	3	3/7	ND	0.9	-	-	4	3	-
Verapamil	13	8	8/13	ND	319	-	-	8	5	-

* Huber et al (2016) measured amlodipine in effluent from two countries (Europe and North America); therefore, the total studies throughout the geographic regions (5) does not match the # times studied (4).

Table 7. Detection frequency and geographic distribution of calcium channel blockers in saltwater.

	Saltwater Detections (ng/L)					Geographic Distribution				
Compound	# Times Studied	# Times Detected	Ratio	Min	Max	Africa	Asia-Pacific	Europe	North America	South America
*Amlodipine	5	1	1/5	ND	< 4.17	-	-	4	2	
Clevidipine	0	0	0/0	ND	ND	-	-	-	-	-
Dehydronifedipine	1	0	0/1	ND	ND	-	-	-	1	-
Desmethyldiltiazem	2	1	1/2	ND	1.7	-	-	-	2	-
Diltiazem	6	6	6/6	ND	23.5	-	1	2	3	-
Felodipine	0	0	0/0	ND	ND	-	-	-	-	-
Flunarizine	0	0	0/0	ND	ND	-	-	-	-	-
Isradipine	0	0	0/0	ND	ND	-	-	-	-	-
Mibefradil	0	0	0/0	ND	ND	-	-	-	-	-
Nicardipine	0	0	0/0	ND	ND	-	-	-	-	-
Nifedipine	1	1	1/1	ND	3.7	-	-	-	1	-
Nimodipine	0	0	0/0	ND	ND	-	-	-	-	-
Nisoldipine	0	0	0/0	ND	ND	-	-	-	-	-
Nitrendipine	0	0	0/0	ND	ND	-	-	-	-	-
Norverapamil	2	0	0/2	ND	ND	-	-	1	1	-
Verapamil	4	2	2/4	ND	3	-	-	2	2	-

* Huber et al (2016) measured amlodipine in saltwater from two countries (Europe and North America); therefore, the total studies throughout the geographic regions (6) does not match the # times studied (5).

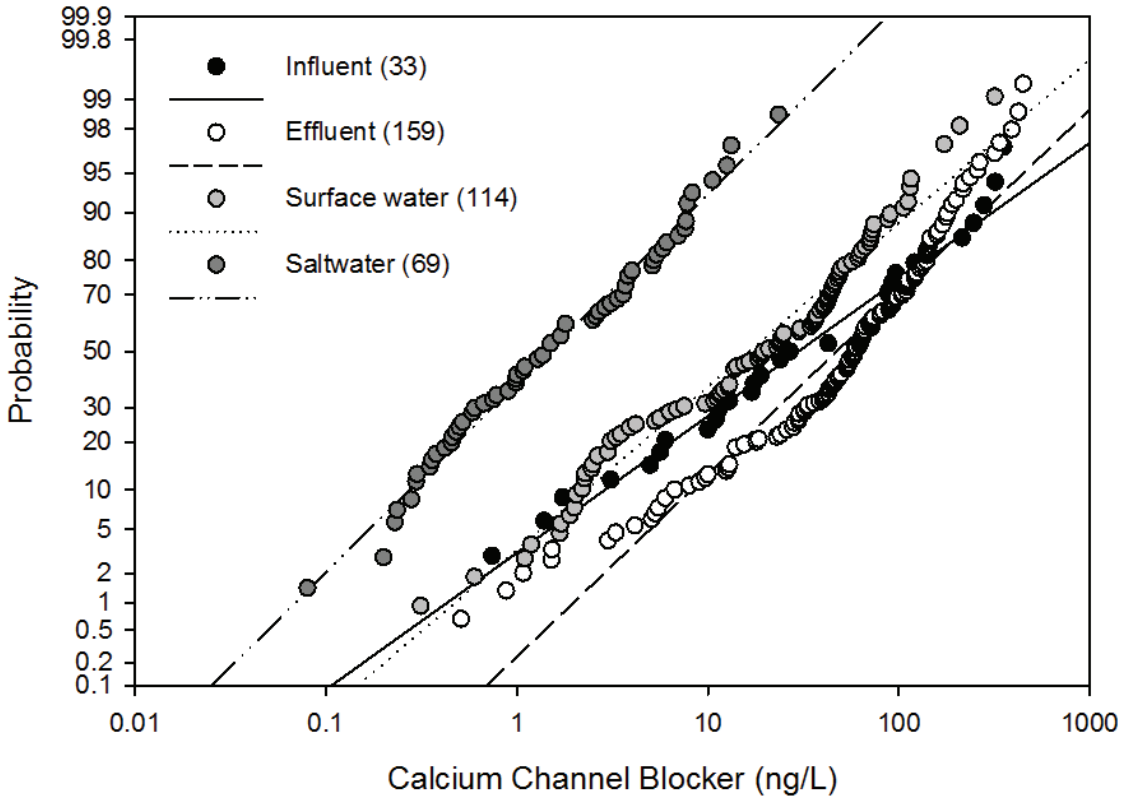

Figure 3: Global calcium channel blocker (CCB) environmental exposure distributions of maximum measured environmental concentrations in influent sewage, effluent, freshwater, and saltwater. Numbers within parenthesis indicate the number of detections in each matrix. Four different CCBs, which were detected throughout different geographic regions, are included in each matrix EED.

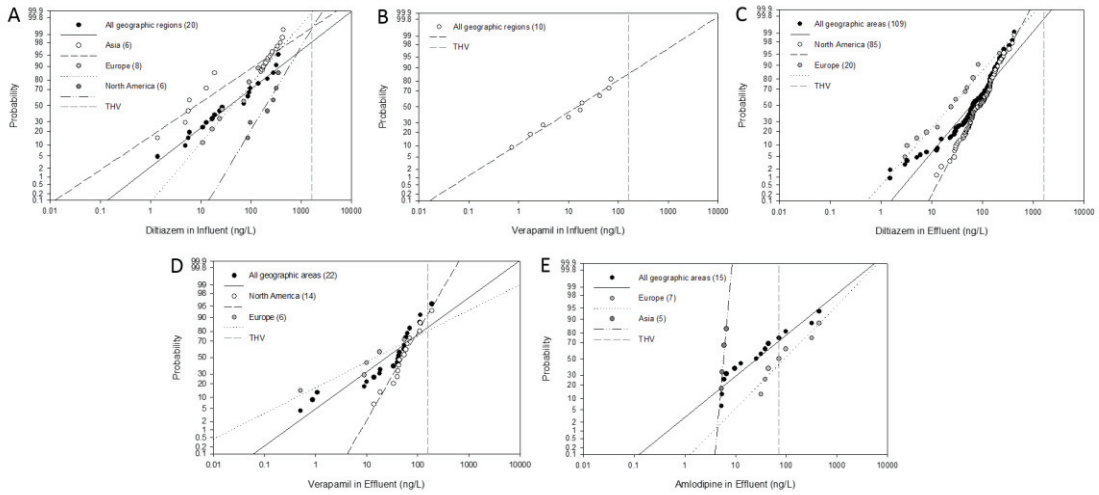

Figure 4: Environmental exposure distributions of maximum measured effluent concentrations for diltiazem, verapamil, and amlodipine across all and within specific geographic regions. Numbers within parenthesis indicate the number of detections in each geographic region. Vertical short dashed lines (red) represent the therapeutic hazard value (THV) for a calcium channel blocker.

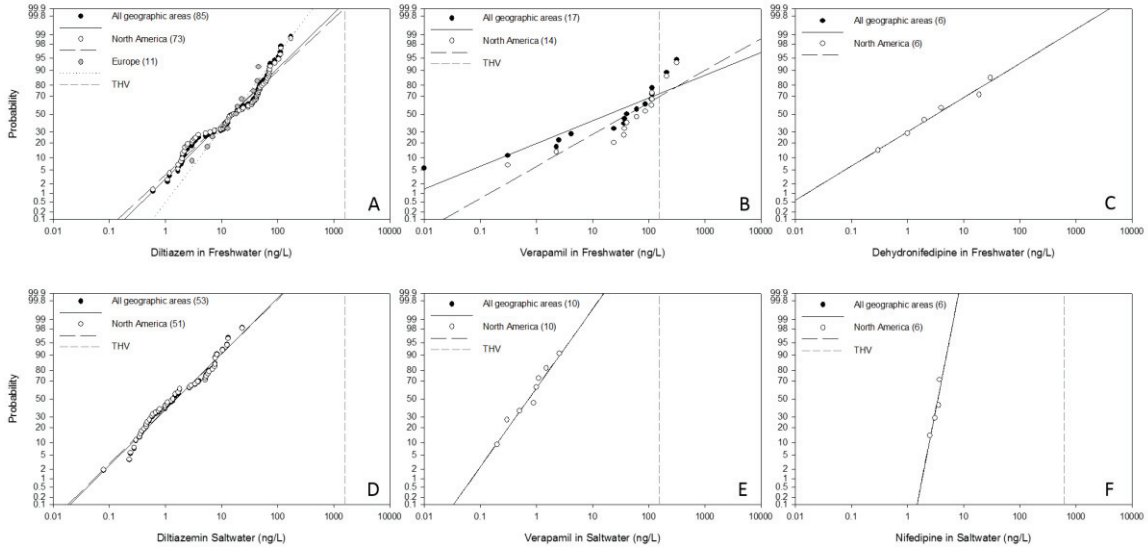

Figure 5: Environmental exposure distributions of maximum measured freshwater and saltwater concentrations for diltiazem, verapamil, and nifedipine and its metabolite dehydronifedipine across all and within specific geographic regions. Numbers within parenthesis indicate the number of detections in each geographic region. Vertical short dashed lines (red) represent the therapeutic hazard value (THV) for a calcium channel blocker.

3.2 *Calcium channel blockers in influent*

Four CCBs and several metabolites were studied in influent sewage, and only three were detected several times throughout all geographic regions (Table 4; Supplementary: Table 17). The most frequently studied compounds in influent were diltiazem (15), verapamil (9), and nifedipine (7). Most of the publications studying occurrence of CCBs in influent were from Europe (17) and North America (15). No refereed studies were found detailing the investigation of these compounds in Africa or Antarctica; only one publication was observed from South America. Diltiazem and verapamil were the most frequently studied CCBs in North America and Europe, respectively (Table 4). Additionally, over half of the studies evaluating amlodipine were from Europe. Concentrations of all CCBs in influent ranged from no detects to 1800 ng/L (diltiazem; Du et al., 2014). Whereas exposure to pharmaceuticals from untreated wastewater influent (e.g., sewage) is less commonly observed in developed countries, as noted above the majority (80%) of global sewage is released untreated to the environment (http://www.unesco.org/new/en/natural-sciences/environment/water/wwap/wwdr/).

3.3 Calcium channel blockers in effluent

Seven CCBs, including metabolites, have been reported in reclaimed wastewater effluents (Supplementary: Table 17). All seven of these compounds were detected in at least one study. Similar to influent, the most studied CCBs were diltiazem (30), verapamil (14), nifedipine (9), and amlodipine (7; Table 5). Interestingly, the primary metabolite of all four of these compounds except amlodipine have been examined (e.g., 5-2) and detected (e.g., 4-2; Table 5). Though several CCBs were studied globally,

regional occurrence differences were evident. In North America, the number of studies examining diltiazem exceeded other CCBs by a magnitude of three while other compounds were similarly studied in wastewater effluent. Diltiazem and verapamil were equally the two most studied compounds in Europe, while nifedipine was slightly more extensively examined in Asia-Pacific. Similar to influent sewage, only one study examined a CCB, nifedipine, in South America. Though amlodipine was less frequently studied among geographic regions, it had the highest occurrence concentration in effluent (448 ng/L; Huber et al., 2016) followed by diltiazem (425 ng/L; Meador et al., 2016). Subsequently, both of the highest CCB concentrations discharged in effluent were to marine systems in which elevated amlodipine was reported from a Faroe Island hospital with limited treatment before discharging to the ocean (Huber et al., 2016).

3.4 Calcium channel blockers in surface water

Similar to effluent, a total of seven CCBs and metabolites have been analyzed in global freshwater ecosystems; all were detected in at least two studies (Table 6; Supplementary: Table 17). Here again, the number of studies (37) and detections of diltiazem (30) were more than twice any other compound, followed by verapamil when compared to amlodipine and nifedipine. CCBs in freshwater were examined more often in North America (55) followed by Europe (25) (Supplementary: Table 17). However, CCBs sans diltiazem were studied more frequently in Europe (e.g., amlodipine, nifedipine, verapamil) and in Asia-Pacific (e.g., amlodipine, nifedipine) than in North America. Interestingly, in North America, more papers analyzed dehydronifedipine, the nifedipine metabolite, than the parent compound. Whereas diltiazem received the

majority of attention in total across all geographic regions, verapamil was detected at the highest concentration in freshwater (319 ng/L; Choy et al., 2016).

Though most of the global surface water occurrences of CCBs resulted from freshwater studies, seven compounds and metabolites were also reported in coastal and marine systems (Table 7). A total of 21 different studies analyzed CCBs, including diltiazem (6), amlodipine (5), and verapamil (4) (Supplementary: Table 17). All seven compounds were examined in North America followed by four substances reported from Europe. Diltiazem was the most commonly studied compound in North American coastal and marine systems, while amlodipine (4) was more commonly examined in Europe. Diltiazem was detected in six studies with the highest occurrence (23.5 ng/L; Cantwell et al., 2016) among CCBs in coastal and marine waters.

3.5 Aquatic toxicology of calcium channel blockers

Toxicity of three CCBs have been studied in non-target aquatic organisms, including amlodipine, diltiazem, and verapamil (Supplementary: Table 18). The majority of these studies have been conducted with verapamil followed by diltiazem and amlodipine. A number of standardized and non-standard experimental methods and endpoints have been employed to characterize the effects of CCBs to bacteria, invertebrates, and fish. However, the majority of these studies evaluated standard ecotoxicity endpoints (e.g., survival, growth, or reproduction), while others aimed to determine biochemical and subcellular responses to CCBs. Thus, most studies reported standard calculated toxicity threshold values (e.g., NOEC, LOEC, or EC/LC50) because the majority of these papers examined multiple CCB concentrations; conversely, several

studies evaluated less than three CCB concentrations or reported inconsistent dose-response relationships.

In the present study, sufficient data allowed for development of a species sensitivity distribution (SSD) for verapamil, but this SSD included only acute toxicity data (e.g., 96 h) (Supplementary: Table 17, Supplementary: Table 18). The 5th and 20th centiles of the verapamil SSD predicted 95% and 80% of the species would be protected from acute lethality at 0.134 mg/L and 0.895 mg/L, respectively (Supplementary: Figure: 24). Interestingly, invertebrates were more sensitive than vertebrates following acute exposures to amlodipine, verapamil and diltiazem (Supplementary: Table 18). For example, *Brachionus calyciflorus* was the most sensitive species of the reported acute verapamil toxicity studies (Supplementary: Table 18). Verapamil was the most acutely toxic CCB to vertebrates (*Oncorhynchus mykiss* 96 h LC_{50} = 2.72 mg/L) and invertebrates (*Streptocephalus proboscideus* 24 h LC_{50} = 0.5 mg/L). However, such an exercise may have limited utility because environmental concentrations of human pharmaceuticals, as confirmed here with CCBs, are well below acutely lethal levels (Berninger and Brooks, 2010).

Various sublethal endpoints were evaluated, ranging from standard growth and reproduction bioassays to feeding/ingestion rate, luminescence inhibition, and morphological changes (Supplementary: Table 18). Additionally, a number of studies reported antioxidant enzyme activity (Li et al., 2010; Steinbach et al., 2016), and haematological and blood biochemical (Keller, 2017; Steinbach et al., 2016), behavior (Kania et al., 2015) and histology (Keller, 2017; Steinbach et al., 2016) responses following acute and chronic exposures to verapamil and diltiazem. Across all endpoints

studied, verapamil, a first generation CCB, was observed to cause responses in aquatic organisms at concentrations ranging from 0.3 mg/L to 1704.8 mg/L (Supplementary: Table 18) (Lilius et al., 1994; Overturf et al., 2012). Chronic studies examining verapamil induced effects on *Pimephales promelas* growth were as sensitive as acute lethality studies (e.g., 0.6 mg/L 28 d growth LOEC; Overturf et al., 2012). Chronic exposures to verapamil elicited the most sensitive responses by vertebrates (*Pimephales promelas* 28 d growth LOEC ¼ 0.6 mg/L), while amlodipine caused the most sensitive responses by invertebrates (*Hydra vulgaris* 17 d regeneration LOEC ¼ 0.01 mg/L; Supplementary: Table 18). Unfortunately, insufficient sublethal and chronic toxicity data was available to develop chronic SSDs for CCBs. Further, CCB studies robustly examining sublethal responses linked mechanistically to therapeutic modes and mechanisms of action (Berninger and Brooks, 2010) within an adverse outcome framework (Ankley et al., 2010), which has been recommended for ecotoxicology studies of pharmaceuticals (Brausch et al., 2012), are lacking.

3.6 Probabilistic environmental hazard assessments

Sufficient CCB occurrence data for several environmental matrices among multiple geographic regions allowed for PEHAs to be conducted. EEDs were created for diltiazem and verapamil occurrences in influent sewage (Figure 4A and B) and amlodipine, diltiazem, and verapamil in effluent (Figure 4C-E). Diltiazem (109) and verapamil (22) had the highest number of occurrences followed by amlodipine (15). When comparing each CCB distribution across all geographic regions, the 20th centile value for diltiazem (22.43 ng/L) was approximately four times higher than either

amlodipine (6.10 ng/L) or verapamil (4.73 ng/L). Based on the available reported MECs, amlodipine, diltiazem, and verapamil were subsequently examined among various geographic regions. Herein, the 20th centile (29.95 ng/L) of amlodipine from Europe was three times higher than the 20th percentile (5.26 ng/L) for Asia-Pacific (Table 8). Similarly, ~5 x and ~18 x differences were observed for diltiazem and verapamil 20th values in North America compared to Europe, respectively. Subsequently, there was a greater likelihood of observing diltiazem compared to amlodipine or verapamil across all geographic regions examined (Table 8). Interestingly, sufficient data were reported for three CCB metabolites including desmethyldiltiazem (19), norverapamil (7), and dehydronifedipine (6) in WWTP effluent, but these detections were only published from North America (Table 8).

Environmental exposure distributions were created for CCBs in surface waters separately for freshwater and saltwater PEHAs (Table 8). Diltiazem had the greatest number of detections in freshwater (85) followed by verapamil (17) across all geographic regions; however, these two distributions in freshwater were very similar (20th centile values of 4.54 ng/L and 4.08 ng/L, respectively). 20th centile values were slightly higher in Europe (6.46 ng/L) compared to North America (4.17 ng/L). Sufficient verapamil occurrences were only available to create an additional distribution for North America; its 20th centile value was slightly higher than that from across all geographic regions. Here again, occurrence of desmethyldiltiazem (51) was only reported in North American freshwaters (Table 8). In saltwater systems, diltiazem (53) had the greatest number of detections followed by verapamil (10) and nifedipine (6). The 20th centile value from each CCB saltwater distribution was the lowest predicted concentration of all the

environmental matrices. Across all geographic regions, the 20th value for nifedipine (2.75 ng/L) was approximately 6 x and 9 x higher than diltiazem (0.48 ng/L) and verapamil (0.31 ng/L), respectively. Only one diltiazem detection each was reported in Europe and Asia-Pacific (Table 8).

To consider the aquatic hazards of sublethal CCB exposure to fish, PEHAs were performed to identify the probability of exceeding THVs, because mechanistic sublethal studies associated with evolutionarily conserved pharmacological targets and molecular initiation events are lacking for CCBs (Supplementary: Table 18). As noted above, THVs are predicted pharmaceutical water concentrations expected to bioaccumulate in fish plasma at a human therapeutic level (Brooks, 2014). THVs appear to present a useful diagnostic approach to identify pharmaceuticals for future research (Caldwell et al., 2014; Brooks, 2014), to examine water quality hazards of effluents from different technologies (Du et al., 2014), to monitor spatiotemporal surface water quality changes (Scott et al., 2016), and to perform global chemical scanning of environmental matrices among geographic regions (Kristofco and Brooks, 2017). Future studies are needed to examine the usefulness of this THV approach for other classes of pharmaceuticals and aquatic organisms. PEHAs using THV values for CCBs were thus initially performed across all geographic regions in all environmental water matrices (e.g., influent, effluent, freshwater, and saltwater; Table 9). While approximately 85% of all medicines are ionizable and the pH specific influence on bioaccumulation and toxicity of ionizable pharmaceuticals have been demonstrated (Valenti et al., 2009; Berninger et al., 2011; Nichols et al., 2015), we could not account for site specific pH conditions based on inconsistent information provided in the literature; thus, log K_{OW} were used for fish

plasma modeling. Recent studies have demonstrated that measured pharmaceutical uptake was better predicted by using log K_{OW} than log D (Patel et al., 2016; Nichols et al., 2015). However, multiple factors have been demonstrated to influence comparative pharmacokinetics (e.g., metabolic enzymes; Connors et al., 2013) and pharmacodynamics in fish, which presents uncertainty during environmental assessments of pharmaceuticals (Brooks, 2014; Facciolo et al., 2012; Huerta et al., 2016; Margiotta-Casaluci et al., 2014). Thus, an assessment factor of 1000 has been suggested by Huggett et al. (2003) to account for such uncertainties within and among species. Though we did not employ this recommended assessment factor in the current study, doing so would have appreciably increased the percent exceedance of CCB THVs in effluent discharges and surface waters. Future research is clearly necessary to understand ionizable chemical bioaccumulation and associated hazards to non-target aquatic organisms.

In the present study, THVs were calculated based on both minimum (C_{min}) and maximum (C_{max}) human therapeutic concentrations; these values ranged from 3 to 30 ng/mL and 15-250 ng/mL, respectively, for four CCBs (Table 9). Based on sufficient MEC data availability, predicted percent exceedances were estimated in all four water matrices for diltiazem and verapamil, but only for amlodipine and nifedipine in effluent and saltwater, respectively. CCB THVs (C_{min}, C_{max}) with the greatest likelihood of exceedance in influent was verapamil (14.4, 1.3) followed by amlodipine in effluent (28.1, 6.5) and verapamil in freshwater (27.5, 9.2). However, almost no exceedances were observed in saltwater for diltiazem, nifedipine and verapamil. Of the four CCBs examined, a THV (based on C_{min}) for amlodipine had the highest predicted percent exceedance in effluent of all the water matrices assessed. Though effluent was the only

matrix with sufficient data availability for amlodipine, detections were reported in freshwater ranging from 0.26 to 25 ng/L (de Solla et al., 2016; Huerta-Fontela et al., 2011; Varga et al., 2012). Future research should examine the aquatic hazards of this second generation CCB to non-target species based on such PEHA observations, which are influenced by its relatively high log K_{OW}, low C_{min} and C_{max} values, and the lowest THV of the four CCBs examined (Table 9).

Percent exceedances of diltiazem and verapamil THVs were observed; however, a relatively larger number of reported occurrences of diltiazem was available within all four water matrices. Though diltiazem THV exceedance was only minimally predicted in influent (1.9, 0.2), effluent (0.3, ~0), and freshwater and marine systems, its detection in fish plasma near or exceeding the C_{min} and C_{max} has been reported (Fick et al., 2010a; Scott et al., 2016; Tanoue et al, 2015; Lazarus et al., 2015; Du et al., 2014). Specifically, diltiazem and verapamil represent the only CCBs that have been examined in plasma from multiple fish species in effluents and surface waters (Table 10). Only one study was found examining the occurrence of verapamil and diltiazem in fish plasma from wastewater effluent in Europe (verapamil 0.7 ng/mL; diltiazem 0.9 ng/mL (Fick et al., 2010b)). Though the occurrence of diltiazem in fish plasma sampled from both freshwater and marine systems were predominantly reported from North America, the exceedance probability of a human C_{min} (30 ng/mL) in fish plasma from all available surface water data was 18% (Figure 6); however, such observations were strongly influenced by data from saltwater studies (Table 10; Figure 6). Thus, future research is warranted to understand the comparative pharmacokinetics and dynamics of diltiazem and other CCBs in aquatic organisms, particularly in coastal and marine systems.

Similar research appears necessary for verapamil. In the present study, predicted verapamil THV percent exceedances in influent, effluent, and freshwater were between 14.4-27.5% and 1.2-9.2% for C_{min} and C_{max}, respectively, compared to a nearly zero percent exceedance likelihood in saltwater (Table 9). The highest percent exceedance for verapamil was predicted in freshwater systems (C_{min} = 27.5%, C_{max} = 9.2%). However, only Fick et al. (2010b) has studied the occurrence of verapamil in fish plasma following exposure to WWTP effluent. In this previous effort, detections ranged from below the limit of quantification to 0.7 ng/mL (Fick et al., 2010b). Previous laboratory studies have examined bioconcentration of verapamil in freshwater fish species, in which measures in plasma and relevant pharmacological tissues were reported (Nallani et al., 2016; Steinbach et al., 2013). Here again, CCB THV exceedances of verapamil were only identified in the present study when occurrence data was sufficiently available. Future studies are necessary to address the aforementioned data gaps to enhance CCB and other pharmaceutical bioaccumulation hazards, especially in rapidly developing regions of developing countries where access to medicines are occurring faster than sustainable water resource management systems, including WWTP treatment infrastructure and resource recovery, are being implemented.

Table 8: Equations for regression lines and concentration values corresponding to various centiles for various environmental exposure distributions of maximum reported calcium channel blocker (CCB) concentrations (ng/L) in influent, effluent, freshwater, and saltwater. For each distribution, 'n' represents the number of CCBs reported and used in that specific water matrix. Based on data availability, geographic regions and individual CCBs were categorized and equation descriptors are provided.

							Centile value (ng/L)						
Matrix	**Compound**	**Geographic region**	**n**	**r^2**	**Slope**	**Intercept**	**1%**	**5%**	**10%**	**20%**	**50%**	**95%**	**99%**
Influent	All Compounds	All regions	33	0.97	1.26	-1.86	0.43	1.49	2.88	6.43	29.85	309.07	2077.86
	Diltiazem	All regions	20	0.96	1.27	-2.00	0.56	1.92	3.71	8.22	37.71	740.63	2543.20
		Asia-Pacific	6	0.90	1.10	-1.00	0.06	0.26	0.55	1.39	8.05	250.52	1041.01
		Europe	8	0.93	1.94	-3.16	2.69	6.06	9.33	15.75	42.88	303.60	683.09
		North America	6	0.88	2.72	-6.25	27.54	48.99	66.59	96.57	196.65	789.42	1404.11
	Verapamil	All regions	10	0.97	1.05	-1.22	0.09	0.40	0.88	2.32	14.75	547.89	2450.08
Effluent	All compounds	All regions	159	0.93	1.67	-2.82	1.97	5.01	8.23	15.01	47.41	448.73	1138.72
	Amlodipine	All regions	15	0.92	1.33	-1.89	0.47	1.52	2.85	6.10	26.19	451.53	1468.86
		Asia-Pacific	5	0.96	18.44	-14.13	4.37	4.76	4.98	5.26	5.84	7.17	7.81
		Europe	7	0.91	1.67	-3.28	3.74	9.57	15.79	28.95	92.32	890.40	2277.16
	Diltiazem	All regions	109	0.92	1.95	-3.48	3.90	8.71	13.36	22.43	60.45	419.58	936.34
		Europe	20	0.97	1.85	-2.58	1.37	3.20	5.03	8.70	24.84	192.93	451.09
		North America	85	0.99	3.09	-5.98	15.24	25.33	33.21	46.09	86.29	294.02	488.61
	Desmethyldiltiazem	All regions	19	0.89	2.82	-4.39	5.39	9.40	12.64	18.09	35.95	137.54	239.81
		North America	19	0.89	2.82	-4.39	5.39	9.40	12.64	18.09	35.95	137.54	239.81
	Norverapamil	All regions	7										

		North America	7										
	Verapamil	All regions	22	0.84	1.19	-1.64	0.27	1.00	2.02	4.73	24.14	584.45	2188.44
		Europe	6	0.91	0.83	-0.97	0.02	0.15	0.42	1.41	14.70	1430.17	9529.47
		North America	14	0.96	2.83	-4.84	7.73	13.46	18.09	25.87	51.32	195.76	340.90
	Dehydronifedipine	All regions	6	0.98	1.00	-0.54	0.02	0.08	0.18	0.48	3.33	145.44	695.26
		North America	6	0.98	1.00	-0.54	0.02	0.08	0.18	0.48	3.33	145.44	695.26
Freshwater	All compounds	All regions	114	0.97	1.51	-1.83	0.47	1.34	2.33	4.55	16.44	202.29	572.29
	Diltiazem	All regions	85	0.96	1.62	-1.91	0.55	1.45	2.43	4.54	14.97	154.36	405.91
		Europe	11	0.95	2.17	-2.60	1.34	2.75	4.05	6.46	15.80	90.60	186.80
		North America	73	0.95	1.53	-1.79	0.45	1.24	2.15	4.17	14.82	176.62	493.12
	Desmethyldiltiazem	All regions	51	0.75	2.07	-1.05	0.24	0.51	0.77	1.26	3.22	20.11	42.98
		North America	51	0.75	2.07	-1.05	0.24	0.51	0.77	1.26	3.22	20.11	42.98
	Verapamil	All regions	17	0.91	0.99	-1.45	0.13	0.64	1.47	4.08	28.68	1293.38	6267.35
		North America	14	0.78	0.94	-1.54	0.15	0.78	1.89	5.57	44.00	2497.52	13312.24
Saltwater	All compounds	All regions	69	0.99	1.75	-0.30	0.07	0.17	0.27	0.49	1.48	12.93	31.76
	Diltiazem	All regions	53	0.98	1.63	-0.31	0.06	0.15	0.26	0.48	1.55	15.78	41.21
		North America	51	0.97	1.61	-0.29	0.05	0.14	0.24	0.45	1.52	16.09	42.77
	Nifedipine	All regions	6	0.78	8.31	-4.49	1.82	2.20	2.43	2.75	3.47	5.47	6.61
		North America	6	0.78	8.31	-4.49	1.82	2.20	2.43	2.75	3.47	5.47	6.61
	Verapamil	All regions	10	0.95	2.31	0.32	0.07	0.14	0.20	0.31	0.72	3.74	7.38

	North America	10	0.95	2.31	0.32	0.07	0.14	0.20	0.31	0.72	3.74	7.38

Table 9: Exceedance percentages of therapeutic hazard values (THV) by measured concentrations of four calcium channel blockers in different environmental matrices. Predicted percent exceedances of the THVs were calculated using both the minimum and maximum human therapeutic level (C_{min}, C_{max}). (-) indicates an environmental exposure distribution contained < 5 data values and therefore the THV percent exceedance was not be calculated.

						Predicted percent exceedance of THV across all regions (%)			
Compound	**Log K_{OW}**	**$P_{B:W}$**	**C_{min} (ng/mL)**	**C_{max} (ng/mL)**	**THV (ng/L)**	**Influent**	**Effluent**	**Freshwater**	**Saltwater**
Amlodipine	3.30	41.9	3.0	15.0	71.6	-	28.1, 6.5	-	-
Diltiazem	2.80	18.5	30.0	130.0	1617.6	1.9, 0.2	0.3, ~0	~0/~0	~0/~0
Nifedipine	3.27	39.9	25.0	150.0	627.3	-	-	-	~0/~0
Verapamil	3.97	127.4	20.0	250.0	157.0	14.4, ,1.3	16.7, 1.2	27.5, 9.2	~0/~0

Table 10: Equations for regression lines and concentration values corresponding to various centiles for internal diltiazem fish plasma levels (ng/L) from all available surface water data, or from freshwater or saltwater systems. For each distribution, 'n' represents the number of reported fish plasma concentrations. Based on data availability, geographic regions and individual CCBs were categorized and equation descriptors are reported. Exceedance probabilities of minimum human therapeutic dose (C_{min}) in fish plasma are included.

								Centile value (ng/ml)						
Matrix	**Compound**	**Geographic region**	**n**	**r^2**	**Slope**	**Intercept**	**C_{min} Exceedance (%)**	**5%**	**10%**	**20%**	**50%**	**80%**	**90%**	**95%**
Plasma-Surface Water	Diltiazem	All regions	51	0.82	0.59	0.06	17.9	0.001	0.005	0.029	0.803	21.97	123.8	516.5
Plasma-Freshwater	Diltiazem	All regions	23	0.86	2.59	4.32	~0	0.005	0.007	0.010	0.021	0.045	0.067	0.09
Plasma-Saltwater	Diltiazem	All regions	28	0.90	2.23	-2.61	24.6	2.705	3.935	6.194	14.76	35.16	55.35	80.51

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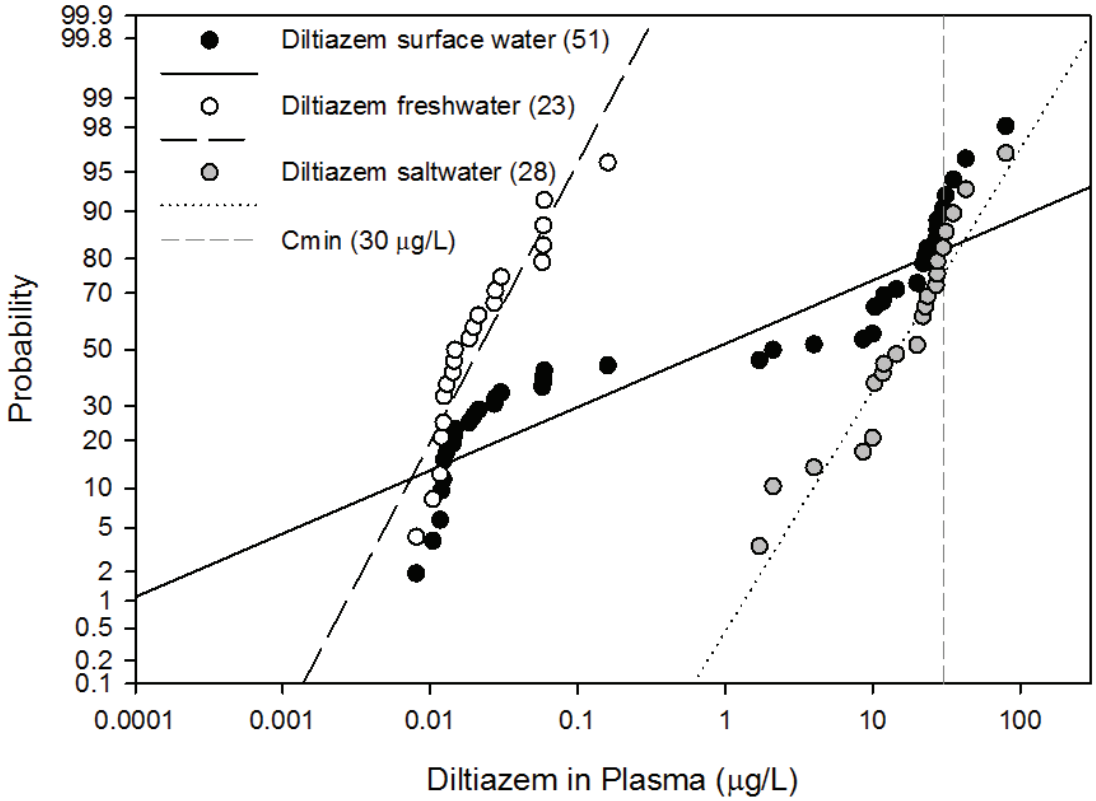

Figure 6: Probabilistic hazard assessment of mean measured fish plasma diltiazem concentrations from several freshwater and saltwater species across all geographic regions. Numbers within parenthesis indicate the number of detected diltiazem concentrations across all geographic regions within each water matrix. The vertical small dashed line (red) represents the diltiazem minimum human therapeutic plasma level (C_{min} = 30 ng/mL).

3.7 Probabilistic hazard assessments of CCB mixtures

Though seventy-nine studies have examined the occurrence of CCBs in global water matrices, only six studies examined the same CCBs within a common matrix. Diltiazem and verapamil were specifically examined 39 and 24 times in studies analyzing their occurrence in effluent in which the number of detections were 27 and 20, respectively. Geographically, the majority of studies examining and detecting both diltiazem and verapamil of effluent were from North America (3) followed by Europe (2)

and Asia (1). Across all percentiles, diltiazem effluent hazard concentrations were almost 3 times higher than verapamil (Table 11). However, the calculated THV for verapamil (157 ng/L) was approximately 10 times lower than diltiazem (1618 ng/L) and thus contributed a greater percentage to the additive THR (Table 11; Figure 7). At the 80th percentile of diltiazem and verapamil distributions, the additive THR exceeded 1.0 corresponding to 321.2 and 127.1 ng/L, respectively. At these two concentrations, diltiazem and verapamil contributed 19.9 and 81.0%, respectively, to the THR of 1.0, corresponding to 425 and 190 ng/L, respectively. Conversely, at the 20th centile of diltiazem and verapamil distributions, the additive THRs were 0.06 or 6%, respectively (Table 11). CA predictions have been reported for pharmaceutical mixtures with compounds of the same mechanism of action (Backhaus, 2014; Christensen et al., 2007; Cleuvers, 2004, 2005; Fent et al., 2006). These studies used standard model organisms and endpoints with crustaceans, algae, and in vitro assays to support CA modeling, while few studies have characterized the alternative sub-lethal effects (e.g., therapeutic) of these pharmaceutical mixtures. Clearly future research should assess pharmacological sub-lethal endpoints corresponding to therapeutic and side effect mechanisms and modes of action (Boxall et al., 2012; Rudd et al., 2014).

Table 11: Diltiazem and verapamil 5th, 20th, 50th, 80th, and 95th percentiles with corresponding concentrations (ng/L) are presented from environmental exposure distributions. Therapeutic hazard ratios (THR) of predicted calcium channel blocker concentrations to compound specific therapeutic hazard values (THV) were calculated. A THR value > 1.0 indicates an exceedance of an additive THR. Diltiazem THV (ng/L) = 1618; Verapamil THV (ng/L) = 157.

	Predicted calcium channel blocker concentration (ng/L)		Predicted calcium channel blocker concentration:THV ratio		
Percentile	**Diltiazem**	**Verapamil**	**Diltiazem**	**Verapamil**	**Additive THRs (%)**
5th	4.7	1.7	0.003	0.011	0.014
20th	18.5	6.9	0.011	0.044	0.055
50th	77.1	29.7	0.048	0.189	0.237
80th	321.2	127.1	0.199	0.810	1.009
95th	1254.5	510.2	0.776	3.249	4.025

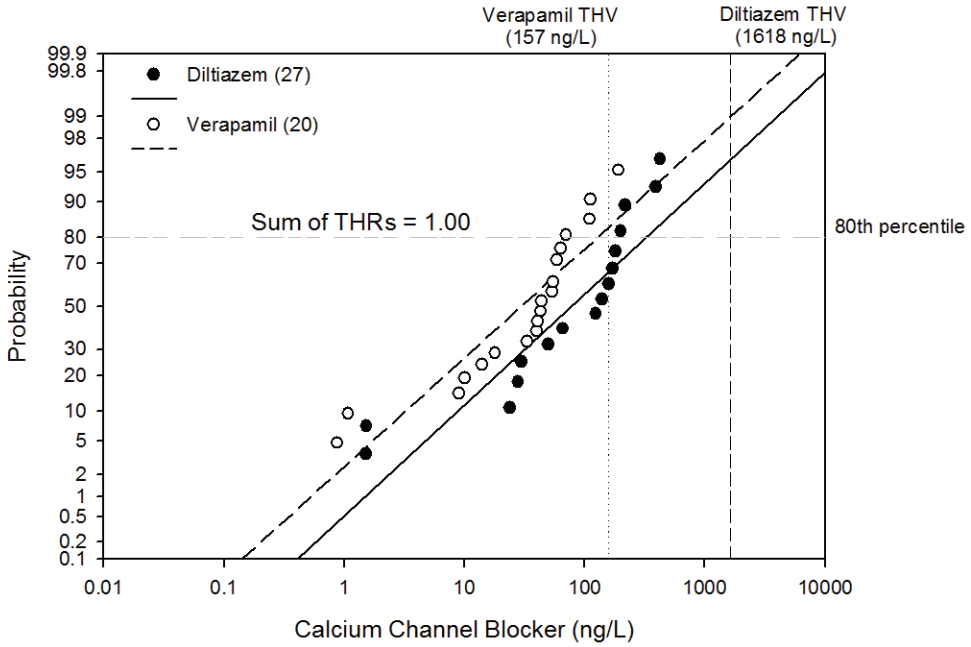

Figure 7: Probabilistic hazard assessment of maximum measured environmental concentrations for diltiazem and verapamil in effluent reported from the same study across all geographic regions. Numbers within parenthesis indicate the number of each detected CCB across all geographic regions. Vertical dotted lines (black) represents the therapeutic hazard value (THV) for verapamil (157 ng/L) and the vertical short dashed line (black) represents the THV for diltiazem (1618 ng/L). Horizontal short-short-long dashed lines (gray) represent percentile value when an additive therapeutic hazard ratio (THR) equaled 1.

4. Conclusions

Here we examined refereed literature on the occurrence and ecotoxicology of CCBs in wastewater and surface water matrices. One hundred and sixty one primary literature articles reported the examination, occurrence, and effects of four CCBs in effluent, sediment, sludge, and aquatic systems. Approximately half of the matrices studied for the occurrence of CCBs were for water from North America, Europe, and Asia-Pacific. Environmental occurrence of these compounds were scarce and nonexistent in South America and Africa, respectively. In addition, studies examining CCBs in influent and coastal and marine systems were relatively limited. Whereas occurrence of diltiazem and verapamil have been routinely examined in water matrices, studies of other

first and second generation CCBs (e.g., nifedipine, amlodipine) are lacking. Concentrations of CCBs in non-target organism were minimally examined, except for diltiazem (e.g., tissue and plasma), which has been reported to accumulate in fish plasma above human therapeutic plasma levels. Thus, further studies are necessary to understand comparative bioaccumulation and toxicity of CCBs in nontarget aquatic organisms.

Unfortunately, very few studies have examined the pharmacological cardiovascular effects of CCBs in non-target aquatic organisms, but recent efforts have investigated tissue specific oxidative, metabolic, antioxidant, or histological induce effects in several teleosts. Calcium and calcium channels play a role in multiple biological processes within all organisms (Reuter, 1983). As medications for treatment of high blood pressure and cardiac arrhythmias CCBs have been specifically designed to target smooth muscle tissue calcium channels and elicit therapeutic benefits in humans (Goodman, 1996). However, a common drug target for CCBs (e.g., diltiazem) is predicted to be 70-76% conserved in teleost species (Gunnarsson et al., 2008). Such functional conservation of drug targets in non-target organisms remains an understudied topic, yet the application of mammalian to fish biological read across (Brooks et al., 2009) has been demonstrated with select pharmaceuticals (Brodin et al., 2013; Huerta et al., 2016; Huggett et al., 2003; Margiotta-Casaluci et al., 2014, 2016; Rand-Weaver et al., 2013; Valenti et al., 2012). It is important to note, however, that each CCB has multiple targets, varying with known and relatively unstudied pharmacological actions (Wishart et al., 2006). Therefore, while CCBs are specifically designed to elicit cardiac therapeutic effects in humans, these diverse molecular initiation events require future comparative

ecotoxicological study, particularly focusing on linkages (or lack thereof) to ecologically important adverse outcomes.

When data availability was sufficient, assessments estimating aquatic hazards of CCBs were performed. EED 5th and 95th percentiles for all CCBs were 1.5 and 309.1 ng/L in influent, 5.0 and 448.7 ng/L for effluent, 1.3 and 202.3 ng/L in freshwater, and 0.17 and 12.9 ng/L in saltwater, respectively. Because sublethal chronic toxicity information for CCBs were limited, we employed THVs during PEHAs. We observed both amlodipine in effluents and verapamil in freshwaters to exceed THVs without a safety factor 28% of the time, highlighting the need to understand ecological consequences of exposure to these CCBs. We then employed an additive THR approach to examine CCB mixtures. Based on currently available data, an additive THR exceeded 1 approximately 20% of the time, which suggests these CCBs and other pharmaceutical mixtures with common molecular initiation events deserve future investigation. Ecological implications of such occurrence and toxicity gaps for CCBs are unknown but deserve further study, particularly in rapidly urbanizing regions that are vulnerable to climate change. This global scanning approach identified the utility of global assessments to identify specific CCBs, mixtures with common mechanisms of action, and geographic locations for which environmental monitoring and assessment efforts appear warranted.

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CHAPTER FOUR

Influence of Diltiazem on Fathead Minnows Across Dissolved Oxygen Gradients

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ABSTRACT

Water resources in many arid to semi-arid regions are stressed by population growth and drought. Growing populations and climatic changes are influencing contaminant and water chemistry dynamics in urban inland waters where flows can be dominated by, or even dependent on, wastewater effluent discharge. In these watersheds, interacting stressors such as dissolved oxygen (DO) and environmental contaminants (e.g., pharmaceuticals) have the potential to affect fish physiology and populations. Recent field observations from our group identified the calcium channel blocker diltiazem in fish plasma exceeding human therapeutic doses (e.g., C_{min}) in aquatic systems impaired due to nonattainment of DO water quality standards (WQS) and criteria. Thus, our study objectives examined: 1) standard acute and chronic effects of DO and diltiazem to fish, 2) influences of DO, at criteria levels deemed protective of aquatic life, on diltiazem toxicity to fish, and 3) whether sublethal effects occur at diltiazem water concentrations predicted to cause a human therapeutic level in fish plasma (therapeutic hazard value, THV). DO x diltiazem co-exposures significantly decreased survival at typical stream, lake, and reservoir WQS of 5.0 and 3.0 mg DO/L. DO and diltiazem growth effects were observed at 2x and 10x their LC50 values (1.7 and

28.2 mg/L, respectively). Larval fathead minnow swimming behavior following all DO and diltiazem exposures generally decreased and significantly reduced light:dark bursting distance traveled, number of movements, and duration at concentrations as low as the THV. Individual and population level consequences of such responses are not yet understood; however, these observations suggest that assessments with pharmaceuticals and other contaminants may underestimate the effects in fish across DO levels considered protective of aquatic life.

INTRODUCTION

Surface water quality assessment and management in urban areas is challenging, particularly in watersheds receiving wastewater treatment plant (WWTP) discharges and nutrient enrichment (Brooks et al., 2006; Berninger et al., 2011; Haggard et al., 2005; Nakamura et al., 2008; Taylor, 2002; Valenti et al., 2009; Waiser et al., 2011). Excessive nutrients in conjunction with climate change exacerbates select harmful algal blooms and causes eutrophication that depletes dissolved oxygen (DO) levels in freshwater and marine ecosystems (Breitburg, 2002; Waiser et al., 2011). In these urban systems, diverse organic contaminants, including pharmaceuticals, are continuously released from WWTP, which results in life cycle exposures to aquatic organisms especially in effluent-dominated and dependent ecosystems (Brooks et al., 2006). Such watersheds often experience hypoxic events, which has received increasing attention in marine and coastal systems. Unfortunately, hypoxia events have received relatively little attention in freshwater ecosystems (Pollock et al., 2007). Our recent research (Saari et al., 2018) identified exceedances of low DO thresholds and differential implementation of DO

criteria in a geographic region experiencing rapid population growth and severe droughts over the past decade. Unfortunately, interactive effects of low DO and pharmaceuticals have received even less study.

Understanding aquatic responses to chemical and nonchemical stressors was recently highlighted as a priority research question to understand risks of pharmaceuticals in the environment (Boxall et al., 2012). Fish responses to chemical stressors (e.g., ammonia, metals, polycyclic aromatic hydrocarbons) have been shown to be influenced by low DO (Fleming and Di Giulio, 2011; Hattlink et al., 2005; Lyu et al., 2013; Matson et al., 2008; Prokkola et al., 2015). Recently, our group and others have observed concentrations of the calcium channel blocker diltiazem in fish plasma approaching and even exceeding human therapeutic plasma levels (Du et al., 2014; Fick et al., 2010a; Scott et al., 2016; Tanoue et al., 2015). Consequences of such observations are unknown, but indicate therapeutic risks to fish. For example, approaches have been developed to predict steady state fish plasma uptake and internal doses of pharmaceuticals. These approaches were developed from physiological-based pharmacokinetic modeling and the functional conservation of pharmaceutical targets across vertebrates (Brooks, 2014; Du et al., 2014; Fitzsimmons et al., 2001; Gunnarsson et al., 2008; Huggett et al., 2003). Using these models, therapeutic hazard values (THV) can be derived to identify water concentrations predicted to result in fish plasma levels of medicines equaling human therapeutic doses (Berninger et al., 2011; Brooks, 2014; Fick et al., 2010b). Recently, Saari et al (2017) examined global occurrence and associated hazards of calcium channel blockers in multiple environmental matrices. Interestingly, environmental exposure distributions of untreated sewage was the only matrix predicted to exceed the diltiazem THV (C_{min} =

1.9%; C_{max} = 0.2%), yet diltiazem in fish plasma levels from the field exceeded C_{min} doses 17% of the time (Saari et al., 2017). Whether such human therapeutic plasma concentrations in fish result in adverse outcomes are unknown, particularly in urbanized watersheds already impaired due to nonattainment of DO water quality standards (Brooks et al., 2006; Scott et al., 2016).

The objectives of the present study were to examine whether DO influences toxicity of diltiazem in fish. We initially investigated individual responses of the larval fathead minnow (*Pimephales promelas*), a common fish model, to DO and diltiazem. We then examined whether DO, at current water quality criterion values for inland waters, influenced acute and chronic toxicity of diltiazem.

MATERIAL AND METHODS

To address our study objectives, the following subsections describe how experimental conditions were maintained and traditional morphometric (e.g., mortality, growth) and nontraditional sublethal (e.g., heart rate, feeding rate, photo-locomotor behavior) responses of *Pimephales promelas* to DO, diltiazem or DO x diltiazem mixtures were designed and measured. Standard DO experimental systems and protocols are lacking and thus typical acute and chronic toxicity methods were used with minor modifications. Herein, appropriately manipulating DO represented a critically important experimental consideration. DO water concentrations were regulated by mixing both nitrogen gas and air that were then infused in each experimental chamber, conceptually similar to approaches described by Ho and Burggren (2012) and Zhou et al (2000). Gas was regulated using RiteFlow meters (Scienceware Bel-Art Products, Wayne, NJ, USA).

Two to five different DO concentrations were maintained consistently and continually with individual nitrogen and air Riteflow meters per treatment level. Each N2-air regulator pair delivered gas that was mixed in sealed PVC chambers (10.2 x 61.0 x 0.3 cm; 4" x 24" x 1/8") filled with bioballs. A single outflow line ran mixed gas to a climate controlled walk-in incubator to a 6-port manifold where gas infusion levels were manually adjusted to achieve desired DO treatment levels. Gas was then bubbled in semi-sealed experimental chambers (e.g., 750 mL Mason jars, 20 L glass tanks).

Larval Pimephales promelas *Experiments*

In the present study, all acute and chronic experiments were performed with reconstituted hard water (RHW) made according to U.S. Environmental Protection Agency (EPA) methods (U.S., 2002a; b). All experiments were carried out in a climate controlled environmental chamber at a constant temperature of 25 ± 1 ^{0}C with a 16:8 h light-dark cycle on a backup power supply. Water chemistry analyses were performed at initiation of each study and on renewal days of chronic studies according to standard methods (Association, 1989). For experiments with DO, measurements were taken multiple times daily with a YSI ProODO optical DO sensor (YSI Inc., Yellow Springs, Ohio, USA). Experimental treatment levels of DO and diltiazem were informed from the literature and preliminary acute range finding studies with larval *Pimephales promelas* reared at Baylor University in accordance with Institutional Animal Care and Use Committee guidelines.

Acute studies

Triplicate 48 h toxicity studies were conducted individually with DO and diltiazem (U.S. EPA, 2002a). Briefly, each experiment was carried out using 750 mL glass chambers (semi-sealed Mason jars) with 10 organisms per unit and four replicates per treatment level. Nominal DO (mg/L) treatment levels included 8.2 (control), 5.0, 3.0, 2.0, 1.0, and 0.5. Nominal diltiazem treatment levels were 0, 0.00015 (diltiazem THV), 15, 30, 45, 60 mg/L. Primary endpoints for these acute studies with either DO or diltiazem included mortality and heart rate. Photo-locomotor behavioral responses (PLR) to these DO treatment levels were also examined.

After completing these triplicate experiments with either DO or diltiazem, individual acute 48 h studies examining DO x diltiazem interactions were then separately completed with identical diltiazem treatment levels (0, 0.00015, 15, 30, 45 mg/L), under normoxic DO levels (8.2 mg DO/L) and DO manipulated at either 5.0 or 3.0 mg DO/L. Hereafter, 8.2 mg DO/L, 5.0 mg DO/L, and 3.0 mg DO/L treatment levels will be described as normal, moderate, and low DO when referring to interactive studies. The 0.00015 mg/L diltiazem treatment level was equal to the THV, which is a water concentration predicted to bioconcentrate in fish plasma to a human therapeutic level (Berninger et al., 2011; Brooks, 2014). This conceptual approach was first proposed by Huggett et al (2003) to estimate fish plasma steady state pharmaceutical levels resulting from aqueous exposures. Initial plasma modeling by Fitzsimmons et al. (2001) predicted fish blood:water partition coefficeints of hydrophobic compounds. Diltiazem is a weak base; therefore, the initial fish uptake model in the present study was modified to account for the experimental pH (8.3) by using log D instead of log P (Berninger et al., 2011).

The human diltiazem minimum (C_{min} = 30 ng/mL) therapeutic level was obtained from Schulz et al (2012). Thus, for diltiazem THV calculations, a water concentration predicted to bioconcentrate to the human C_{min} was employed (Brooks, 2014; Du et al., 2014).

Experimental units for acute studies were each filled with 500 mL of treatment water. Less than 24 h post hatch (hph) *P. promelas* were used in each study. Fish were fed newly hatched brine shrimp (*Artemia sp*.) nauplii 2 hours before each study and were not fed throughout each experiment. After 48 h exposure, 5 fish from each replicate were randomly selected for heart rate measurements. Fish were anesthetized with 50 mg/L MS-222 and 100 mg/L sodium bicarbonate for 3 minutes. When unresponsive, fish heart rates were counted visually via dissection microscope by recording ventricular beats for 10 seconds during three separate measurements (Finn et al., 2012). Preliminary studies indicated no significant differences in fish heart rates between 3 minute anesthetized and non-anesthetized fish. Replicate units were examined individually within approximately five minutes. The remaining 5 fish in each replicate were used for PLR behavior evaluations under a 2-cycle light-dark assay.

Similar to previously published methods from our laboratory by Kristofco et al (2016) and Steele et al. (2018), behavioral observations were recorded in quantization mode using Zebrabox and accompanying Zebralab tracking software (ViewPoint, Lyon, France). Calibration parameters for the plate and pixel detection thresholds were: plate width = 125 mm; pixel detection thresholds = black; movement thresholds: resting = < 5 mm/s; cruising = 5-20 mm/s; bursting = > 20 mm/s; data bin = one minute. To reduce background noise from reflections on well walls, tracking was set to refresh after each

one minute bin and movement thresholds were set at ≥ 2 pixels. Treatment water from each replicate was added (2 mL) to respective plate wells and one fish per well was added randomly from each treatment replicate to a 24-well plate. All treatment levels were included on each plate. Fish behavior was recorded for 50 minutes and included a 10 minute dark acclimation period followed by 2 light-dark cycles, 10 minutes per light or dark period. Data recorded during the 10 minute acclimation period were not included in analyses. All observations were collected in afternoon to evening hours.

Chronic studies

Short-term 7 day chronic *P. promelas* studies were then conducted using 24 hph fish according to U.S. EPA methods with minor modifications (U.S. EPA, 2002b). Individual experiments were conducted separately for DO, diltiazem, and DO x diltiazem. Initial *P. promelas* chronic studies were conducted with DO in 20 L tanks with 3 L water volumes. These observations informed diltiazem and DO x diltiazem interactive studies, which were performed in 750 mL experimental units. Chronic studies consisted of daily static renewals with either DO adjusted water or diltiazem stock solutions (prepared at time zero, stored in the dark at 4 °C). For the chronic DO study, nominal DO treatment levels (mg/L) were 8.2 (control), 5.0, 4.0, 3.0, and 2.0. Chronic diltiazem treatments were derived from a mean 48 h LC50 value, based on the triplicate studies described above, and subsequent nominal concentrations were determined following 10-fold dilution, including 0, 0.03394, 0.3394, 3.394, 33.94, 339.4, 3394, 33940 µg/L.

Two interactive nominal DO x diltiazem studies were conducted at normal control (8.2 mg DO/L) and either moderate (5.0 mg DO/L) or low (3.0 mg DO/L) treatment levels across five diltiazem treatment levels including 0, 0.3394, 33.94, 339.4, and 3394 µg/L. Fish were fed newly hatched brine shrimp (*Artemia sp*.) nauplii twice daily. Study endpoints included survival, growth, heart rate, and feeding and PLR behavior. Briefly, heart rates were observed as described above with four fish from each replicate and anesthetized with 67 mg/L MS-222 and 133 mg/L sodium bicarbonate for 3 minutes. Again, each beaker was processed within approximately five minutes. Four fish from each replicate were then used for PLR observations under a 2-cycle light-dark assay following the methods introduced above or feeding rates. Fish from heart rate and PLR observations were then employed for traditional growth (dry weight) measurements following previously reported methods (Stanley et al., 2007; U.S. EPA, 2002b).

Feeding rates were assessed following 7 days of exposure according to previous methods (Stanley et al., 2007). Food was withheld from fish 24 h prior to study initiation. Feeding rates were determined by enumerating brine shrimp nauplii consumed over 15 minutes. Two fish per treatment were randomly selected from each of four replicates. Individual fish from each treatment were placed in 100 mL beakers of clean RHW for an hour before adding 25 brine shrimp nauplii. After 15 minutes, fish were removed and the remaining brine shrimp were recorded. Feeding rates were calculated as the number of consumed brine shrimp nauplii per minute. The number of artemia consumed by two randomly selected fish per replicate was used to calculate mean feeding rate. Fish used in feeding rate studies were not included in growth measurements.

Chemical and Analytical Quantification of Treatment Levels

Diltiazem hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO, USA; CAS # 33286-22-5; purity: ≥ 99%). Experimental treatment levels in each acute and chronic diltiazem experiment were analytically verified by liquid chromatography tandem mass spectrometry (LC-MS/MS) on an Agilent Infinity 1260 autosampler/quaternary pumping system, Agilent jet stream thermal gradient electrospray ionization source, and model 6420 triple quadrupole mass analyzer. Briefly, a 500 µL aliquot of undiluted or diluted stock solution were combined with 450 µL of 0.1% formic acid (w/w) and spiked with 50 µL of an internal standard (diltiazem-d3) in a standard 2 mL analytical vial (Agilent Technologies, Santa Clara, CA, USA) before analyses. A gradient mobile phase condition that resulted in the elution of diltiazem at 4.2 minutes was identified. Salts and other highly polar sample constituents were diverted to waste and away from the MS/MS during the first minute of each sample run. Chromatography was performed using a 10 cm × 2.1 mm Poroshell 120 SB-AQ column (120Å, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA) preceded by a 5 mm × 2.1 mm Poroshell 120 SB-C18 attachable guard column (120Å, 2.7 µm, Agilent Technologies, Santa Clara, CA, USA). The ionization mode, monitored transitions, and instrumental parameters for diltiazem/diltiazem-d3 were as follows: ESI+ diltiazem 415.2 > 150, fragmentor = 140, collision energy = 50, and diltiazem-d3 418 > 177.9, fragmentor = 135, collision energy = 28.

Limit of detection (LOD) and limit of quantification (LOQ) were determined by running several method blanks and calculating the standard deviation. LOD and LOQ for diltiazem were determined to be 0.009 µg/L and 0.026 µg/L respectively. Ten standards,

ranging in concentration from below LOQ to 500 µg/L, were used to construct a linear calibration curve ($r2 \geq 0.998$). Instrument calibration was monitored over time via analysis of continuing calibration verification (CCV) samples, which were run every five samples, with an acceptability criterion of ±20%. Calibration standards were prepared in RHW and calibration verification samples were prepared in 0.1% formic acid (v/v). Ultrapure water, Thermo Barnstead Nanopure Diamond UV (Dubuque, IA, USA) water purification system with 18 MΩ, RHW, and 0.1% formic acid were run to validate the purity of solutions and as method blanks.

Statistical analyses

Diltiazem and DO lethal concentration (LC50) values, based on analytically verified observations, were calculated using the Toxicity Relationship Analysis Program (TRAP; version 1.30). Sigma Plot 11.0 software (Systat Software 323 Inc., San Jose, CA, USA) was used for all other statistical analyses. Prior to analysis, data normality and equal variance tests were performed. If normality and equal variance assumptions were not met, data values were transformed (e.g., log, square root). Experimental responses were evaluated using $\alpha = 0.05$. Lowest-observable-effect concentrations (LOEC) were identified using one-way analysis of variance (ANOVA) with Dunnett's post hoc test to identify treatment level effects. Mean behavioral responses such as distance traveled, number of movements (counts), and duration were calculated across 1 minute intervals (bins). Total (e.g., distance traveled and count) and individual behavioral responses of separate and interactive DO and diltiazem studies were determined across light and dark photoperiods and compared. For data not meeting ANOVA assumptions following

transformation, ANOVA on ranks were performed. Interactive effects of DO x diltiazem exposure were determined by Two-Way ANOVA with Holm-Sidak post-hoc tests relative to treatment control.

RESULTS

Acute studies

Treatment levels of DO and diltiazem were verified within 95-140% and 17-92% (Supplementary: Table 19), respectively, of nominal concentrations. Therefore, all toxicity point estimates were calculated using measured concentrations of diltiazem and DO. Water quality parameters from each study were within acceptable ranges (Supplementary: Table 20). Control survival was 100% for all 48 h acute studies. Acute toxicity studies showed dose-dependent responses to DO, diltiazem, and DO x diltiazem treatment levels. For individual studies with DO or diltiazem, mean (±SD; n=3 studies) 48 h LC50 values were 1.7 (±0.1) and 35.1 (±0.9) mg/L, respectively (Figure 8A-B). For interactive DO x diltiazem studies, LC50 values at normal and moderate DO levels were 30.5 mg/L and 16.0 mg/L, whereas diltiazem LC50 values were 28.2 mg/L and 8.3 mg/L at normal and low DO levels, respectively (Figure 8C). No significant differences in heart rate were observed in DO treatments down to 2.3 mg/L (< 90% survival at 1.1 mg/L; SI Figure 25A) but 12 mg/L diltiazem significantly ($p<0.05$) decreased fish heart rates (LOEC; Table 12; SI Figure 25B). Decreases in heart rate were similarly reproduced at 13 mg/L diltiazem (LOEC) at both normal and moderate DO concentrations, and at 12.5 mg/L at normal and low DO levels. Interactive effects of DO on heart rate across diltiazem treatments were insignificant (SI Figure 26A-B; $p > 0.05$; Two-way ANOVA).

Mean PLR behavioral responses of unexposed or naïve *P. promelas* were visualized from five acute studies across multiple speed categories and endpoints/metrics (e.g., total distance and count, resting duration) over 2-cycle light:dark periods. Mean (±SE) naïve fish distance traveled across 2-light cycles was slightly greater (13.4±1.1 mm/s) than dark (11.2±1.2; Figure 9A). Similar light and dark trends were observed in distance traveled, total number of movements (counts), and duration of activity across stimulatory, cruising and refractory speed categories. Fish behavioral responses across all DO conditions were generally significant different from controls ($p > 0.05$), although decreasing trends were observed for fish activity in the light and dark (e.g., total number of movements, duration, bursting distance traveled; Figure 10A-C, SI Figure 27A-C and 28A-C). Interactive DO x diltiazem significantly ($p< 0.05$) decreased activity (e.g., distance traveled, counts, duration) across both light and dark, although responses in the light and dark were not always monotonic (e.g., normal and moderate DO; Figure 12A-F and 13A-F, SI Figure 31-34A-F). Significant differences in activity were generally more sensitive in the dark (e.g., LOEC) than the light with significant responses observed at the THV and 26297 µg/L, respectively (Table 13).

Table: 12. Acute (48 h) LC_{50} values, and chronic (7 d) Lowest Observed Effect Concentrations (LOEC) and No Observed Effect Concentrations (NOEC) for heart rate, feeding rate and growth responses following dissolved oxygen (DO), diltiazem (DZM), and DO x DZM studies with *Pimephales promelas*.

		LOEC, NOEC concentrations (µg/L)			
	Mean 48 h LC_{50} (mg/L)	**48 h Heart Rate (beats/min)**	**7 d Growth (mg/org)**	**7 d Feeding Rate (prey/min)**	**7 d Heart Rate (beats/min)**
DO	[a]1.7 ±0.1	[b]NA, 2.3	4.3, 5.1	[b]NA, < 3.2	4.3, 5.1
DZM	[a]35.1 ±0.9	[c]12000, 0.1	2356, 278	>2356, >2356	[d]NA
8.2 mg DO/L x DZM	30.5	13000, 0.11	2215, 259	>2215, >2215	[e]259, 23
5.0 mg DO/L x DZM	16.0	13000, 0.11	2215, 259	>2215, >2215	259, 23
8.2 mg DO/L x DZM	28.2	12506, 0.084	2348, 277	>2348, >2348	277, 26
3.0 mg DO/L x DZM	8.3	>0.084, >0.084	2348, 277	>2348, >2348	2348, 277

(a) Indicates mean (±SD, N=3 studies). (b) Indicates a LOEC was not calculated because the concentration above the NOEC resulted in significant mortality (1.1 mg/L). (c) Indicates mean heart rates for 3 studies. (d) Insufficient data for statistical analysis (N=2 per concentration). (e) Significant increase in heart rate at 0.259 µg/L (LOEC), thus the NOEC was 23 µg/L; however, a significant decrease in heart rate was observed at 2215 µg/L.

Table: 13. Acute (48 h) LC_{50} values, and chronic (7 d) Lowest Observed Effect Concentrations (LOEC) and No Observed Effect Concentrations (NOEC) for photo-locomotor behaviors following dissolved oxygen (DO), diltiazem (DZM), and DO x DZM studies with *Pimephales promelas*. Arrows indicate whether responses were significantly ($p< 0.05$) increased or decreased relative to control.

Acute Behavioral Response LOECs (Light / Dark; µg/L)											
	TD	**RD**	**CD**	**BD**	**TC**	**RC**	**CC**	**BC**	**RD**	**CD**	**BD**
DO	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	(↓) 3.4 / -	- / -	- / -
DZM	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
8.2 mg DO/L x DZM	(↓) 26297 / (↑) 13000	(↓) 26297 / (↓) 26297	(↓) 26297 / (↓) 0.112	(↓) 26297 / (↓) 26297	(↓) 26297 / (↓) 0.112	- / -	(↓) 26297 / (↓) 0.112	- / (↓) 26297	- / -	(↓) 26297 / (↓) 0.112	- / (↓) 26297
5.0 mg DO/L x DZM	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
8.2 mg DO/L x DZM	(↓) 12506 / -	(↓) 20418 / (↓) 12506	(↓) 12506 / (↓) 0.084	- / -	(↓) 12506 / (↓) 0.084	(↓) 12506 / (↓) 0.084	(↓) 12506 / (↓) 0.084	(↓) 12505 / -	- / (↑) 0.084	(↓) 12506 / (↓) 0.084	(↓) 12506 / -
3.0 mg DO/L x DZM	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
Chronic Behavioral Response LOECs (Light / Dark; µg/L)											
DO	(↑) 3.4 /	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
DZM	(↑) 26 / -	- / -	(↑) 26 / -	- / -	(↓) 2356 / (↓) 2356	- / (↓) 2356	(↓) 2356 / (↓) 2356	- / (↓) 2356	(↓) 2356 / (↓) 2356	- / -	- / (↓) 2356
8.2 mg	- / -	- / (↑)	- / -	- / -	- / -	- / -	- / -	(↓) 23 /	- / -	- / -	(↓) 23 / -

DO/L x DZM		23						(↓) 2215			
5.0 mg DO/L x DZM	- / -	- / -	- / -	- / -	- / -	- / -	- / -	(↓) 0.1 / -	- / -	- / -	(↓) 23 / -
8.2 mg DO/L x DZM	- / -	(↑) 26 / (↑) 277	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -	- / -
3.0 mg DO/L x DZM	- / -	- / -	- / -	- / -	- / -	- / -	- / -	(↓) 0.1 / (↓) 0.1	- / -	- / -	(↓) 0.1 / (↓) 0.1

DO: dissolved oxygen; DZM: diltiazem; 8.2: normal 8.2 mg DO/L; 5.0: moderate 5.0 mg DO/L; 3.0: low 3.0 mg DO/L. (-): indicates no significant effects observed. TD: sum total distance traveled per minute across all speed categories; RD: resting (<5 mm/s) distance traveled per minute; CD: cruising (5-20 mm/s) distance traveled per minute; BD: bursting (>20 mm/s) distance traveled per minute. TC: total number of movements (counts) per minute across all speed thresholds; RC: resting (<5 mm/s) number of movements (counts) per minute; CC: cruising (5-20 mm/s) number of movements (counts) per minute; BC: bursting (> 20 mm/s) number of movements (counts) per minute. RD: resting (<5 mm/s) duration (sec.) per minute; CD: cruising (5-20 mm/s) duration (sec.) per minute; BD: bursting (>20 mm/s) duration (sec.) per minute.

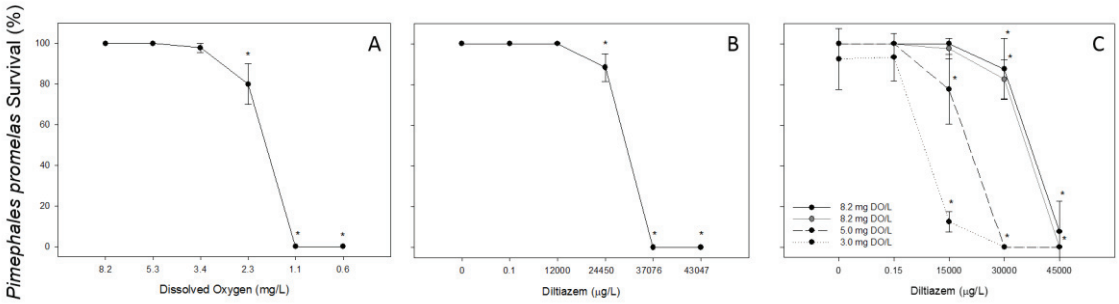

Figure: 8. Mean (±SD, N=3 studies) percent survival by *Pimephales promeals* larvae following 48 h A) dissolved oxygen (DO), B) diltiazem or C) DO x diltiazem studies. Moderate and low DO x diltiazem studies were conducted in separate experiments (C) with a normal DO treatment (black circles = normal and moderate DO x diltiazem study; gray circles = normal and low DO x diltiazem study). *: $p < 0.05$.

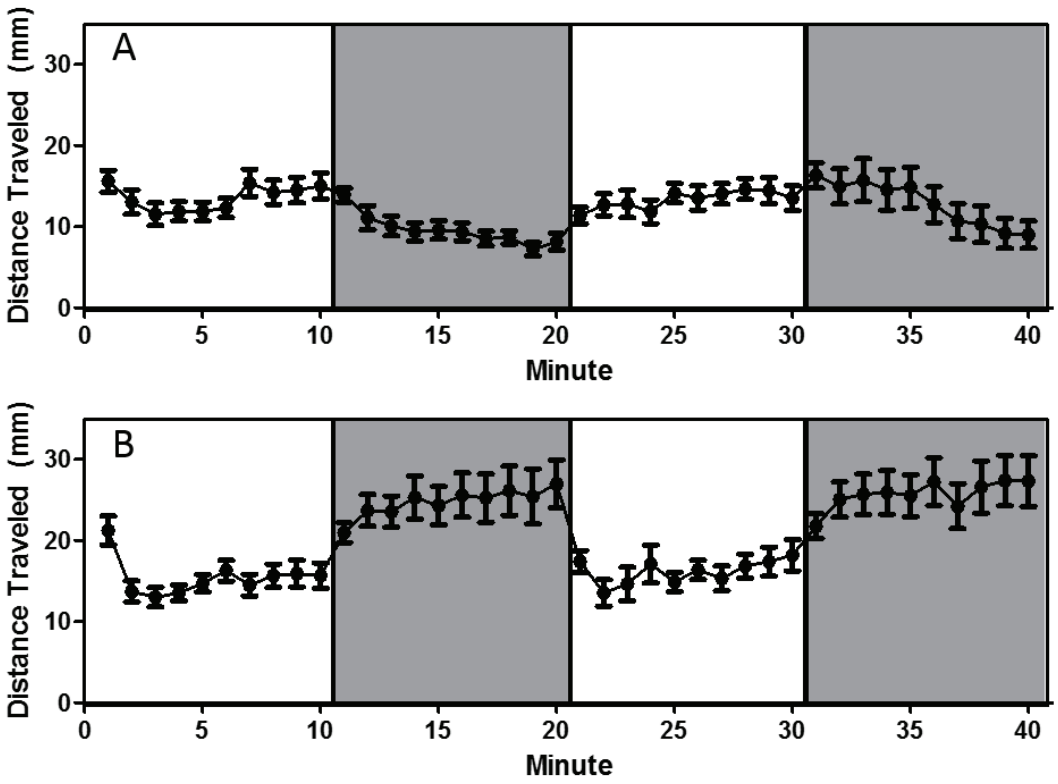

Figure: 9. Baseline behavior activity of unexposed *Pimephales promelas* larvae. Mean (±SE) total distance traveled per minute by *P. promelas* larvae following A) 48 h or B) 7 d studies. Two dark and two light photoperiod responses were measured. A total of 72 (18 replicates each of 4 larvae) and 64 (16 replicates of 4 fish) *P. promelas* from 48 h and 7 d studies, respectively, were used for each baseline behavioral observation. Data presented as total distance traveled by unexposed larval fish across three speed categories in the light (white background) or dark (gray background).

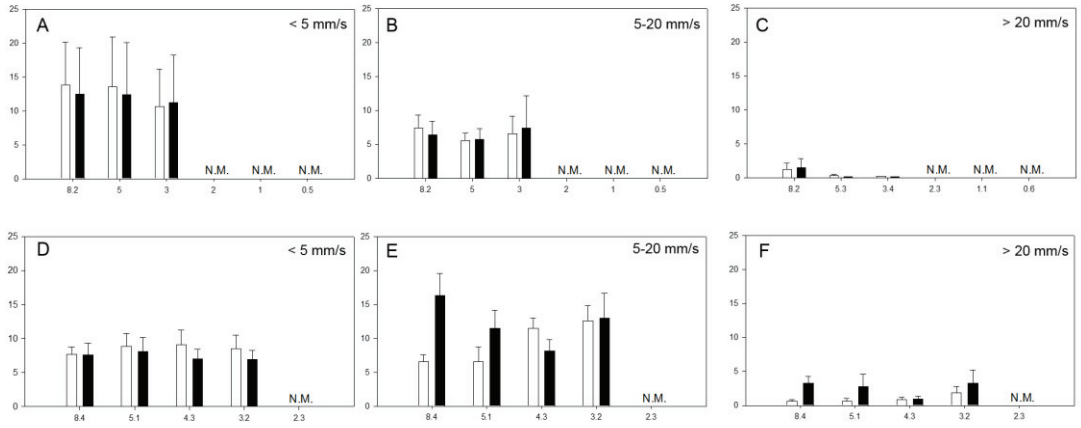

Figure 10. Mean (±SE) distance traveled per minute by *Pimephales promelas* larvae following 48 h (panels A, B, C; N=3 studies) or 7 d (panels D, E, F; N=4, n=4-5) studies across dissolved oxygen (DO) gradients. Data presented as distance traveled in speed (mm/s) categories for resting (< 5 mm/s; panels A, D), cruising (5-20 mm/s; B,E), and bursting (>20 mm/s; C, F) behaviors. Distance traveled was observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at DO concentrations causing significant mortality. *: p < 0.05.

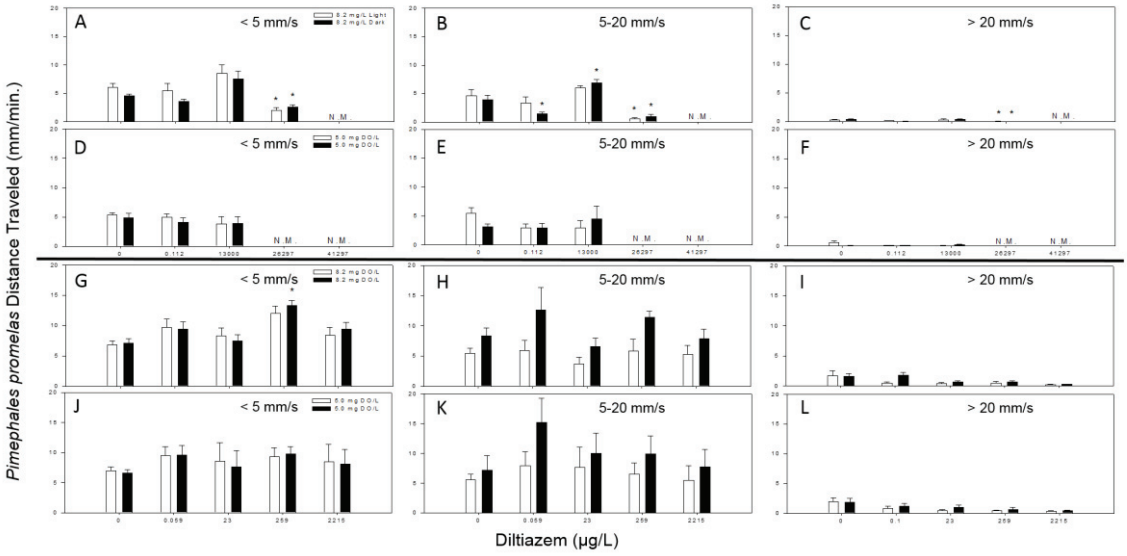

Figure: 12. Mean (±SE) distance traveled per minute by *Pimephales promelas* larvae following 48 h (panels A, B, C, D, E, F; N=4, n=4-5) or 7 d (panels G, H, I, J, K, L; N=4, n=4-5) studies across normal and moderate dissolved oxygen (DO) x diltiazem interaction treatments. Data presented as distance traveled in speed (mm/s) categories for resting (< 5 mm/s; panels A, D, G, J), cruising (5-20 mm/s; B, E, H, K), and bursting (>20 mm/s; C, F, I, L) behaviors. Distance traveled was observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at DO concentrations causing significant mortality. *: p < 0.05. Normal DO: 8.2 mg DO/L; Moderate DO: 5.0 mg DO/L.

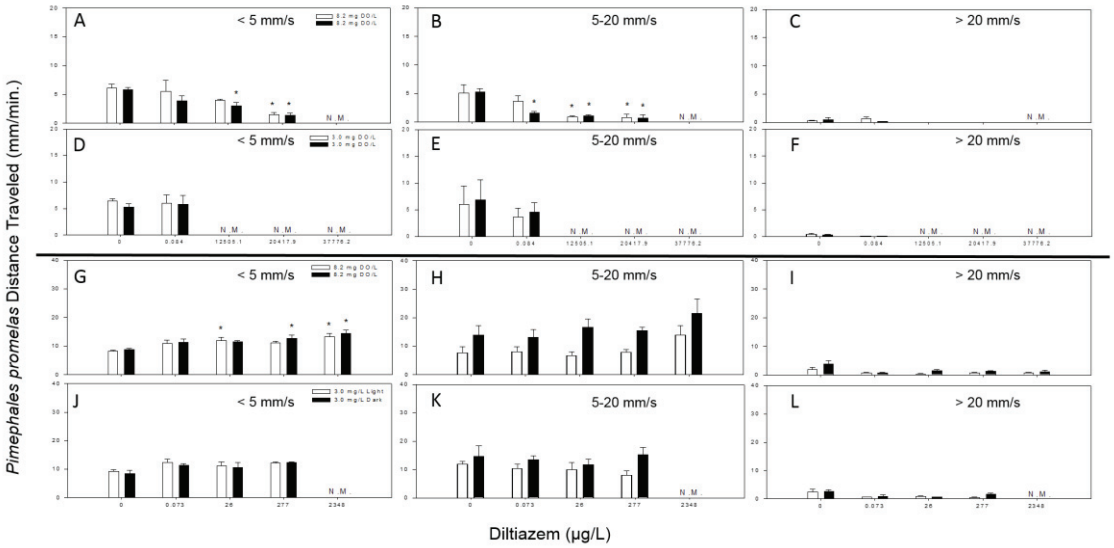

Figure: 13. Mean (±SE) distance traveled per minute by *Pimephales promelas* larvae following 48 h (panels A, B, C, D, E, F; N=4, n=4-5) or 7 d (panels G, H, I, J, K, L; N=4, n=4-5) studies across normal and low dissolved oxygen (DO) x diltiazem interaction treatments. Data presented as distance traveled in speed (mm/s) categories for resting (< 5 mm/s; panels A, D, G, J), cruising (5-20 mm/s; B, E, H, K), and bursting (>20 mm/s; C, F, I, L) behaviors. Distance traveled was observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at DO concentrations causing significant mortality. *: $p < 0.05$. Normal DO: 8.2 mg DO/L; Low DO: 3.0 mg DO/L.

Chronic studies

Similar to acute studies, treatment levels of DO and diltiazem were measured or analytically verified within 100-113% and 17-82% of nominal concentrations, respectively (Supplementary: Table 19). Therefore, all toxicity point estimates were calculated using measured concentrations of diltiazem and DO. Similar to acute experiments, water quality parameters from each study were within acceptable ranges (Supplementary: Table 20) and control survival was 100% in each chronic experiment. Interactive DO x diltiazem experiments resulted in significant decreases in mean (±SD; 55.0±33.2) survival at low DO and 2348 µg/L diltiazem. Additionally, chronic endpoints such as growth demonstrated dose-dependent reduction across all individual and interactive studies. Individual DO and diltiazem fish growth LOEC values were 4.3 mg

DO/L and 2356 µg/L diltiazem, respectively (Figure 14A-B). DO x diltiazem treatments across normoxic, moderate (5.4 mg DO/L) DO levels significantly ($p < 0.05$) decreased growth only in the highest diltiazem treatments at 2215 and 2348 µg/L, respectively; however, no interactive effects of DO were observed ($p = 0.254$; Table 12; Figure 15A-B). Similar to individual DO influences on growth, heart rates were significantly ($p < 0.05$) reduced by low DO (SI Figure 35A-B). Interactive DO x diltiazem studies also significantly ($p < 0.05$) altered heart rates across diltiazem concentrations at low DO and 2348 µg/L (SI Figure 36A-B), and no significant differences in feeding rates were observed in either chronic studies (SI Figure 37A-B and 38A-B).

Similar to acute studies, mean PLR behavioral responses of unexposed or naïve *P. promelas* were visualized from four chronic studies across multiple speed categories and endpoints/metrics (e.g., total distance and count, resting duration) across 2-cycle light:dark periods. In each chronic study, distances traveled across light:dark cycles were inverted, relative to 48 hph light:dark fish behavior (Figure 9B), with greater activity in the dark than the light. Mean (±SE) total distances traveled of unexposed fish across 2-light cycles were lower (15.9±1.1 mm/s) than distances traveled in the dark (25.0±2.3; Figure 9B). This preferential dark activity was consistent across all behavioral speed categories and endpoints/metrics. In individual DO studies, low oxygen levels significantly increased ($p< 0.05$) total distance traveled in the light, while nonmonotonic responses were observed in the dark and across other endpoints (Figure 10D-F, SI Figure 27D-F and 28D-F). Individual diltiazem behavior responses were also nonmonotonic yet light and dark activity was significantly reduced across several endpoints ($p< 0.05$; Figure 11A-C, SI Figure 29A-C and 30A-C) Likewise, interactive DO x diltiazem

treatments significantly ($p < 0.05$) decreased activity (e.g., distance traveled, counts, duration) across both light and dark, although responses in the light and dark were not always monotonic (e.g., normal and moderate DO; Figure 12G-L and 13G-L; SI Figure 31-34G-L). LOEC values indicated activity in the dark (e.g., LOEC) was generally more sensitive than the light. Light and dark differences in sensitivity were demonstrated by significant ng/L (THV) level effects observed in the dark versus higher (µg/L) diltiazem concentrations in the light (Table 13). Dissolved oxygen did not significantly affect behavioral responses to diltiazem.

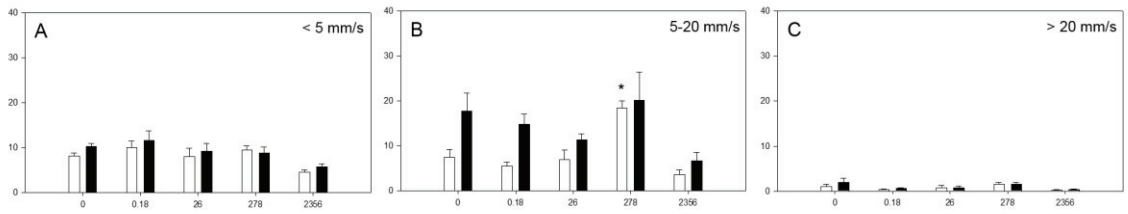

Figure: 11. Mean (±SE) distance traveled per minute by *Pimephales promelas* larvae following 7 d (panels A, B, C; N=4, n=4-5) diltiazem studies. Data presented as distance traveled in speed (mm/s) categories for resting (< 5 mm/s; panels A), cruising (5-20 mm/s; B), and bursting (>20 mm/s; C) behaviors. Distance traveled was observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at DO concentrations causing significant mortality. *: $p < 0.05$.

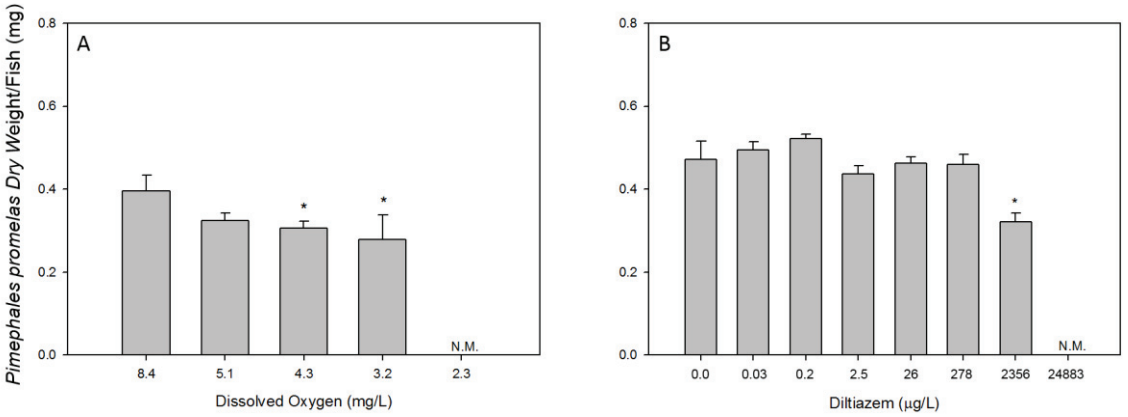

Figure: 14. Mean (±SE, N=4, n=6-8) dry weights (growth) by *Pimephales promeals* larvae following 7 d A) dissolved oxygen (DO) and B) diltiazem studies. No growth measurements were observed (N.M.: Not Measured) at DO or diltiazem concentrations causing significant mortality. *: $p < 0.05$.

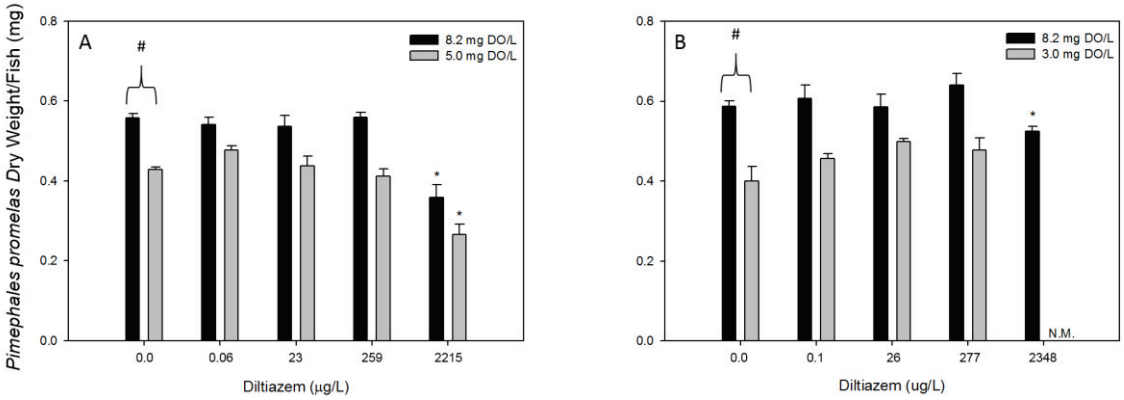

Figure: 15. Mean (±SE, N=4, n=6-8) dry weight (growth) by *Pimephales promeals* larvae following 7 d experiments across A) normal and moderate dissolved oxygen x diltiazem and B) normal and low DO x diltiazem studies. No growth measurements were observed (N.M.: Not Measured) at DO or diltiazem concentrations causing significant mortality. *: $p < 0.05$. Normal DO: 8.2 mg DO/L; Moderate DO: 5.0 mg DO/L; Low DO: 3.0 mg DO/L.

DISCUSSION

In the present study we employed standard acute and chronic toxicity studies to examine individual and interactive toxicological effects of DO and diltiazem to larval *P. promelas*. Few studies have characterized the effects of diltiazem in fish. Although the effects of DO have been reported over the last six decades, previous DO studies failed to calculate standard toxicity endpoints following standardized procedures. The present study reports individual DO and diltiazem LC50 values and demonstrates DO x diltiazem markedly decreases *P. promelas* survival across both acute and chronic studies at typical DO water quality criteria (WQC) levels. Neither 48 h acute nor 7 day chronic traditional or nontraditional endpoints consistently showed significant interactive effects across normal, moderate, or low DO levels at diltiazem concentrations below levels decreasing survival (< 90%). 48 h DO x diltiazem LC50 values decreased at moderate (5.0 mg/L) and low (3.0 mg/L) DO concentrations relative to normoxic levels by a factor of 2.1 and 3.5, respectively. Both moderate and low DO concentrations are common freshwater high

aquatic life use WQC values for streams and reservoirs in Texas, USA, often dominated or dependent on WWTP effluent containing pharmaceuticals such as diltiazem (Brooks et al., 2006; Du et al., 2015; Scott et al., 2016). Inland waters of Texas and other U.S. states are notorious for being listed on the U.S. Clean Water Act 303(d) list due to non-attainment of DO WQC (Brooks et al., 2008; CRASR, 2006). Therefore, the present study demonstrates the toxicological influence of DO to a model contaminant and pharmaceutical in fish, which deserves further attention at a time of increasing global hypoxia (Breitburg et al., 2018; Watson et al., 2016).

The necessity of DO to aquatic life, such as teleosts, has been reviewed previously (Wu, 2002) and has been suggested to be the major factor, coinciding with temperature, impacting fish populations under global climate change (Pörtner, 2010; Pörtner, 2002; Pörtner and Knust, 2007). Literature reviews and studies have outlined the physiological and biochemical strategies fish employ to cope with less than optimal DO concentrations (Richards, 2009; Wu, 2002). Too little oxygen initiates a well-coordinated response to increase DO uptake and a subsequent defense against the metabolic consequences of limited ATP production leading to a finite substrate-dependent duration of survival (Richards, 2009). Initially, the ventilator response accompanies detected decreased DO levels in fish to enhance respiratory water flow across the gills. Previous studies have shown increased oxygen uptake positively correlating with higher chemical accumulation (e.g., endrin, tetrachlorobenzene, EE2; Blewett et al., 2013; McKim and Goeden, 1982; Yang et al., 2000). Increasing chemical uptake at less than optimal DO levels can thus add to the physiological and biochemical perturbations already occurring in fish trying to maintain and/or cope with a lack of oxygen. Multiple aquatic organism

(e.g., daphnia, fish) have been shown to have greater chemical sensitivity at less than optimal DO concentrations across multiple levels of biological organization. For example, similar DO x chemical experiments with ammonia, 1, 2, 4-trichlorobenzene, sweet crude oil, cadmium, and copper in daphnia and several fish species (Carlson, 1987; Dasgupta et al., 2015; Fitzgerald et al., 2016; Hattlink et al., 2005; Lyu et al., 2013) have been shown to decrease survival consistent with our observations. At the subcellular level, hypoxia and the pharmaceutical diclofenac were shown to induce CYP1A activity (EROD) above diclofenac activity alone in the three-spined stickleback, which is opposite to other studies predominantly reporting an inhibitory effect on CYP1A activity (Matson et al., 2008; Prokkola et al., 2015; Rahman and Thomas, 2012). Conversely, LDH activity increased in response to hypoxia but was suppressed to control levels following co-exposures. To date, enzymatic specific responses to DO x chemical exposures are commonly studied with PAHs but remain understudied with contaminants of emerging concern like pharmaceuticals. A few studies, for example, with hypoxia x bisphenol A (BPA) and cardiovascular responses were altered and caused significant decreases in survival, red blood cell density, tissue vascularization, and development in *Danio rerio* embryos (Cypher et al., 2015). Clearly these studies demonstrate empirical evidence indicating DO can significantly increase fish sensitivity to chemical perturbations under co-exposure conditions.

To our knowledge, this is the first *P. promelas* 48 h acute study to assess the effect of DO to this common model aquatic organism. An LC50 of 2.00 (± 0.23) mg/L for *P. promelas* is greater than that of *Daphnia magna* (0.6-0.7 mg/L) (Nebeker et al., 1992) but similar to that reported for adult common smelt (1.83 mg/ L), and rainbow

trout parr (1.62 mg/L) (Landman et al., 2005). Few standard calculated DO LC50 values in freshwater fish have been reported because most lethal studies only report DO concentrations causing mortality. Similarly, few diltiazem and other CCB standard toxicity values have previously been reported. Acute diltiazem 48 h LC50 values of 25.6 and 28.0 mg/L for *Oryzias latipes* and *Daphnia. magna*, respectively, are similar to mean (±SD) 48 h LC50 of 30.5±1.2 mg/L estimated in the present study (Kim, 2007). The acute toxicity of other CCBs such as verapamil have been similarly reported in embryo and larval common carp (*Cyprinus carpio*) and juvenile rainbow trout (*Oncorhynchus mykiss*), in which 96 h LC50 values reported by Steinbach et al (2013) and Li et al (2010) were 16.3-4.8 mg/L and 2.7 mg/L, respectively. Short-term survival and growth of early life stage fish (e.g., fathead minnow) have been predictive endpoints to determine contaminant concentrations causing adverse effects relevant to ecological risk assessment (Norberg and Mount, 1985; U.S. EPA, 2002b). Further, the present study reports the first individual DO and diltiazem standard lethal toxicity value for larval *Pimephales promelas*, a common regulatory model organism. While extreme DO and diltiazem levels can cause impacts to fish survival, 7 d chronic growth effects were far more sensitive than mortality.

Long term low dose effects of DO have been broadly reported in the literature and particularly used to derive national ambient WQC guidelines. Short and long-term growth studies have been extensively conducted in cold water (salmonid) teleosts with fewer conducted in warm water (non-salmonid) species (Saari et al., 2018). The *P. promelas* chronic growth LOEC (4.3 mg DO/L) in the present study is comparable to the reduced growth EC10 for juvenile largemouth bass (4.4 mg DO/L) (JRB, 1984; Saari et al., 2018).

Non-salmonid or warm freshwater fish laboratory and field studies reviewed in the development of the U.S. Ambient WQC for Dissolved Oxygen concluded fish production to be moderately impaired at 4.0 mg DO/L (U.S., 1986). DO dependent decreases in growth have been observed in many species such as plaice (*Pleuronectes platessa*), dab (*Limanada limanada*) (Petersen and Pihl, 1995), Atlantic cod (*Gadus morhua*) (Chabot and DUTIL, 1999), sockeye salmon (*Oncorhynchus nerka*) (Brett and Blackburn, 1981), northern pike (*Esox lucius*) (Adelman and Smith, 1970), largemouth bass (*Micropterus salmoides*) (Brake, 1972; Stewart et al., 1967), coho salmon (*Oncorhynchus kisutch*) (Brett and Blackburn, 1981; Herrmann et al., 1962), channel catfish (*Ictalurus punctatus*) and yellow perch (*Perca flavescens*) (Carlson et al., 1980); however, the interactive effects of DO x chemical on growth and across other levels of biological organization are poorly understood .

Interactive DO x diltiazem chronic studies indicated significant DO influences on fish growth and heart rate. Chronic growth decreases measured in DO studies were replicated in interactive experiments and similar significant reductions in growth at moderate and low DO treatments were measured only in diltiazem controls. The effects of diltiazem on larval fish growth were not dependent on the DO level. Again, survival was the most sensitive endpoint measured across acute DO x diltiazem studies, and similarly significant interactive effects on survival were observed at low DO and the highest diltiazem treatment level. Percent survival under normal DO levels throughout chronic interactive exposures were 100% even up to 2215-2348 µg/L diltiazem. Very few chronic fish studies with early life stages have focused on the growth effects of CCBs. A study by Steinbach et al (2013) observed no significant effects of verapamil in common

carp following 31 day exposure at 0.463-463 µg/L. Conversely, Overturf et al. (2012) reported 28 day chronic exposure to 600 µg/L verapamil significantly decreased larval fathead minnow growth rate (Overturf et al., 2012).

Diltiazem, a benzothiazepine, and verapamil, a phenylalkylamine, belong to the group of CCBs highly prescribed to treat angina, hypertension, and arrhythmia (Romero et al., 2003). Both diltiazem and verapamil have small bioconcentration potentials but of the two the physiochemical properties of verapamil relative to diltiazem would predict higher bioaccumulation potentials with a higher log P (4.2 and 2.8, respectively). Thus, diltiazem and verapamil bioconcentration factors (BCF) from laboratory studies range between 0.5-194 (Steinbach et al., 2016b) and 0.7-75 (Nallani et al., 2016; Steinbach et al., 2013), respectively in various fish tissues. Whether hypoxia increased CCB bioconcentration is not known. Internal fish plasma modeling predicts the hazard of biologically active pharmaceuticals in fish based on the conservation of drug targets between mammalian and teleost species (Brooks, 2014; Fick et al., 2010b; Gunnarsson et al., 2008; Huggett et al., 2003). Internal fish plasma levels of diltiazem have been reported approaching and even exceeding human therapeutic levels (Fick et al., 2010a; Scott et al., 2016; Tanoue et al., 2015). Several compounds have linked internal fish tissue concentrations to specific pharmacological effects (e.g. antidepressants, steroids, anxiolytics, and nonsteroidal anti-inflammatory) (Cuklev et al., 2011; Huerta et al., 2016; Margiotta-Casaluci et al., 2014; Patel et al., 2016; Runnalls et al., 2015; Valenti et al., 2012), while others lack sufficient data necessary to validate fish plasma modeling and read-across approaches (Rand-Weaver et al., 2013).

Other non-traditional sublethal endpoints (e.g. heart rate, locomotor activity, enzyme activity) predictive of adverse effects to toxicants have also been reported (U.S. EPA, 2002b). In the present study, heart rates displayed a dose-dependent trend in acute DO x diltiazem studies at normal and moderate DO levels similar to previous CCB studies with verapamil (Steinbach et al., 2013). Conversely, nonmonotonic trends in 7 day DO x diltiazem exposures were observed with significant decreases in heart rate at 2215-2348 µg/L diltiazem across all three interactive DO treatment levels and significant increases observed at 259-277 µg/L at normal and moderate DO. Decreasing heart rates are consistent with the pharmacological action of diltiazem. Studies with other CCBs in larval zebrafish have shown reduced heart rate following exposure to mg/L verapamil concentrations and thus demonstrate a pharmacological effect in fish. Similar effects were seen in 4 dpf zebrafish following exposure to verapamil resulting in decreased heart rate, surrogate stroke volumes and even cessation of blood flow at higher concentrations (Parker et al., 2014). Steinbach et al (2016a) demonstrated histological changes in the heart and blood vessels of rainbow trout livers suggesting vasodilation following long-term µg/L diltiazem concentrations (Steinbach et al., 2016a). Vasodilation can lead to reflex tachycardia triggered by the sympathetic nervous system in mammals to re-establish normal blood pressure (Scholz, 1997). Whether increased heart rates observed in separate chronic DO x diltiazem exposures at 259-277 µg/L at normal and moderate DO levels represent tachycardia is unknown. Diltiazem represents an intermediate vasodilator and cardio depressant in humans and while the drug target is relatively conserved its complete function in fish is understudied (Gunnarsson et al., 2008; Rottbauer et al., 2001). Using the ECOdrug (http://www.ecodrug.org/) database, the

diltiazem drug target (voltage-dependent calcium channel L-type α-1C, α-1D, α-1F, α-1s subunits) is predicted to be 70.4-78.3% and 64.1-81.1% conserved in zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), respectively (Gunnarsson et al., 2008; Verbruggen et al., 2017). Thus, pharmacological responses in fish referenced above can be expected. Additional research with non-traditional regulatory endpoints relevant to the biological activity of pharmaceuticals is necessary to advance ecotoxicological read-across models with highly conserved fish drug targets.

Fish behavior relative to other standardized toxicity metrics can be a sensitive indicator of chemical exposure in which effects often occur at lower concentrations (Little and Finger, 1990; Melvin and Wilson, 2013). Therefore, PLR behavior following acute and chronic exposures were measured to detect potential alterations in behavior. The use of zebrafish as a model system for developmental, biomedical, and toxicological research has spurred the need to understand their behavior and have been the typical model organism studied (MacPhail et al., 2009). *P. promelas*, on the other hand, have been typically used by U.S. regulatory agencies focused on survival and growth endpoints but recent studies have begun to investigate their behavioral sensitivity following contaminant exposures) (Colón-Cruz et al., 2018; Steele et al., 2018). Colon-Ruiz et al. (2018) demonstrated naive 6 dpf fathead minnows, similarly used in our study, displayed high activity in the light compared to the dark following observations of their photo-dependent swimming activity. This general light:dark behavior was identical to that observed in our acute studies and similarly identified for fathead minnows by Steele et al. (2018) following acute 96 h behavior toxicity studies. In contrast, larval zebrafish, display high activity in the dark than light and with less variability (Kristofco et al., 2016;

MacPhail et al., 2009). Remarkably, the present study demonstrates naïve 7 dph (11 dpf) fathead minnow behavior changes and mimics the light:dark photo-dependent behavior in zebrafish. The cause of this behavioral change in fathead minnow with development is unclear but may be related to an early life stage lack of innervation leading to minimal light:dark sensory perception and/or circadian rhythm (Farrell, 2011). The light:dark activity preference of 7 dph fish is consistent with adult fathead minnow behavior reported by Valenti et al (2012). In the Valenti et al (2012) study, adult male fathead minnow shelter-seeking behavior similarly demonstrated movement preferences in the dark versus the light in which sertraline increased light cruising activity consistent with its human therapeutic effect (Valenti et al., 2012).

Behavioral observations in both acute and chronic experiments displayed a general decrease in activity. Acute behavior following DO exposure tended to decrease larval activity in both the light and dark, although few observations were statistically significant. Fish activity under minimal DO conditions varies depending on developmental stage, species-specific strategies to increase oxygen uptake, and metabolic tolerance (Chapman and McKenzie, 2009; Pelster, 2002; Wu, 2002). If mechanisms to increase oxygen uptake and/or avoid hypoxia fail, reductions in activity are typically used to conserve energy (Chapman and McKenzie, 2009; Richards, 2009). In the present study, acute DO exposure behavior is generally consistent with the aforementioned trend and were reproducible as observed in DO x diltiazem studies. Consistent patterns from chronic behavior measurements following DO and diltiazem exposures were difficult to determine. Regardless, both DO and diltiazem chronic observations demonstrated decreasing trends and/or significant decreases in bursting distance traveled, number of

movements (counts), and duration per minute. Bursting activity, on average, comprises < 1.0 second/minute of their activity in which fish travel 1-3 mm/minute, which is divided into 2-10 individual movements (greater activity in dark than light). Whether decreases in the above mentioned activity demonstrates physiological or ecological adverse outcomes deserves additional attention, but decreases in spontaneous swimming activity at minimal DO concentrations is consistent with previously published literature (Chapman and McKenzie, 2009; Domenici et al., 2013). Again, this type of decreased activity merits additional study, especially when Robb and Abrahams (Robb and Abrahams, 2002) reported fathead minnow feeding response in the presence of a predator (yellow perch) were reduced under non-lethal hypoxic versus normoxic conditions.

The present study also investigated whether THV concentrations of diltiazem exhibited toxicological or pharmacological effects in larval fish following acute and chronic exposures. The THV describes the water concentration predicted to bioconcentrate in fish plasma to an equivalent human therapeutic level (Berninger et al., 2011; Brooks, 2014). In acute and chronic studies containing the diltiazem THV, no significant effects were observed across acute or chronic exposure endpoints except behavior. Both 48 h and 7 day studies, significant decreases and decreasing trends in number of movements (counts), distance traveled, and duration across both light and dark conditions were observed. Kristofco et al [43] observed the antihistamine diphenhydramine (DPH) significantly decreased distance traveled below THV concentrations (18.6 µg/L at pH 7) across specific larval zebrafish development stages. It is not clear why larval fish behavioral responses below THVs are markedly more sensitive than other toxicological endpoints. Feeding behavior has also been shown to

decrease following chronic DPH exposure below its THV (Berninger et al., 2011), and as introduced above Valenti et al (2012) observed significant increases in adult fathead minnow light activity well below other standard ecotoxicological endpoints and above human therapeutic plasma levels (Valenti et al., 2012). Although internal diltiazem plasma concentrations of fish larvae could not be measured in the current study, literature associating pharmaceutical tissue concentrations to relevant human therapeutic effects is growing. As previously mentioned, the effects of low dose pharmaceutical exposure have been linked to sublethal non-standard pharmacological endpoints with numerous compounds (e.g. antidepressants, steroids) (Cuklev et al., 2011; Huerta et al., 2016; Margiotta-Casaluci et al., 2014; Patel et al., 2016; Runnalls et al., 2015; Valenti et al., 2012). These effects have been shown to be pH-dependent, another abiotic factor, in which uptake and toxicity has been shown to increase with increasing pH related to the non-ionized chemical species (Berninger et al., 2011; Nichols et al., 2015; Valenti et al., 2009).

Diltiazem uptake into the gulf killifish (*Fundulus grandis*) has similarly been demonstrated to be pH-dependent (Scott et al. unpublished data) with rapid gill uptake occurring within hours. Bioconcentration and metabolism of diltiazem in juvenile rainbow trout indicated the highest and lowest accumulated doses were in the kidney and plasma, respectively (Steinbach et al., 2016b). The calculated half-life for diltiazem across whole body tissues analyzed ranged from 1.5 h (liver) to 49 h (muscle) [33]. In the same and follow up studies, 17 phase I diltiazem metabolites were detected in rainbow trout (Koba et al., 2016; Steinbach et al., 2016b). In humans, diltiazem has three main metabolites that are produced by phase I cytochrome p450 enzymes (CYP) 3A4 and 2D6

among others (Law et al., 2013; Wishart et al., 2006). Hypoxia has been shown to inhibit CYP activity (e.g. CYP1A) in multiple fish (Fleming and Di Giulio, 2011; Rahman and Thomas, 2012) and mammalian model species (Fradette et al., 2007; Fradette and Souich, 2004). The toxicokinetic effects of hypoxia were not investigated in this study but deserve future research to understand potential influences on internal pharmaceutical bioavailability, clearance, and ensuing potential affects in fish.

Though acute and chronic survival were the most sensitive endpoints in the present study, the long term low dose effects of diltiazem are yet to be understood. Similar pharmacological effects in fish have been observed following diltiazem or other CCB exposures illustrating conservation of CCB drug targets exists between mammals and telesosts (Berghmans et al., 2008; Li et al., 2011; Steinbach et al., 2016a; Steinbach et al., 2013; Verbruggen et al., 2017). Saari et al (2017) recently performed a global hazard assessment of CCB in multiple environmental matrices to predict their concentration across geographic regions. Environmental exposure distribution 5th and 95th centiles for all CCBs were 5.0 and 448.7 ng/L in effluent and 1.3 and 202.3 ng/L in freshwater, respectively (Saari et al., 2017). Furthermore, based on the publicly reported diltiazem fish plasma concentrations from freshwater and marine systems, the human minimum therapeutic plasma level (C_{min} = 30 ng/mL) was shown to be exceeded 17% of the time. The full pharmacological and toxicological effects of diltiazem to aquatic organisms, particularly in adult fish, in environmental matrices is unknown. While several studies have reported the effects of CCBs and other heart medications (e.g. β-blockers), additional research is necessary especially with co-exposures involving other pharmaceuticals and stressors.

Human population growth and climate-induced physical habitat changes are altering the physical, chemical, and biological characteristics of aquatic ecosystems (Hartmann et al., 2013; Staudt et al., 2013). Global hypoxic occurrences in marine and freshwater ecosystems introduce physiological constrains on fish populations (Breitburg et al., 2018; Pörtner and Farrell, 2008; Pörtner and Knust, 2007; Whitney et al., 2016). In the United States WQC are established to protect surface waters and their designated uses, which include aquatic life. Recently published articles have demonstrated surface water DO thresholds affecting organism growth and survival above the typical hypoxic threshold (e.g. 2.0 mg DO/L) (Elshout et al., 2013; Saari et al., 2018; Vaquer-Sunyer and Duarte, 2008). The DO concentrations used in the present study demonstrate typical high aquatic life use WQC in the State of Texas, USA for streams and reservoirs which are consistently impaired due to noncompliance of DO regulatory standards (Saari et al., 2018). Multiple nonchemical and chemical stressors interacting in these aquatic systems represent uncertainties to water quality assessments and thus ecosystem protection goals. The present study evaluated the impacts of two common stressors in fish which represent natural field conditions in urbanized surface waters throughout the world (Saari et al., 2017).

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CHAPTER FIVE

Low Dissolved Oxygen Increases Uptake of a Model Calcium Channel Blocker By its Effects on Adult *Pimephales promelas*

Abstract

Human population growth accompanied with urbanization has created an urban water cycle where surface waters in some regions are dominated or dependent on wastewater treatment plant discharges. These watersheds represent worst case scenarios for exposure to multiple stressors such as down the drain chemicals (e.g., pharmaceuticals) and other environmental stressors (e.g., temperature, dissolved oxygen (DO)). Multiple stressors have the potential to affect chemical exposure dynamics and fish cardiac physiology leading to undesirable population trajectories. Recent field observations from my laboratory group identified the calcium channel blocker diltiazem in fish plasma exceeding human therapeutic doses (e.g., C_{min}) in coastal estuaries impaired due to nonattainment of DO water quality standards (WQS). Thus, objectives of the present study were two-fold: 1) to examine whether DO influences diltiazem uptake by fish; and 2) to determine whether changes in DO-dependent toxicokinetics influence fish physiological and biochemical responses. My results identified that internal steady state diltiazem concentrations in adult fathead minnows were reached at ~6 h versus ~24 h under low (3.0 mg DO/L) and normal (8.2 mg DO/L) DO conditions, respectively. Low DO levels approximately doubled diltiazem uptake in fish relative to normoxic conditions. Increased internal diltiazem concentrations were associated with significant ($p<0.05$) increases in fish ventilation rate to enhance oxygen uptake at these low DO

levels. During a subsequent study, decreased burst swim performance (U_{burst}) of adult fathead minnows was significantly ($p<0.05$) altered by low versus normal DO. DO x diltiazem studies resulted in U_{burst} reductions between 13-31% from controls, though diltiazem effects were not dependent on DO ($p = 0.06$). Significant ($p<0.05$) increases in plasma lactate levels between DO treatments was indicative of alterations in aerobic metabolic energy demand-supply dynamics and supports the observed reductions in fish swim performance. Physiological responses in fish exposed to diltiazem alone were minimal; however, in co-exposure with low DO, decreasing trends in U_{burst} were measured and were inversely related to plasma lactate levels. The physiological consequences of such trends in adult fathead minnows remain unknown but may indicate potential interactive cardiac effects under low DO conditions at human therapeutic diltiazem plasma concentrations. Such physiological responses to these multiple stressors, when paired with internal tissue concentrations, identify the utility of employing biological read across approaches to identify adverse outcomes of heart medications and potentially other cardiotoxicants impacting fish cardiovascular function across DO gradients.

Introduction

The continued global growth of the human population has created an urban water cycle characterized by high chemical use, which enters the environment through wastewater treatment plant (WWTP) effluent discharge to surface waters (Brooks, 2014; Brooks et al., 2006; Postel, 2010). Coincidentally, 70% of the human population reside in cities, typically located in close proximity to waterbodies, where local water resources are

stressed from climate change, nutrient enrichment and contaminant loading (Brooks et al., 2006; Heathwaite, 2010; Hooper, 2013). These stressors have been demonstrated to influence chemical exposure scenarios and toxicological effects in aquatic organisms (McKim and Erickson, 1991). Effluent-dominated or dependent aquatic systems represent worst case scenarios for exposure to pharmaceuticals and other environmental stressors (e.g., low dissolved oxygen; DO). Unfortunately, pharmaceutical occurrence in surface waters (Halling-Sorensen et al., 1998; Kolpin et al., 2002; Ternes, 1998) and biota (Brooks et al., 2005; Lazarus et al., 2015; Ramirez et al., 2009) are increasingly reported in the literature causing concern to environmental managers due to their physiochemical attributes and high biological activity (Ankley et al., 2007). Effective water management is essential as our access to medicine is increasing faster than WWTP infrastructure is implemented, particularly in developing regions. Further, excessive nutrients accompanied with climate change exacerbates eutrophication and harmful algal blooms that deplete DO in freshwater and marine ecosystems (Breitburg, 2002; Waiser et al., 2011). Therefore, surface water integrity is often challenged by multiple stressors. Unfortunately, influence of multiple stressors on chemical exposure dynamics and effects in fish remain understudied (Armitage et al., 2017; Boxall et al., 2012; McKim and Erickson, 1991).

Understanding how environmental factors influence partitioning of chemicals to fish and how these organisms respond to multiple stressors has been previously examined with persistent organic pollutants (POPs; McKim and Erickson, 1991) and then more recently with pharmaceuticals (Nichols et al., 2015). My laboratory research group and others also recently identified concentrations of the calcium channel blocker (CCB)

diltiazem in fish tissue approaching and exceeding human therapeutic levels in fish plasma sampled from urbanized watersheds (Du et al., 2014a; Fick et al., 2010a; Scott et al., 2016; Tanoue et al., 2015). The extent of such conditions were examined in a global probabilistic hazard assessment of CCBs, in which diltiazem was reported to exceed minimum human therapeutic plasma levels (C_{min}) in fish 17% of the time in fish from the field (Saari et al., 2017). Consequences of such observations remain unknown but predictive pharmaceutical assessment approaches (Huggett et al., 2003; Brooks, 2014) indicate therapeutic risks to fish are likely due to drug target conservation of CCBs across vertebrates.

Fish plasma modeling approaches, which were developed from physiological based toxicokinetic modeling from empirically derived blood:water partition coefficients ($P_{B:W}$) in rainbow trout (*Oncorhynchus mykiss*) (Fitzsimmons et al., 2001; Huggett et al., 2003) have been used to predict steady state internal pharmaceutical concentrations. Using these models, therapeutic hazard values (THV) can identify water concentrations predicted to bioconcentrate in fish plasma to a human therapeutic dose (Berninger et al., 2011; Brooks, 2014; Fick et al., 2010b), and then can be employed to examine hazards of surface waters, sewage and effluent discharges (Du et al., 2014; Kristofco et al., 2017; Saari et al., 2017). In fact, I recently reported the global occurrence and hazards of CCBs in various aquatic matrices (Saari et al., 2017). Diltiazem environmental exposure distributions (EEDs) indicated WWTP effluent concentrations were the only water matrix (e.g., freshwater, saltwater, influent) predicted to exceed a THV but only in approximately 2.0% of the scenarios. However, diltiazem fish plasma concentrations sampled in the field exceeded the minimum human therapeutic dose 17% of the time,

suggesting that a more advanced understanding of diltiazem uptake and sublethal effects to fish is necessary. The significance of such therapeutic plasma concentrations in fish are unknown, particularly when such exposures co-occur with other environmental stressors typically found in urbanized watersheds historically impaired due to nonattainment of DO water quality standards (Scott et al., 2016).

The development of medicines altering ion entry to cells has enhanced our basic understanding of calcium's role in important biological processes and has been instrumental for various disease treatments (Braunwald, 1982; Reuter, 1983). Diltiazem is a CCB regularly prescribed to treat hypertension and angina, which acts as a vasodilator and cardiodepressant (Spedding and Paoletti, 1992; Wishart et al., 2006). Similar to verapamil, diltiazem shows greater sensitivity and activity in cardiac cells than the peripheral vasculature thereby making it applicable for use to treat arrhythmias in addition to hypertension (Spedding and Paoletti, 1992; Wishart et al., 2006). This therapeutic agent primarily targets one of five-calcium channels in humans by blocking the L-type channel, the major channel in muscle cells mediating contraction, and preventing an influx of calcium into cardiomyocytes (Braunwald, 1982; Wishart et al., 2006). Calcium influx inhibition decreases the contractile activity of cardiomyocytes resulting in decreased force of contraction by the heart. Additionally, calcium channel antagonism affects cardiac action potentials by decreasing conduction and increasing the refractory period between contractions, which thus results in its use for treatment of atrial fibrillations (Wishart et al., 2006). Thus, CCBs have the ability to treat a broad array of cardiovascular disorders in humans. Based on comparative physiology and the conservation of CCB drug targets in vertebrates, the plausibility of diltiazem to interact

with fish calcium channels exists, particularly when levels approach and exceed human C_{min} plasma levels in fish plasma (Scott et al., 2016).

Several comprehensive reviews are available for the molecular and biochemical, metabolic, physiological, and adaptive strategies in fish to oxygen limitations (Farrell and Richards, 2009; Perry et al., 2009; Richards, 2009; Wells, 2009). Fish cardiovascular responses to reduced oxygen are specific to individual fish regulation strategies that are ultimately coordinated to balance cardiac metabolic energy supply and demand (e.g., adenosine triphosphate; ATP), including mechanisms to cope with metabolic waste products (Richards, 2009; Stecyk, 2017). Mismatch ATP production and demand leads to ATP-dependent ion pump failure (e.g., Na+/K+-ATPase) resulting in a disruption of cellular membrane resting potentials and ionic integrity (Boutilier, 2001). Uncontrolled calcium influx through voltage-gated channels initiates calcium-dependent breakdown of proteins and phospholipids, ultimately leading to cell death (Boutilier, 2001). Thus, disruption of calcium ion channels and homeostasis can lead to cardiac failure, reduced swimming performance, and adverse outcomes in fish (Claireaux et al., 1995; Claireaux et al., 2000; Herbert and Steffensen, 2005; van Raaij et al., 1996a; van Raaij et al., 1996b).

Recent studies examining the acute and chronic effects of DO x diltiazem exposure to larval fathead minnows showed significant decreases in survival and growth at typical water quality standard levels, including 3.0 mg DO/L (Saari et al, Accepted with revision). Swim behavior studies with larval fish indicated general decreases in swim behavior and significantly reduced bursting distance traveled, number of movements, and duration in response to low DO or mg/L diltiazem concentrations. Other

studies have reported pharmacological action following acute and chronic diltiazem and verapamil exposures in larval zebrafish (*Danio rerio*) and fathead minnows (*Pimephales promelas*) and juvenile rainbow trout (*Oncorhynchus mykiss*) at µg/L to mg/L concentrations. Such effects observed were in CCB target tissues and were plausibly similar to the mode of actions of these CCBs, resulting in reduced fish heart rate (Saari et al, Accepted with revision; Parker et al., 2014) and stroke volume (Parker et al., 2014) and histological changes in heart and liver blood vessels that were suggestive of vasodilation (Steinbach et al., 2016). The cardiovascular and organism level consequences of such responses in fish are poorly understood, particularly in conjunction with environmental factors such as DO, which share molecular and biochemical perturbation pathways in fish. Therefore, the objective of the present study was two-fold: 1) to examine whether DO influences diltiazem uptake by fish; and 2) to determine whether changes in DO-dependent toxicokinetics result in changes in adult fathead minnow burst swim performance and biochemical endpoints.

Materials and Methods

Experimental Animals

Fathead minnows (*P. promelas*) used in all studies were obtained from cultures at Baylor University originating from the U.S. Environmental Protection Agency (EPA) laboratories in Duluth, MN and Cincinatti, OH and Environmental Consulting & Testing in Superior, WI. Adult male fathead minnows were cultured in dechlorinated tap water according to US EPA recommendations. Fish were cultured at 25 ± 1 ℃ on a 16:8-h light:dark cycle and were daily fed flake food (~ 1.5% body weight) in the morning

followed by artemia and flake food in the evening. A summary of the ages and mean weight of fish used from each treatment is provided in Table 14.

Dissolved Oxygen Regulation and Diltiazem Treatment Levels

Waterborne DO x diltiazem exposures were conducted with multiple semi-flow through exposure systems previously employed in our laboratory (Nichols et al., 2015) under identical light and temperature conditions used during culture. Dechlorinated tap water, as noted above, was used for all experiments due to large volumes requirements. Routine water chemistry parameters were measured throughout each study including DO, temperature, pH, conductivity, chlorine, ammonia, nitrite, alkalinity, and hardness according to established methods (American Public Health Association et al., 1998). Diltiazem hydrochloride was obtained from Sigma-Aldrich (St. Louis, MO, USA; CAS # 33286-22-5; 99% purity).

DO water concentrations were regulated by mixing both nitrogen gas and air that were then infused in each experimental chamber as detailed previously (Saari et al., Accepted with revision). Briefly, gas was regulated using RiteFlow meters (Scienceware Bel-Art Products, Wayne, NJ, USA). DO treatment levels were maintained consistently and continually with individual nitrogen and air Riteflow meters per treatment level. Each N2-air regulator pair delivered gas that was mixed in sealed PVC chambers (4” x 24” x 1/8”) filled with bioballs. A single effluent line ran gas to a climate controlled walk-in incubator where gas infusion levels were manually adjusted from a 6-port manifold to achieve desired DO treatment levels. Gas was then bubbled into experimental exposure headboxes. DO x diltiazem control and chemical exposures were conducted in semiflow-through recirculating exposure systems. Headboxes were infused with gas, as

described above, to maintain desired DO levels. Each exposure system headbox provided a single diltiazem concentration to 3 (bioconcentration study) or 4 (swim performance study) replicate aquariums. To maintain stable pH and aqueous concentrations of diltiazem, study solutions were partially renewed every 12 h by approximately 50%. Daily water replacement was approximately 100%. Samples for traditional water chemistry parameters and for analytical verification of diltiazem treatment levels were taken at the start and end of each study and before and after each water renewal. DO and diltiazem water concentrations were selected from preliminary uptake experiments and previous acute and chronic studies with juvenile fathead minnows (Saari et al., Accepted with revision). Diltiazem water treatments were based on empirical data, fish plasma modeling, and therapeutic hazard values (THV) employed previously in our laboratory (Brooks, 2014; Du et al., 2014a). The THV is the pharmaceutical water concentration expected to bioconcentrate in fish plasma within the human therapeutic range (e.g., C_{min} - C_{max}; Brooks, 2014).

Fathead minnows were acclimated to DO conditions prior to introduction to experimental systems. Fish from culture tanks were transported to experimental systems in 10 gallon aquaria. Thus, acclimations were done in 10 gallon tanks with 30 L dechlorinated tap water. An airstone bubbling a mixture of N_2-air gas (described below) was placed in each tank and DO levels were decreased by 0.8 mg/L per 15 minutes to approximately 3.25 mg DO/L. Based on preliminary study observations of fathead minnows to decreasing DO, DO conditions for fish were lowered to 3.25 mg DO/L over 1.5 h, similar to previous fathead minnow hypoxia studies (Robb et al, 2003). Fathead minnows have been reported to initiate aquatic surface respiration at 9% DO saturation

(Gee et al, 1978). Further, we previously identified 48 h DO LC_{50} values at 1.7 mg DO/L in larval *P. promelas*; therefore, experimental DO levels were selected above such values to prevent fish mortality.

Dissolved Oxygen x Diltiazem 96 h Bioconcentration Study

Two experiments were performed to determine whether DO influences diltiazem uptake by adult fathead minnows. These conditions included either 8.2 or 3.0 mg DO/L during separate experiments. Since water pH has been demonstrated to influence the uptake of weak base pharmaceuticals by fish, pH was monitored during this study. As mentioned above, DO levels were maintained by infusing air or mixed gas in headboxes of the experimental system. Headboxes circulated normal (8.2 mg DO/L) or low (3.0 mg DO/L) oxygenated water via submersible pumps in 3 replicate 20 liter glass aquaria according to previously described methods in our laboratory (Nichols et al., 2015). Experiments were initiated at identical times with the same batch of water and conducted for 96 h. Fish from 3 replicate aquaria were sampled at 0, 1, 6, 24 and 96 h, then anesthetized with tricaine methanesulfonate (MS-222), weighed, measured for total length, and blood collected from the caudal artery using heparinized microhematocrit capillary tubes. Plasma was separated via centrifugation at 8,000 x g for 10 minutes at 4˚C and was stored at -80 ˚C until processed. Fish plasma and tissue were pooled from two fish per experimental unit giving 3 replicate samples for each exposure system and duration.

To compare the differences in diltiazem accumulation across DO treatments, bioconcentration factors (BCFs) were calculated from whole body homogenates as

previously described (Arnot and Gobas, 2006). BCFs are defined as the ratio of target analyte (diltiazem) detected in biota and the associated water concentration. Furthermore, blood-water partition coefficients ($P_{B:W}$) were predicted and calculated for each exposure duration from analytically verified plasma levels and mean exposure water concentrations, similar to previous studies (Margiotta et al, 2014). Using the whole body tissue BCF and $P_{B:W}$ values (V_D) was calculated in 96 h studies similar to previous methods (Nichols et al., 2015) examining the utility of pharmacokinetics.

Measured fish plasma concentrations were compared to predicted internal concentrations by the fish plasma model. This model predicts fish steady state plasma concentrations ($F_{SS}PC$) starting from a given water concentration, which is based on equations previously described (Huggett et al, 2003), to calculate the Log $P_{Blood:Water}$ partition coefficient using the Log K_{OW}. However, pH has been previously reported to significantly affect ionization, bioavailability, and toxicity of model weak base pharmaceuticals (Valenti et al, 2009; Berninger et al, 2011). For this reason, Log D_n (n = mean experimental pH) was used to run the model instead of Log K_{OW}. $F_{SS}PC$ were determined by multiplying the water concentrations and $P_{Blood:Water}$. Then, the analytically verified diltiazem water concentration was used to compare measured versus predicted plasma concentrations.

Dissolved Oxygen x Diltiazem Swim Tunnel Studies

DO and diltiazem exposures were conducted in identical experimental systems mentioned above, and all studies were performed separately due to the intensive time requirement of swim performance observations. DO and diltiazem concentrations were

similarly sampled as mentioned above. Experiments were conducted over 24 h and were performed on separated days due to the intensive time requirements of swim performance observations. Two fish from 4 separate aquaria were sampled and measured for ventilation rate, burst swimming performance, hematocrit, L-lactate, and diltiazem tissue accumulation. Each specific endpoint and measurement protocol are described in detail below. Plasma was sampled and separated as previously described above. Individual fish plasma samples were split into two aliquots for biochemical analysis and analytical determination. Analytical fish plasma samples were then pooled from each replicate and stored at -80 ˚C until processed.

Ventilation rates were examined after 24 h exposure to DO and diltiazem by GoPro (Hero black 5; GoPro Inc. 2017) video recording each fish from all replicate aquaria prior to swim performance trials for 15 minutes. Fish videos were individually observed using VLC Media Player (3.0.3 Vetinari; VideoLAN Organization) to slow down (e.g., 40-50%) normal video speed to accurately quantify opercular movement. Videos were observed and after a five-minute camera acclimation time, individual fish opercular movements were recorded 3 times approximately 1 minute apart at 10-15 second intervals (depending on fish orientation in tanks) to calculate ventilation rates (beats/second). After video recording ventilation rates, fish were individually exercised to determine burst swimming performance.

After recording fish ventilation rates, one fish was randomly selected per replicate to measure burst swimming performance (U_{burst}) in a Brett-type swim tunnel (Brett, 1964) previously described in our laboratory (Brooks, 2002; Stanley, 2006). Briefly, a single swim tunnel was immersed in a 40-gallon aquarium of water corresponding to the studied

exposure conditions. The swim tunnel consisted of a 450 mm x 75 diameter acrylic tube with 1 mm mesh at each end. Water was pumped through the tunnel using a 0.5 horsepower centrifugal pump at a maximum speed of ~70 cm/s. Fish were anesthetized with 60 mg/L MS-222 buffered with 120 mg/L sodium bicarbonate for 1 minute and transferred into a black tarp enclosed swim tunnel to acclimate undisturbed for 30 minutes (Tierney et al, 2011). Flow rates were determined using a March-McBirney (Frederick, MD, USA) flow meter. The acclimation flow rate was set at 2.3 cm/s (e.g., 0.08 ft./s) or ~0.3 BL/s (e.g., 7.0 cm total length) in order to remove waste while allowing fish to rest on the bottom (Tierney et al, 2011).

To examine fathead minnow burst swimming performance, a constant acceleration test was used at a 10 cm/s and 1 minute step height and length, respectively. The initial flow rate was 10 cm/s and fish were exercised until fatigued. Fatigue was determined by the fish ceasing to swim and being caught against the mesh after several gentle prods (Brett, 1964). Critical burst swimming speed was calculated as

$$U_{burst} = u_1 + (t_1 / t_2 \times u_2) \quad (1)$$

where u_1 = last step height completed by fish (cm/s), u_2 = the step height (cm/s), t_1 = the time fish swum at fatigue speed, and t_2 = the step length (seconds; Brett, 1964, Tierney et al, 2011). Critical burst swimming speed of each fish was normalized to body length (cm; Brett, 1964; Tierney et al, 2011). After swim performance trials, fish were anesthetized in 200 mg/L MS-222 and 400 mg/L sodium bicarbonate and measured for total length and weight. Fish blood was collected, as mentioned above, and separated into red blood cells and plasma which was aliquoted into two microcentrifuge tubes prior to storage at -80 ˚C for analytical and biochemical (e.g., lactate) analysis. Immediately after centrifugation,

red blood cells were used to determine hematocrit according to previously methods (Hesser, 1960).

L-Lactate Plasma Concentration

Individual fish plasma samples collected after swim performance trials were analyzed for L-lactate using an assay kit from Cayman Chemical Company (Ann Arbor, MI, USA). Fish plasma from three tank replicates were examined for L-lactate. In this assay, lactate was measured by NADH production and reaction with a fluorescent substrate yielding a highly fluorescent product. Lactate dehydrogenase catalyzes the oxidation of lactate to pyruvate and reduces NAD+ to NADH. Fathead minnow plasma samples were removed from storage at -80 ˚C and prepared for assaying according to kit manufacturer instructions. Based on preliminary plasma lactate examinations, samples were diluted 1:7 using Ultrapure water (Cayman Chemical Company). Fluorescence was analyzed using an excitation and emission wavelengths at 530 and 590 nm, respectively. Plasma lactate (units) was then calculated and compared across treatment levels.

Analytical Methods for Water and Tissue

Experimental water treatments and tissue samples from each uptake and swim performance study were analytically verified following previously reported methods (Saari et al, In Press; Haddad et al., In press). Water and tissue (e.g., plasma and whole body-homogenates) samples were analyzed following extraction by isotope-dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) using previously reported instrumental parameters (Bean et al., 2018; Haddad et al., In press).

Statistical Analyses

Data is presented as mean ± standard deviation (SD; x studies) or standard error (SEM; x studies). Differences in uptake across time, treatments, and DO conditions were examined using general linear models in SPSS (IBM SPSS Statistics 25, Chicago, IL, USA). Significant differences within each DO or diltiazem treatment were determined using one-way analysis of variance (ANOVA) in Sigma Plot 11.0 software (Systat Software 323 Inc., San Jose, CA, USA). Similarly, differences in physiological endpoints across DO x diltiazem studies were determined using Two-way ANOVA with multi-comparison Holm-Sidak Test to examine whether DO significantly influenced fish responses across diltiazem treatment levels.

Results

DO x Diltiazem 96 h Bioconcentration Studies

Water quality parameters among all exposure systems were within acceptable levels (Supplementary: Table 21). Fish survival in normal DO x diltiazem studies was 100% but mortalities were observed under low DO x diltiazem conditions in three separate studies (Table 14). Mean (±SE) normal and low DO concentrations across all four experimental durations were 7.23 ± 0.2 mg DO/L and 3.07 ± 0.1 mg DO/L (Table 15). For normal and low DO treatments, measured diltiazem concentrations were 0.84 ± 0.03 µg/L and 0.83 ± 0.04 µg/L, respectively (Table 15). Fig 16A and 16B describe the bioconcentration of diltiazem in plasma and whole-body tissues of fathead minnow following the uptake experiments. Fish exposed to both DO treatments significantly ($p < 0.05$) accumulated diltiazem across nearly all experimental durations, even after 1 h

exposure (Fig. 16A and Fig. 16B). Internal steady state levels of diltiazem were rapidly established at 24 h and 6h at normal and low DO conditions, respectively (Table 15). Diltiazem accumulation in fish plasma and whole-body tissues were significantly ($p < 0.05$) different across DO conditions. Time specific diltiazem accumulation data was used to calculate whole-body bioconcentration factors (BCFs), blood:water partition coefficients ($P_{B:W}$), and volume of distribution (V_D) values. Measured BCF values increased with increasing exposure duration and low DO treatment ranging from 2.0-6.2 and 3.7-16.8 for normal and low DO levels, respectively (Table 15). At steady state, fish at low DO levels had ~2X higher BCF values than fish at normal DO concentrations. $P_{B:W}$ values also increased with increasing exposure duration at low DO levels compared to normoxic conditions. Conversely to BCF and $P_{B:W}$ value trends at normal and low DO concentrations, apparent V_D were similar across in fish under normal DO levels (1.8-3.4) and low DO conditions (1.0-2.7; Table 15)

Table 14. Mean (±SE) values for fathead minnow *(Pimephales promelas*) weight, total body length (BL), and age of fish used in DO x diltiazem bioconcentration (N=3, n=2) and burst swim performance (N=4, n=2) studies. Fish in DO x diltiazem studies were measured immediately after each bioconcentration study or swim tunnel trial. Normal: 8.2 mg DO/L. Low: 3.0 mg DO/L.

	Time Point (h)	Weight (g)	BL (cm)	Age (months)	N/n
Bioconcentration study					
Normal DO x DZM	0	[a]3.77 ± 0.3	6.51 ± 0.1	18	3/9
	1	12.8 ± 0.8	9.62 ± 0.3	18	3/6
	6	10.8 ± 0.9	9.38 ± 0.4	18	3/6
	24	10.0 ± 1.3	8.83 ± 0.2	18	3/6
	96	11.7 ± 0.8	9.28 ± 0.3	18	3/6
Low DO x DZM	0	[b]11.5 ± 0.8	8.90 ± NA	18	1/1
	1	12.5 ± 1.8	9.64 ± 0.5	18	[c]3/5
	6	8.20 ± 0.5	8.52 ± 0.2	18	[d]3/5
	24	14.4 ± 2.0	9.78 ± 0.3	18	[e]3/3
	96	11.2 ± 0.3	9.79 ± 0.2	18	3/6
DO x diltiazem burst swim performance study					
Normal DO x DZM control	24	5.41 ± 0.2	7.65 ± 0.2	8	4/8
Normal DO x DZM 3.0 µg/L	24	5.61 ± 0.4	7.61 ± 0.2	8	4/8
Normal DO x DZM 30 µg/L	24	6.19 ± 0.1	7.81 ± 0.0	8	4/8
Low DO x DZM control	24	5.74 ± 0.3	7.74 ± 0.1	8	[f]4/7
Low DO x DZM 3.0 µg/L	24	6.34 ± 0.5	8.06 ± 0.2	8	[g]4/7
Normal DO x DZM 30 µg/L	24	6.20 ± 0.3	7.76 ± 0.1	8	4/8

(a) Indicates female fish were used as controls for tissue analyses. (b) Indicates one male fish was used control for tissue analyses due to a limited supply of male fathead minnows. (c) One fish euthanized after handling error (flopped out of net onto floor) during 0 h introduction to exposure tank. (d) One fish died approximately 3 hours into exposure. (e) One fish from each replicate died approximately 6 h into 24 h DO x diltiazem exposure. The remaining fish in replicate tanks displayed normal fish behavior; therefore, the remaining fish were included in the study. (f) One fish was euthanized while loading into replicate tank #3 due to 0 h loading error (fish flopped out of net onto floor). (g) One fish died overnight after ~12 h exposure to low DO x diltiazem 3.0 µg/L.

Table 15: Concentrations of diltiazem (DZM) in exposure water, plasma, and whole-body tissue of adult male fathead minnow (*Pimephales promelas*) following normal and low dissolved oxygen (DO) x DZM bioconcentration and burst swim performance studies. Calculated bioconcentration factors (BCF), blood;water partition coefficients ($P_{B:W}$) and apparent volume of distribution (V_D) values are also provided. In bioconcentration (nominal 1 µg/L diltiazem) and burst swim performance (nominal 0, 3, 30 µg/L diltiazem) studies, fish were sampled at 0, 1, 6, 24, and 96 h and 24 h, respectively. Normal: 8.2 mg DO/L; Low: 3.0 mg DO/L; NA: sample size (n) insufficient to calculate standard deviation. SD: standard deviation. N: number of replicates. n: number of samples.

Mean Measured DO (mg/L; SD, N)	Time Point (h)	DZM Mean Measured Water (µg/L, SD, n)	Whole-Body DZM (ug/kg; SD, n)	Plasma DZM (ng/mL; SD, n)	BCF	$P_{B:W}$	V_D
Bioconcentration study							
7.23 (0.5, 4)	0	0	0	0	-	-	-
	1	0.81 (NA, 2)	1.6 (0.5, 3)	0.9 (0.2, 3)	2.0	1.1	1.8
	6	0.89 (NA, 2)	3.3 (0.7, 3)	1.2 (0.2, 3)	3.7	1.3	2.8
	24	0.87 (0.11, 4)	5.4 (0.4, 3)	1.6 (0.2, 3)	6.2	1.8	3.4
	96	0.77 (0.09, 16)	4.6 (0.7, 3)	1.9 (0.1, 3)	6.0	2.5	2.4
	Mean	*[a]0.84 (0.03, 4)*					
3.07 (0.2, 4)	0	0	0	0	-	-	-
	1	0.94 (NA, 2)	3.5 (0.2, 3)	2.6 (1.1, 3)	3.7	2.8	1.3
	6	0.73 (NA, 2)	8.3 (1.1, 3)	8.6 (2.8, 3)	11.4	11.8	1.0
	24	0.84 (0.05, 6)	14.1 (5.4, 3)	5.2 (2.6, 3)	16.8	6.2	2.7
	96	0.81 (0.09, 16)	10.1 (1.9, 3)	6.4 (0.8, 3)	12.5	7.9	1.6
	Mean	*[a]0.83 (0.09, 4)*					

Mean Measured DO (mg/L; SD, N)	Time Point (h)	DZM Mean Measured Water (µg/L, SD, n)	Plasma DZM (ng/mL; SD, n)
Burst swim performance study			
7.83 (0.4, 3)	24	0	0
	24	3.2 (0.6, 4)	19.8 (8.8, 4)
	24	36.5 (3.4, 4)	112.7 (24.9, 4)
3.19 (0.1, 3)	24	0	0
	24	3.1 (0.5, 3)	49.7 (17.4, 4)
	24	33.5 (2.9, 4)	288.1 (67.6, 4)

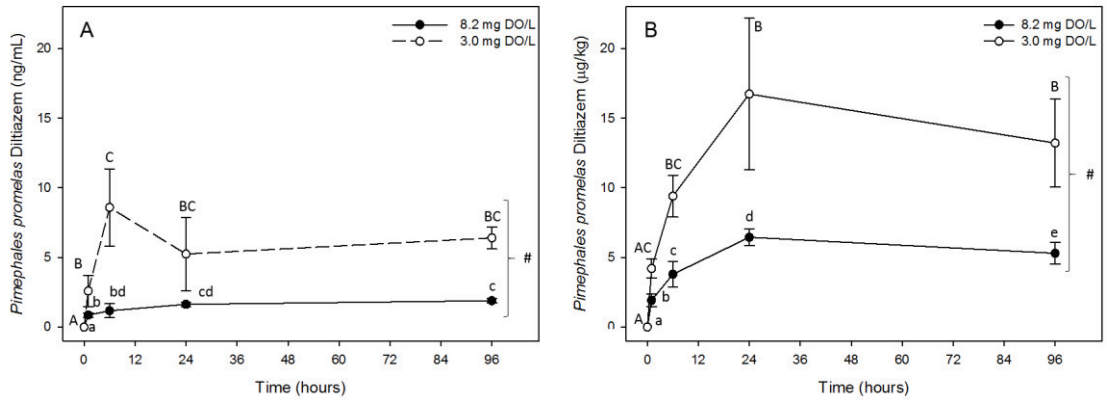

Figure 16: Mean (±SD, N=4, n=1-2) measured concentration of diltiazem in A) plasma and B) whole-body tissue of male adult fathead minnows (*Pimephales promelas)* following 96 h normal and low dissolved oxygen (DO) x diltiazem (1 µg/L) studies. Normal and low DO x diltiazem studies were conducted separately. Tissue samples were collected across five time points throughout each 96 h DO x diltiazem uptake experiment (0, 1, 6, 24, 96 h). Black and white circles represent normal (8.2 mg DO/L) and low (3.0 mg DO/L) levels, respectively. Different letters in the same group (capitalized or not capitalized) correspond to significant ($p < 0.05$) differences. #: significant ($p < 0.05$) influence of DO.

Dissolved Oxygen x Diltiazem Swim Tunnel Studies

Water quality for all experimental units were within acceptable ranges (Supplementary: Table 21). Fish survival in normal DO x diltiazem studies were 100% and one mortality occurred under low DO x diltiazem 3.0 µg/L conditions (Table 14). Mean (±SE) normal and low DO concentrations across all three diltiazem studies were 7.83 ± 0.2 mg DO/L and 3.19 ± 0.1 mg DO/L, respectively (Table 15). For normal and low DO treatments, mean (±SD) measured diltiazem concentrations were 0 (control), 3.2 ± 0.6 µg/L, and 36.5 ± 3.4 µg/L and 0 (control), 3.1 ± 0.5 µg/L, and 33.5 ± 2.9 µg/L, respectively (Table 15). The highest diltiazem treatment is ~70% lower than the 100 µg/L nominal diltiazem concentration, yet still in the human therapeutic range described above. Concentrations of diltiazem in fathead minnow plasma and whole-body tissue are represented in Fig. 17A and 17B, respectively. Similar to the bioconcentration study, fish exposed to both DO conditions accumulated diltiazem at all treatments levels, except

control. Internal diltiazem levels of fish exposed at low DO concentrations were higher than those in normal DO treatments (Table 15). Like the bioconcentration study, data from each diltiazem treatment were used to calculate mean fish tissue BCFs, and $P_{B:W}$ and V_D values. Measured BCF values were higher in low DO treatments ranging from 4.4-5.2 and 9.2-11.6 at normal and low DO levels, respectively. In 24 h exposures, fish at low DO levels had approximately 2X higher BCF values than fish at normal DO conditions. Similarly, $P_{B:W}$ values were higher at low DO concentrations compared to normal DO levels (Table 15). Diltiazem apparent V_D was not different in fish exposed at normal DO levels (0.7-1.7) relative to low DO treatments (0.6-1.3) across all treatments (Table 15).

Fish exposed to 3.1-3.2 µg/L diltiazem at normal and low DO conditions resulted in plasma concentrations below and above the minimum human therapeutic plasma level, respectively. However, both DO treatments levels at 33.5-36.5 µg/L diltiazem resulted in fish plasma levels near and above the maximum human therapeutic level in normal and low DO treatments, respectively. Interestingly, when measured and predicted fish plasma diltiazem levels were compared, models overestimated internal plasma concentrations by 15x and 30x and 6x and 11x at both low and high diltiazem treatment levels under normal and low DO conditions, respectively. Therefore, low DO exposure conditions resulted in fish plasma concentrations above human therapeutic levels and decreased the difference between predicted versus measured fish plasma concentrations.

Ventilation rates measured prior to swim tunnel trials revealed a significant ($p < 0.001$) increase in fish operculum frequency exposed to low DO (Fig. 18A). Low DO increased fish ventilation rates by 143% relative to normal DO conditions when

comparing diltiazem controls. Diltiazem slightly increased ventilation rates across treatment levels at normal DO levels by 5.0% and 32% relative to control (3.2 and 36.5 µg/L, respectively), but such potential increases were not statistically significant ($p = 0.112$; Fig. 18). A significant interactive effect of diltiazem and DO on ventilation rate was not observed ($p = 0.074$); however, rates were lower with increasing diltiazem concentration under low DO conditions by 15% and 61% at 3.1 and 3.5 µg/L diltiazem, respectively.

Burst swimming performance (U_{burst}) was evaluated using an acceleration test to determine whether 24 h exposure to DO x diltiazem impaired fathead minnow bursting ability. DO significantly ($p < 0.001$) decreased U_{burst} relative to normal DO conditions (Fig. 18B). Low DO decreased fish bursting performance by 12.8% relative to normal DO conditions when comparing diltiazem controls. Diltiazem did not significantly ($p = 0.687$) increase U_{burst} across treatment levels at normal DO levels (Fig. 19). Here again, a significant interactive effect of diltiazem and DO on swim performance was not observed ($p = 0.085$), although U_{burst} was lower by 16.4% and 14.5% at 3.1 and 33.5 µg/L at low DO levels relative to diltiazem controls.

Hematocrit was evaluated to determine whether 24 h exposure to DO x diltiazem altered adult fathead minnow red blood cell volume. The effect of diltiazem on hematocrit was dependent on DO conditions ($p = 0.041$; Fig. 19A). In 3.2 and 3.1 µg/L diltiazem treatments, hematocrit significantly ($p = 0.022$) increased by 23% at normal and low DO concentrations. Additionally, under low DO conditions, 3.1 µg/L diltiazem was significantly higher than 0 (control) and 33.5 µg/L by 30.3% and 27.1%, respectively. Fish hematocrit was unchanged ($p = 0.752$) under normal DO conditions (Fig. 19A).

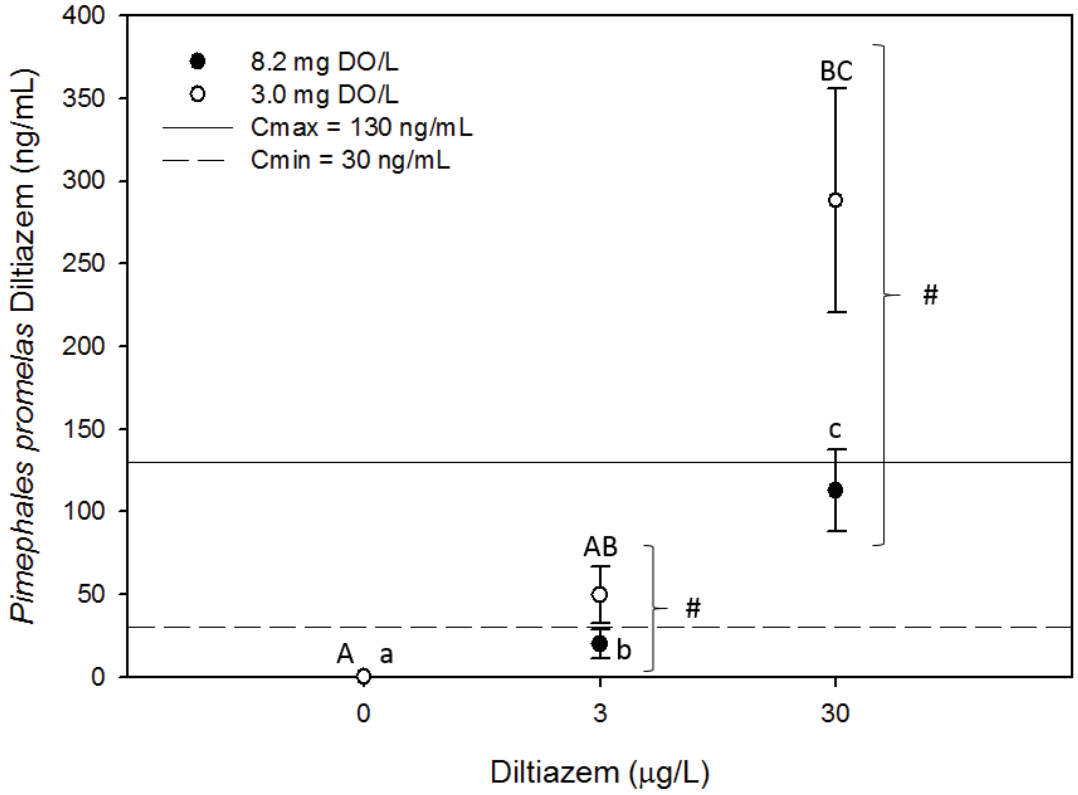

Figure 17: Mean (±SD, N=4, n=1-2) measured concentration of diltiazem in plasma of male adult fathead minnows (*Pimephales promelas*) following 24 h normal and low dissolved oxygen (DO) x diltiazem (0, 3, 30 µg/L) studies. Normal and low DO x diltiazem studies were conducted separately. Black and white circles represent normal (8.2 mg DO/L) and low (3.0 mg DO/L) levels, respectively. Different letters in the same group (capitalized or not capitalized) correspond to significant ($p<0.05$) differences between diltiazem water concentrations. #: significant difference between DO conditions ($p<0.05$).

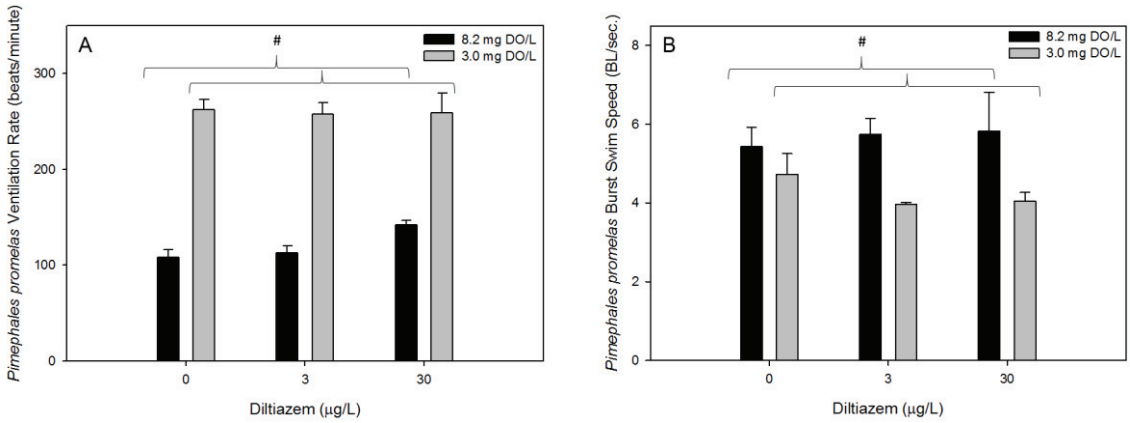

Figure 18: Mean (±SE, N=4, n=1-2) ventilation rate (beats/minute; A) and burst swimming performance (BL/sec.; B) by male adult fathead minnows (*Pimephales promelas)* following 24 h normal and low dissolved oxygen (DO) x diltiazem (0, 3, 30 µg/L) experiments. Normal and low DO x diltiazem studies were conducted separately. DO and diltiazem concentrations are nominal to increase clarity but measured concentrations are in Table 15. Black and gray bars represent normal (8.2 mg DO/L) and low (3.0 mg DO/L) levels, respectively. *: $p < 0.05$ (diltiazem). #: $p < 0.05$ (DO). BL/sec.: body length per second.

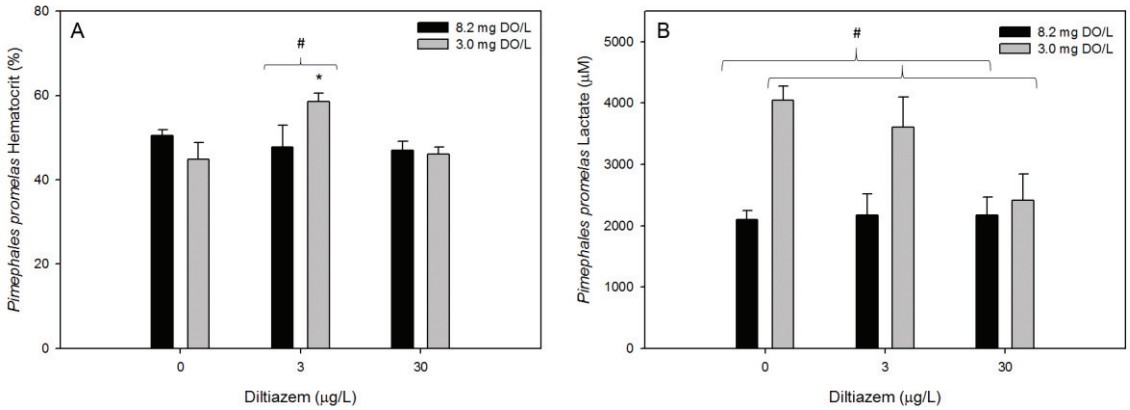

Figure 19: Mean (±SE, N=4, n=1-2) hematocrit (%; A) and plasma lactate (µM; B) of male adult fathead minnows (*Pimephales promelas)* following 24 h normal and low dissolved oxygen (DO) x diltiazem experiments. Normal and low DO x diltiazem studies were conducted separately. DO and diltiazem concentrations are nominal to increase clarity but measured concentrations are in Table 15. Black and gray bars represent normal (8.2 mg DO/L) and low (3.0 mg DO/L) levels, respectively. *: $p < 0.05$ (diltiazem). #: $p < 0.05$ (DO).

L-Lactate Plasma Concentrations

L-lactate (lactate) was measured in plasma to determine whether DO x diltiazem altered energy supply-demand dynamics in fathead minnows. Low DO conditions

significantly ($p = 0.002$) increased lactate concentrations relative to normal DO levels (Fig. 19B) by 92.4% when comparing diltiazem controls. A slight though insignificant ($p = 0.165$) increase in plasma lactate was observed across diltiazem treatments under normal DO conditions relative to control. No significant ($p = 0.131$) interaction effects were observed between diltiazem and DO; however, lactate was lower by 10.7% and 40.3% at 3.1 and 33.5 µg/L diltiazem at low DO levels relative to the low DO diltiazem control (Fig. 19B).

Discussion

Urbanized watersheds represent worst case scenarios for fish exposure to multiple stressors including pharmaceuticals (e.g., diltiazem) and low DO (Breitburg, 2002; Brooks et al., 2006; Waiser et al., 2011). While our understanding of ionizable chemical toxicokinetics and toxicodynamics is growing, empirical evidence demonstrating how environmental factors (e.g., DO, temperature) influence their uptake and toxicity remains poorly understood. In the present study, we examined whether low DO influences the bioconcentration of diltiazem, a model weak base cardioactive medicine, and the physiological effects in adult fathead minnows. Numerous field sampling studies have reported diltiazem accumulation in fish and birds inhabiting urbanized watersheds (Du et al., 2014b; Fick et al., 2010a; Lazarus et al., 2015; Ramirez et al., 2009; Tanoue et al., 2015). Furthermore, this CCB has approached and exceeded minimum human therapeutic levels in fish plasma sampled from urban estuaries along the Texas Gulf of Mexico (Scott et al., 2016). These same watersheds have a history of fish kills attributed to hypoxic conditions and have been registered on the Texas 303(d) list for nonattainment of DO

water quality criteria (Brooks et al., 2008; Thronson, 2008). Unfortunately, the influence of low DO on chemical exposure dynamics and effects in fish remain understudied. The present study demonstrated that DO influences diltiazem uptake and altered some physiological responses in fish.

Bioconcentration of diltiazem in fathead minnows was highly dependent on DO conditions in the present study. Steady state plasma and tissue concentrations were achieved by approximately 24 h in normal conditions, but were observed in just 6 h in low DO treatments. Measured internal diltiazem concentrations were significantly ($p<0.05$) higher in fish under low DO conditions, but similar to diltiazem muscle concentrations in 21 d and 42 d normoxic bioconcentration studies with rainbow trout (Steinbach et al, 2016). Whole body BCFs were ~2X higher at low DO levels relative to normal DO conditions. Thus, low DO conditions influenced the bioconcentration of diltiazem, a model weak base, similar to previous studies with POPs (Blewett et al, 2013; Brauner et al., 1994; McKim and Erickson, 1991; McKim and Goeden, 1982; Yang et al., 2000). Metabolic oxygen consumption (MO_2) has been positively correlated with increased chemical uptake of ethinylestradiol (EE2), 1,2,4,5-tetrachlorobenzene, and endrin (Blewett et al, 2013; Brauner et al., 1994; McKim and Goeden, 1982; Yang et al., 2000). Killifish (*Fundulus heteroclitus*) MO_2 was positively associated with increased EE2 uptake during exercise trials but only slight increases were measured following 2 h hypoxia exposure studies (Blewett et al, 2013). Killifish are tolerant to hypoxia and the results reported by Blewett et al. (2013) support this classification; however, EE2 tissue accumulation slightly increased under hypoxic conditions and decreased MO_2. McKim and Goeden (1982) demonstrated brook trout oxygen utilization and endrin uptake

efficiency decreased with increasing ventilation volume under various short term hypoxic studies. Interestingly, endrin uptake increased with decreasing DO and uptake efficiency but plateaued between 50 and 30% oxygen saturation at 11-12 ˚C.

Fish use various physiological and biochemical strategies to cope with less than optimal DO concentrations. Initially, when decreased oxygen levels are detected, fish increase ventilation frequency or amplitude to boost water flow across the gills and perfuse additional gill lamellae to increase oxygen uptake (Randall, 1982). Thus, low DO levels can result in increased ventilation, which increases water flow to gill lamellae, and has been suggested to be one factor influencing chemical uptake for compounds with log P values < 6.0 under normoxic conditions (McKim and Erickson, 1991). As DO levels decrease, fish oxygen consumption and chemical uptake increases but eventually plateaus despite high respiratory volumes due to shorter water residence time in gill lamellar channels (McKim and Erickson, 1991). Under maximum respiratory volumes, chemical uptake shifts and is dictated by chemical diffusivity parameters instead of water flow across gill epithelium (McKim and Erickson, 1991; McKim and Goeden, 1982). It is unknown whether oxygen and diltiazem uptake plateaued in the present study (~36% saturations at 25 ˚C). Fathead minnows are relatively tolerable to hypoxia in comparison to salmonids; for example, aquatic surface respiration by fathead minnows was reported to occur at 9% DO saturation at 24 ˚C (Gee et al., 1978). Therefore, lower DO conditions than those examined in the present study may further influence diltiazem uptake in fathead minnows. Additional studies are necessary to determine modes of increased chemical uptake across fish species (e.g., warm and cold water species) to examine

whether enhanced uptake is attributed to one or both strategies to increase oxygen consumption (e.g., increased water flow or gill lamellae surface area).

Previous studies with environmental factors have also reported differential chemical uptake due to bulk water pH level and pH at the gill microenvironment. In the present study, mean (±SE) bulk exposure water pH was 8.1±0.02 and 8.4±0.01 under normal and low DO levels, respectively. Our laboratory previously demonstrated that uptake of the ionizable weak base diphenhydramine was elevated with increasing pH in 96 h adult male fathead minnow studies (Nichols et al., 2015). Furthermore, pH conditions at the gill microenvironment were reported to influence chemical uptake of weak acids due to the elimination of metabolic acids at gill lamellae, which altered chemical ionization (Erickson et al, 2006). Fish ventilation rates were not measured in 96 h bioconcentration studies of the present manuscript, although visual observations indicated increased ventilation at low DO. Enhanced respiratory volumes increase water flow across gill lamellar channels, which has been suggested to be one of the limiting factors for chemical uptake (McKim and Erickson, 1991). Elevated ventilation rates will result in increased water flow across gill lamellae and minimize the metabolic acid induced decrease in pH at the gill microenvironment. In the present study, bulk water pH conditions in exposure aquaria resulted in 52% and 66% of diltiazem to be non-ionized (pKa = 8.06) at normal and low DO levels, respectively. However, the fish plasma model with log D calculated $P_{B:W}$ values predicted nearly identical internal fish plasma concentrations (53.9 and 53.2 µg/L at normal and low DO levels, respectively) when empirical study conditions were incorporated (e.g., mean pH, mean 96 h water concentration). Therefore, differences in uptake across DO conditions are potentially due

to other factors besides bulk water pH, such as increased water flow, metabolic acid elimination, and lamellae perfusion at the gills. Additional studies using identical bulk water pH conditions across multiple DO levels is necessary to determine which key factor predominantly influences ionizable weak base uptake. This research appears particularly important since diltiazem has been reported to approach and even exceed C_{min} levels in saltwater more frequently than freshwater systems (Saari et al, 2017; Scott et al, 2016).

When oxygen uptake fails to sufficiently match aerobic metabolic demand, reductions in fish activity commonly occur to minimize the metabolic consequences resulting from a lack of oxygen and limited ATP production (Dutil et al., 2007; Jones, 1971; Richards, 2009). Results from the present study revealed impaired fish U_{burst} at low DO levels (~13%; 3.19 ± 0.1 mg DO/L) following 24 h acute studies. Conversely, fish U_{burst} in acute diltiazem studies remained unchanged; however, decreased fish swim performance was observed in DO x diltiazem co-exposures, though insignificantly ($p = 0.085$), and thus requires additional study to determine if higher diltiazem exposure levels would adversely affect this important physiological endpoint. Reductions in swim performance is commonly associated with decreased metabolic scope (Dutil et al., 2007). Such responses have been attributed to increased metabolic stress by either adding costs to routine maintenance (e.g., basal metabolism) or limiting the maximum oxygen consumption (Brett, 1958). Atlantic cod exposed to low DO conditions similarly experienced decreased swim performance which corresponded to measurable metabolic constraints (Chabot and Claireaux, 2008; Claireaux et al., 1995; Dutil et al., 2007; Herbert and Steffensen, 2005). Changes in fish blood constituents and metabolites (e.g.,

lactate, glucose) during hypoxic exposures reflect metabolic constraints to aerobic respiration and are indicative of shifts in energy supply and demand dynamics (Burton and Heath, 1980; Ishibashi et al., 2002; Muusze et al., 1998; van Raaij et al., 1996a; van Raaij et al., 1996b). In support of the above mentioned blood metabolite changes, fathead minnow plasma lactate levels were significantly higher at low versus normal DO levels (Fig. 19), while diltiazem had no significant ($p = 0.165$) effect on plasma lactate. Fathead minnows are moderately tolerant to low DO conditions. Gee et al (1978) reported 50% of fathead minnows exposed to declining DO at 16.5 ˚C initiated surface respiration and decreased activity declined at 13.7 Torr. Saari et al (2018, In press) reported a 48 h LC_{50} value of 1.7 mg DO/L for larval fish. In the present study, plasma lactate levels in fish exposed to DO x diltiazem were not significantly ($p = 0.131$) decreased with increasing diltiazem concentration, contrary to previous results demonstrating DO-dependent lactate tissue accumulation (Burton and Heath, 1980; van Raaij et al, 1996b; Muusze et al, 1998). Interestingly, metabolic alterations were similarly observed in rats under hemorrhagic shock following diltiazem intravenous injection (Maitra et al, 1991). Diltiazem treated rats experienced beneficial reductions in plasma glucose levels and heart rates but were accompanied with increased plasma lactate levels. Whether diltiazem provides similar beneficial effects in fish are unknown. Additional studies examining temporal metabolic metabolite fluctuations is necessary to pinpoint the physiological responses in fish, particularly under the above described conditions. Furthermore, additional research is needed to understand the pharmacodynamic mechanism of these potential physiological responses (e.g., adverse or beneficial). The present study results suggest decreases in swim performance may be due to pharmacological effects in fish,

similarly observed in other fish models following diltiazem exposure (Steinbach et al, 2016). In the present study, such physiological responses were observed at fish plasma concentrations within and above human therapeutic level. The consequences of such responses in fish remain unknown but deserve further research, particularly when pharmacological activity of diverse medicines has been previously reported in fish (Brodin et al, 2013; Cuklev et al, 2011; Huerta et al, 2016; Margiotta-Casaluci et al, 2014; Runnalls et al, 2015; Valenti et al, 2012).

Diltiazem displays cardioselectivity in humans when prescribed to treat hypertension and angina acting both as a vasodilator and cardiodepressant (Law et al., 2013; Wishart et al., 2006). Diltiazem antagonizes smooth epithelium L-type calcium channels to increase blood flow and deliver sufficient oxygen and nutrients to the heart. Additionally, diltiazem decreases the heart workload by inhibiting an influx of calcium into cardiomyocytes thereby reducing cardiac contractility and contractive force (Law et al., 2013). Whether diltiazem is decreasing the fathead minnow contractive force of cardiomyocytes is unknown. However, decreases in fish heart rate have been reported following acute mg/L CCB exposures (e.g., diltiazem, verapamil) in larval zebrafish (Parker et al., 2014) and fathead minnows (Saari et al, 2018, In Press). Steinbach et al. (2016) reported histological changes of heart and liver blood vessels in rainbow trout suggesting vasodilation following long-term studies at µg/L diltiazem levels. Thus, research examining the cardiovascular effects in fish following long term low dose diltiazem exposure is warranted. The present study measured significant increases in hematocrit at low DO x 3.1 µg/L diltiazem, which may indicate hypotensive conditions in fish, although changes in hematocrit were not observed in other DO x diltiazem

treatments. Thus, diltiazem pharmacological effects have been reported in fish at various doses, exposure durations, and species. Further research is needed to determine whether similar mammalian pharmacodynamic mechanisms are occurring in fish, and, if so, how such responses relate to internal plasma doses.

In humans and mammals, CCBs like diltiazem and verapamil have distinct calcium channel binding sites while other CCBs are less specific (Spedding and Paoletti, 1992). The diltiazem target, the voltage-dependent calcium channel L-type α-1C, α-1D, α-1F, α-1s subunits, is approximately 70-78% and 64-81% conserved in zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*; Gunnarsson et al., 2008; Verbruggen et al., 2017), respectively. Whether genetic conservation of diltiazem and other CCB drug targets correspond to similar pharmacological activity in fish remains unknown. As noted above, pharmacological read-across studies have demonstrated comparative physiological responses in fish for several pharmaceuticals (Brodin et al., 2013; Cuklev et al., 2011; Huerta et al., 2016; Margiotta-Casaluci et al., 2014; Margiotta-Casaluci et al., 2016; Runnalls et al., 2015; Valenti et al., 2012). The present study demonstrated a lack in organism level responses to diltiazem alone; however, in DO x diltiazem co-exposures, multiple physiological responses were observed. Similar studies examining the effects of interacting stressors is necessary to determine the interacting effects of other heart medication in fish. As the present study demonstrates, a failure to consider low DO in toxicological assessments of cardioactive pharmaceuticals and potentially other cardiotoxicants may underestimate their impact to non-target organisms. Here again, the present study results demonstrate the predictive ability of the fish plasma model to prioritize compounds for additional study by linking human therapeutic fish plasma

levels to physiological and pharmacological responses in fish (Brooks, 2014; Fick et al., 2010b; Huggett et al., 2003). Additional studies with other pharmaceutical classes are necessary to support and validate the fish plasma model, particularly under environmentally relevant exposure scenarios. Including the influence of DO during pharmaceutical ecological hazard and risk assessment appears necessary due to their influence on bioconcentration and biological effects in fish. Particular research attention should be given to urban eutrophic systems experiencing diel fluctuations in DO, pH, and temperature (Scott et al, 2016; Valenti et al, 2011; Van Wezel, 1998). Under such scenarios, oxygen levels plummet in parallel with decreases in pH, creating less than optimal DO conditions for fish and increasing the percent neutral weak acid in solution. Therefore, ecological hazard assessment of ionizable chemicals failing to consider environmental factors will likely underestimate the fate and biological effects of these contaminants in fish.

Conclusion

Our experimental results suggest low DO influences the uptake and physiological effects of diltiazem in adult fathead minnows. These observed effects may be dictated by physiological responses in fish to enhance oxygen uptake, which increases diltiazem inhalation by increasing respiratory volume and altering pH conditions at the gill microenvironment. Once diffusion across the gills has occurred, diltiazem distribution in fish tissue (V_D) is minimal, similar to humans, as indicated by the steady state BCFs (9 and 16) under normal and low DO conditions, respectively. Exposure water and internal diltiazem plasma concentrations were linked to physiological responses in adult fathead

minnows but only at low DO conditions. Low DO minimized the time to steady state conditions in 96 h bioconcentration studies, which corresponded to increased ventilation rates and water flow across the gills under low DO conditions. Fish swim performance trials indicated alterations in aerobic respiration energy supply-demand dynamics in 24 h DO x diltiazem studies, which corresponded to nonsignificant decreases in fathead minnow U_{burst} and decreasing plasma lactate levels with increasing diltiazem concentration. While the effects of environmental factors on fish toxicity (Lloyd, 1961) and physiological mechanisms of chemical uptake across the gill (McKim and Goeden, 1982) were first studied ~six decades ago, empirical data examining these key factors and associated mechanisms of uptake are limited and largely dependent on previous work with persistent organic pollutants (e.g., endrin, DDT, halogenated benzenes, etc.). Predicting chemical uptake across the gill is complex and is dependent on environmental factors, experimental conditions, and fish species (Blewett et al., 2013b; McKim and Goeden, 1982; Opperhuizen and Schrap, 1987); therefore, additional research is necessary to predict ionizable chemical uptake and physiological responses in fish across multiple environmental exposure scenarios. Studies and assessments failing to consider low DO influences on cardiotoxicants may underestimate ecological risks to fish.

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APPENDICES

APPENDIX A

Supplemental Information for Chapter Two

Supplementary Table 16: Dissolved oxygen toxicity values used to create species sensitivity distributions from both pre-1986 and recently published data on the effects of low DO to freshwater fish and invertebrates. References are given for each study used in SSDs including the type of organisms, species, common name, life stage, test duration, experimental temperature, median lethal concentration or 50% effective concentration (LC50/EC50), and the parameter calculated. Note: GM, (-), and MCT represent geometric mean, an unknown measure of central tendency, and measure of central tendency, respectively.

Species	Common Name	Life Stage	Test Duration	Temperature (°C)	Endpoint	Toxicity Value (mg DO/L)	MCT	Reference
Acute Invertebrate Toxicity Data								
Acroneuria lycorias	Stonefly	Larvae	96 h	19	LC50	3.60	Median	Nebeker 1972
Acroneuria pacifica	Stonefly	-	96 h	6	LC50	1.60	-	Gaufin 1973
Anabolia nervosa	Caddisfly	Nearly grown nymphs	5 h	25	LC50	0.66	-	Jacob et al, 1984
Arcynopteryx aurea	Stonefly	-	96 h	6	LC50	3.30	-	Gaufin 1973
Asellus intermedius	Amphipod	-	24 h	20	LC50	0.03	-	Sprague 1963
Baetis alpinus	Mayfly	Nearly grown nymphs	5 h	12	LC50	8.13	-	Jacob et al, 1984
Baetis alpinus	Mayfly	Nearly grown nymphs	5 h	15	LC50	8.31	-	Jacob et al, 1984
Baetis niger	Mayfly	Nearly grown nymphs	5 h	20	LC50	3.87	-	Jacob et al, 1984
Baetis vernus	Mayfly	Nearly grown nymphs	5 h	12	LC50	2.99	-	Jacob et al, 1984
Baetisca laurentina	Mayfly	Larvae	96 h	19	LC50	3.50	Median	Nebeker 1972
Brachytron hafniense	Dragonfly	Nearly grown nymphs	5 h	30	LC50	1.99	-	Jacob et al, 1984
Callibaetis montanus	Mayfly	-	96 h	6	LC50	4.40	-	Gaufin 1973
Clistoronia manifica	Caddisfly	Instar I	96 h	17	LC50	1.95	-	Nebeker et al, 1996

Clistoronia manifica	Caddisfly	Instar IV	96 h	18	LC50	1.30	-	Nebeker et al, 1996
Cloeon simile	Mayfly	Nearly grown nymphs	5 h	15	LC50	1.12	-	Jacob et al, 1984
Cloeon simile	Mayfly	Nearly grown nymphs	5 h	20	LC50	2.13	-	Jacob et al, 1984
Cloeon simile	Mayfly	Nearly grown nymphs	5 h	25	LC50	2.64	-	Jacob et al, 1984
Cloeon simile	Mayfly	Nearly grown nymphs	5 h	30	LC50	3.44	-	Jacob et al, 1984
Daphnia magna	Water flea	4 d old	48 h	13	LC50	0.65	GM	Nebeker et al, 1992
Daphnia pulex	Water flea	5-6 d	48-96 h	17	LC50	0.51	GM	Nebeker et al, 1992
Diura knowltoni	Stonefly	-	96 h	6	LC50	3.60	-	Gaufin 1973
Drusinus sp.	Caddisfly	-	96 h	6	LC50	1.80	-	Gaufin 1973
Epeorus sylvicola	Mayfly	Nearly grown nymphs	5 h	12	LC50	8.07	-	Jacob et al, 1984
Epeorus sylvicola	Mayfly	Nearly grown nymphs	5 h	15	LC50	8.08	-	Jacob et al, 1984
Epeorus sylvicola	Mayfly	Nearly grown nymphs	5 h	20	LC50	8.75	-	Jacob et al, 1984
Ephemera danica	Mayfly	Nearly grown nymphs	5 h	15	LC50	0.06	-	Jacob et al, 1984
Ephemera danica	Mayfly	Nearly grown nymphs	5 h	20	LC50	0.34	-	Jacob et al, 1984
Ephemera vulgata	Mayfly	Nearly grown nymphs	5 h	15	LC50	0.03	-	Jacob et al, 1984
Ephemera vulgata	Mayfly	Nearly grown nymphs	5 h	20	LC50	0.03	-	Jacob et al, 1984
Ephemera vulgata	Mayfly	Nearly grown nymphs	5 h	25	LC50	0.04	-	Jacob et al, 1984

Ephemerella mucronata	Mayfly	Nearly grown nymphs	5 h	15	LC50	1.22	-	Jacob et al, 1984
Ephemerella mucronata	Mayfly	Nearly grown nymphs	5 h	20	LC50	1.74	-	Jacob et al, 1984
Ephemerella mucronata	Mayfly	Nearly grown nymphs	5 h	25	LC50	4.08	-	Jacob et al, 1984
Ephemerilla doddsi	Mayfly	-	96 h	6	LC50	5.20	-	Gaufin 1973
Ephemerilla grandis	Mayfly	-	96 h	6	LC50	3.00	-	Gaufin 1973
Ephemerilla subvaria	Mayfly	Larvae	96 h	19	LC50	3.90	Median	Nebeker 1972
Gammarus fasciatus	Amphipod	-	24 h	20	LC50	4.30	-	Sprague 1963
Gammarus pseudolimnaeus	Amphipod	-	24 h	20	LC50	2.20	-	Sprague 1963
Gammarus pseudolimnaeus	Amphipod	adult	24h	15	LC50	1.80	GM	Hoback and Barnhart 1996
Gammarus pseudolimnaeus	Amphipod	Juvenile	24h	15	LC50	1.03	GM	Hoback and Barnhart 1996
Hexagenia limbata	Mayfly	-	96 h	6	LC50	1.80	-	Gaufin 1973
Hexagenia limbata	Mayfly	Larvae	96 h	19	LC50	1.40	Median	Nebeker 1972
Hyalella azteca	Amphipod	-	24 h	20	LC50	0.70	-	Sprague 1963
Hydropsyche betteri	Caddisfly	Larvae	96 h	21	LC50	2.90	Median	Nebeker 1972
Hydropsyche betteri	Caddisfly	Larvae	96 h	19	LC50	2.60	Median	Nebeker 1972

Hydropsyche betteri	Caddisfly	Larvae	96 h	17	LC50	2.30	Median	Nebeker 1972
Hydropsyche betteri	Caddisfly	Larvae	96 h	10	LC50	1.00	Median	Nebeker 1972
Hydropsyche sp.	Caddisfly	-	96 h	6	LC50	3.60	-	Gaufin 1973
Leptophlebia nebulosa	Mayfly	Larvae	96 h	19	LC50	2.20	Median	Nebeker 1972
Leptpohlebia marginata	Mayfly	Nearly grown nymphs	5 h	15	LC50	0.14	-	Jacob et al, 1984
Leptpohlebia marginata	Mayfly	Nearly grown nymphs	5 h	20	LC50	0.15	-	Jacob et al, 1984
Leptpohlebia marginata	Mayfly	Nearly grown nymphs	5 h	25	LC50	0.91	-	Jacob et al, 1984
Leptpohlebia marginata	Mayfly	Nearly grown nymphs	5 h	30	LC50	1.92	-	Jacob et al, 1984
Lestes sponsa	Damselfly	Nearly grown nymphs	5 h	20	LC50	0.92	-	Jacob et al, 1984
Limnephilus ornatus	Caddisfly	-	96 h	6	LC50	3.40	-	Gaufin 1973
Nemoura cinctipes	Stonefly	-	96 h	6	LC50	3.30	-	Gaufin 1973
Nemoura cinerea	Stonefly	Nearly grown nymphs	5 h	12	LC50	1.13	-	Jacob et al, 1984
Nemoura cinerea	Stonefly	Nearly grown nymphs	5 h	25	LC50	2.68	-	Jacob et al, 1984
Neophylax sp.	Caddisfly	-	96 h	6	LC50	3.80	-	Gaufin 1973
Neothremma alicia	Caddisfly	-	96 h	6	LC50	1.70	-	Gaufin 1973
Onychogomphus forcipatus	Dragonfly	Nearly grown nymphs	5 h	25	LC50	1.18	-	Jacob et al, 1984
Onychogomphus forcipatus	Dragonfly	Nearly grown nymphs	5 h	30	LC50	1.14	-	Jacob et al, 1984

Paranephrops planifrons	Crayfish	Adult/Juvenile	48 h	17	LC50	0.77	Mean	Landman et al, 2005
Paratya curvirostris	Crayfish	Adult/Juvenile	48 h	15	LC50	0.82	Mean	Landman et al, 2005
Pteronarcella badia	Stonefly	-	96 h	6	LC50	2.40	-	Gaufin 1973
Pteronarcys californica	Stonefly	-	96 h	6	LC50	3.53	GM	Gaufin 1973
Pteronarcys dorsata	Stonefly	Larvae	96 h	19	LC50	2.20	Median	Nebeker 1972
Rhamdia quelen	Silver catfish	Juvenile	24-96 h	27	LC50	0.52	GM	Weiss and Zaniboni-Filho, 2010
Rhamdia quelen	Silver catfish	Juvenile	96 h	26	LC50	0.52	-	Braun et al, 2006
Rhithrogena iridina	Mayfly	Nearly grown nymphs	5 h	12	LC50	5.36	-	Jacob et al, 1984
Rhyacophila obliterata	Caddisfly	Nearly grown nymphs	5 h	15	LC50	7.07	-	Jacob et al, 1984
Sialis lutaria	Alderfly	Nearly grown nymphs	5 h	20	LC50	0.96	-	Jacob et al, 1984
Sialis lutaria	Alderfly	Nearly grown nymphs	5 h	25	LC50	0.89	-	Jacob et al, 1984
Sialis lutaria	Alderfly	Nearly grown nymphs	5 h	30	LC50	0.95	-	Jacob et al, 1984
Silo pallipes	Caddisfly	Nearly grown nymphs	5 h	12	LC50	7.87	-	Jacob et al, 1984
Silo pallipes	Caddisfly	Nearly grown nymphs	5 h	15	LC50	7.99	-	Jacob et al, 1984
Simulium vittatum	Midge	-	96 h	6	LC50	3.20	-	Gaufin 1973
Siphlonurus aestivalis	Mayfly	Nearly grown nymphs	5 h	20	LC50	0.48	-	Jacob et al, 1984

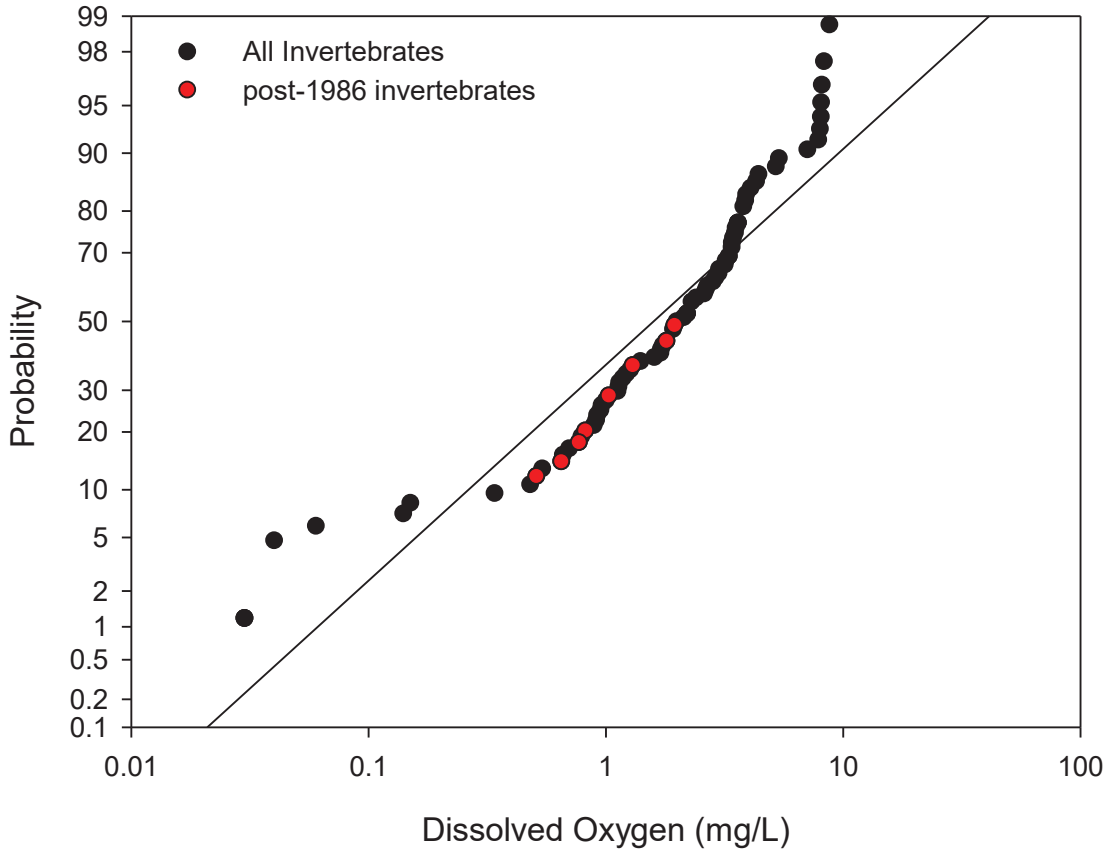
Siphlonurus aestivalis	Mayfly	Nearly grown nymphs	5 h	25	LC50	0.54	-	Jacob et al, 1984
Siphlonurus lacustris	Mayfly	Nearly grown nymphs	5 h	15	LC50	2.83	-	Jacob et al, 1984
Siphlonurus lacustris	Mayfly	Nearly grown nymphs	5 h	20	LC50	3.17	-	Jacob et al, 1984
Siphlonurus lacustris	Mayfly	Nearly grown nymphs	5 h	25	LC50	3.39	-	Jacob et al, 1984
Sympecma fusca	Damselfly	Nearly grown nymphs	5 h	20	LC50	0.79	-	Jacob et al, 1984
Sympecma fusca	Damselfly	Nearly grown nymphs	5 h	25	LC50	1.27	-	Jacob et al, 1984
Sympecma fusca	Damselfly	Nearly grown nymphs	5 h	30	LC50	1.71	-	Jacob et al, 1984
Chronic Invertebrate Toxicity Data								
Baetisca laurentina	Mayfly	Larvae	30 d	19	LC50	5.00	Median	Nebeker 1972
Clistoronia manifica	Caddisfly	Embryo	21 d	17	LC50	2.00	-	Nebeker et al, 1996
Ephemera simulans	Mayfly	Larvae	30 d	19	LC50	4.50	Median	Nebeker 1972
Gammarus lacustris	Amphipod	Adult	168 h	13	LC50	0.49	GM	Nebeker et al, 1992
Pteronarcys dorsata	Stonefly	Larvae	30d	19	LC50	4.80	Median	Nebeker 1972
Pteronarcys dorsata	Stonefly	Larvae	30d	19	LC50	4.40	Median	Nebeker 1972
Acute Fish Toxicity Data								
Anguilla australis	Shortfin eel	Elvers	48 h	15	LC50	0.54	Mean	Landman et al, 2005
Chasmistes brevirostris	Shortnose sucker	Larval	24-96 h	32	LC50	2.09	GM	Saiki et al, 1999
Chasmistes brevirostris	Shortnose sucker	Juvenile	24-96 h	30	LC50	1.34	Mean	Saiki et al, 1999

Deltistes luxatus	Lost River Sucker	Larval (35 d old)	24-96 h	32	LC50	2.10	GM	Saiki et al, 1999
Deltistes luxatus	Lost River Sucker	Juvenile (3-7 months old)	24-96 h	31	LC50	1.62	GM	Saiki et al, 1999
Galaxias maculatus	Inanga	Whitebait	48 h	15	LC50	2.65	Mean	Landman et al, 2005
Gobiomorphus cotidianus	Common bully	Juvenile/Fry	48 h	15	LC50	0.84	GM	Landman et al, 2005
Notropis topeka	Topeka shiner	Adult	96 h	28	LC50	1.26	Mean	Koehle and Adelman, 2007
Oncorhynchus mykiss	Rainbow trout	Parr	48 h	15	LC50	1.62	Mean	Landman et al, 2005
Oncorhynchus mykiss	Rainbow trout	Swim-up-fry	48 h	15	LC50	1.59	Mean	Landman et al, 2005
Retropinna retropinna	Common smelt	Adult	48 h	15	LC50	1.83	Mean	Landman et al, 2005
Chronic Fish Toxicity Data								
Esox lucius	Northern Pike	Juvenile	42-48 d	19	EC50	1.73	-	Adelman and Smith, 1970
Ictalurus punctatus	Channel Catfish	Juvenile	69-71 d	25	EC50	0.79	-	Carlson et al, 1980
Micropterus salmoides	Largemouth Bass	Juvenile	14 d	19	EC50	1.53	GM	Brake, 1972
Micropterus salmoides	Largemouth Bass	Juvenile	11-15.5 d	25	EC50	2.38	-	Stewart, 1967
Micropterus salmoides	Largemouth Bass	Juvenile	11-15.5 d	26	EC50	2.28	-	Stewart, 1967
Perca flavescens	Yellow Perch	Juvenile	25 d	25	EC50	0.55	-	Carlson et al, 1980
Esox lucius	Northern Pike	Juvenile	42-48 d	19	EC10	4.95	-	Adelman and Smith, 1970

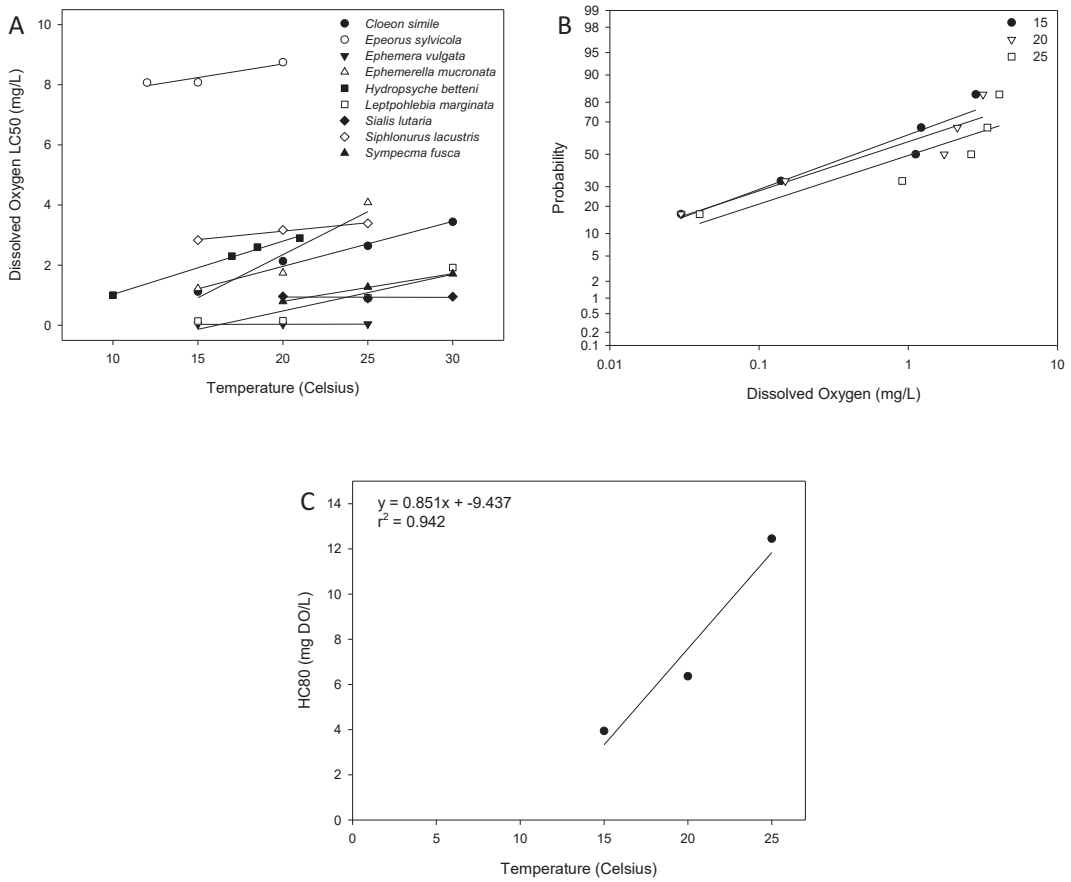
Ictalurus punctatus	Channel Catfish	Juvenile	69-71 d	25	EC10	4.54	-	Carlson et al, 1980
Micropterus salmoides	Largemouth Bass	Juvenile	14 d	25	EC10	3.92	-	Brake, 1972
Micropterus salmoides	Largemouth Bass	Juvenile	11-15.5 d	26	EC10	4.51	-	Stewart, 1967
Micropterus salmoides	Largemouth Bass	Juvenile	11-15.5 d	19	EC10	6.41	-	Stewart, 1967
Perca flavescens	Yellow Perch	Juvenile	25 d	25	EC10	4.74	-	Carlson et al, 1980
Oncorhynchus kisutch	Coho Salmon	Fingerlings	42 d	15	EC50	1.92	-	Brett and Blackburn, 1981
Oncorhynchus kisutch	Coho Salmon	Juvenile	18-20 d	13	EC50	2.26	GM	Warren, 1973
Oncorhynchus kisutch	Coho Salmon	Juvenile	13 d	18	EC50	1.38	-	Warren, 1973
Oncorhynchus kisutch	Coho Salmon	Juvenile	20 d	9	EC50	1.68	-	Warren, 1973
Oncorhynchus kisutch	Coho Salmon	Juvenile	20 d	22	EC50	0.72	-	Warren, 1973
Oncorhynchus kisutch	Coho Salmon	Fingerlings	18 d	18	EC50	2.13	GM	Fisher, 1963
Oncorhynchus kisutch	Coho Salmon	Juvenile	21 d	20	EC50	3.08	GM	Herrmann, 1962
Oncorhynchus mykiss	Rainbow trout	-	54-71 d	12	EC50	2.04	GM	Spoor, 1981
Oncorhynchus nerka	Sockeye Salmon	Fingerlings	42 d	15	EC50	3.03	-	Brett and Blackburn, 1981
Oncorhynchus tshawytscha	Chinook Salmon	Juvenile	20 d	13	EC50	1.77	GM	Warren, 1973
Oncorhynchus tshawytscha	Chinook Salmon	Juvenile	20 d	8	EC50	1.98	-	Warren, 1973

Oncorhynchus tshawytscha	Chinook Salmon	Juvenile	20 d	18	EC50	3.65	GM	Warren, 1973
Oncorhynchus tshawytscha	Chinook Salmon	Juvenile	20 d	22	EC50	4.44	-	Warren, 1973
Salmo trutta	Brown trout	-	60 d	12	EC50	2.26	-	Spoor, 1981
Salvelinus fontinalis	Brook trout	-	62 d	12	EC50	1.96	-	Spoor, 1981
Salvelinus namaycush	Lake trout	-	96-131 d	12	EC50	1.93	GM	Spoor, 1981
Oncorhynchus kisutch	Coho Salmon	Fingerlings	18 d	18	EC10	5.18	GM	Fisher, 1963
Oncorhynchus kisutch	Coho Salmon	Juvenile	21 d	20	EC10	4.48	GM	Herrmann, 1962
Oncorhynchus kisutch	Coho Salmon	Juvenile	18-20 d	13	EC10	4.33	GM	Warren, 1973
Oncorhynchus kisutch	Coho Salmon	Juvenile	13 d	18	EC10	7.79	-	Warren, 1973
Oncorhynchus kisutch	Coho Salmon	Juvenile	20 d	9	EC10	4.78	-	Warren, 1973
Oncorhynchus kisutch	Coho Salmon	Juvenile	20 d	22	EC10	0.97	-	Warren, 1973
Oncorhynchus kisutch	Coho Salmon	Fingerlings	42 d	15	EC10	5.35	-	Brett and Blackburn, 1981
Oncorhynchus mykiss	Rainbow trout	-	54-71 d	12	EC10	6.13	GM	Spoor, 1981
Oncorhynchus nerka	Sockeye Salmon	Fingerlings	42 d	15	EC10	4.41	-	Brett and Blackburn, 1981
Oncorhynchus tshawytscha	Chinook Salmon	Juvenile	20 d	13	EC10	4.74	GM	Warren, 1973
Oncorhynchus tshawytscha	Chinook Salmon	Juvenile	20 d	8	EC10	3.76	-	Warren, 1973

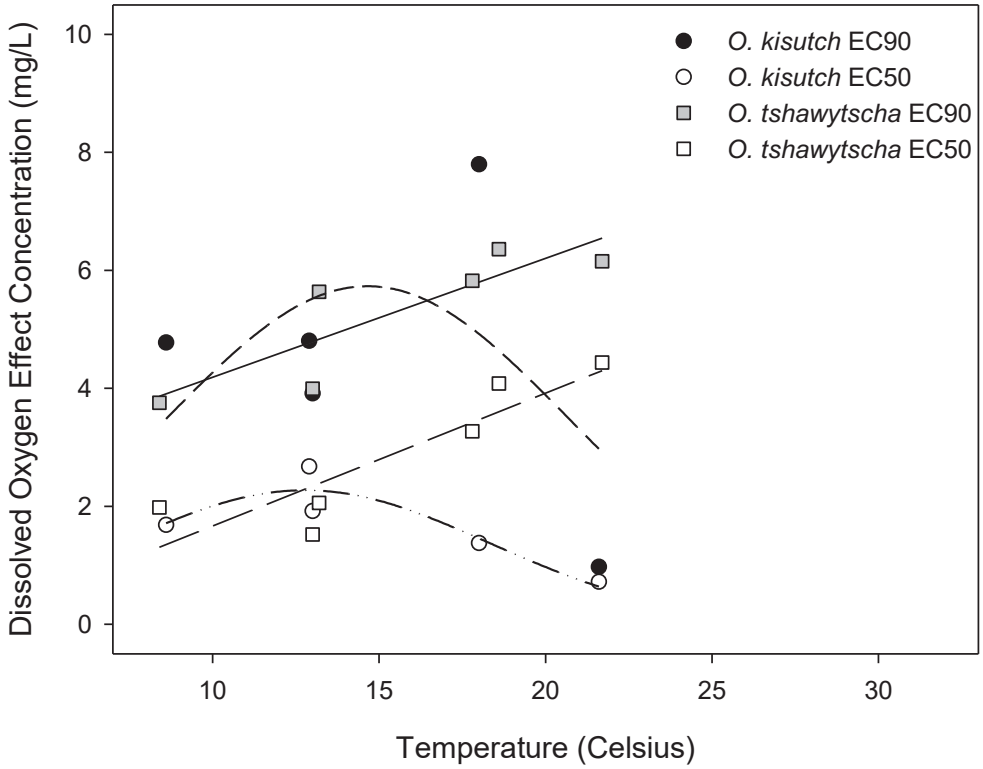
Oncorhynchus tshawytscha	Chinook Salmon	Juvenile	20 d	18	EC10	6.09	GM	Warren, 1973
Oncorhynchus tshawytscha	Chinook Salmon	Juvenile	20 d	22	EC10	6.15	-	Warren, 1973
Salmo trutta	Brown trout	-	60 d	12	EC10	5.39	-	Spoor, 1981
Salvelinus fontinalis	Brook trout	-	62 d	12	EC10	7.57	-	Spoor, 1981
Salvelinus namaycush	Lake trout	-	96-131 d	12	EC10	6.08	GM	Spoor, 1981



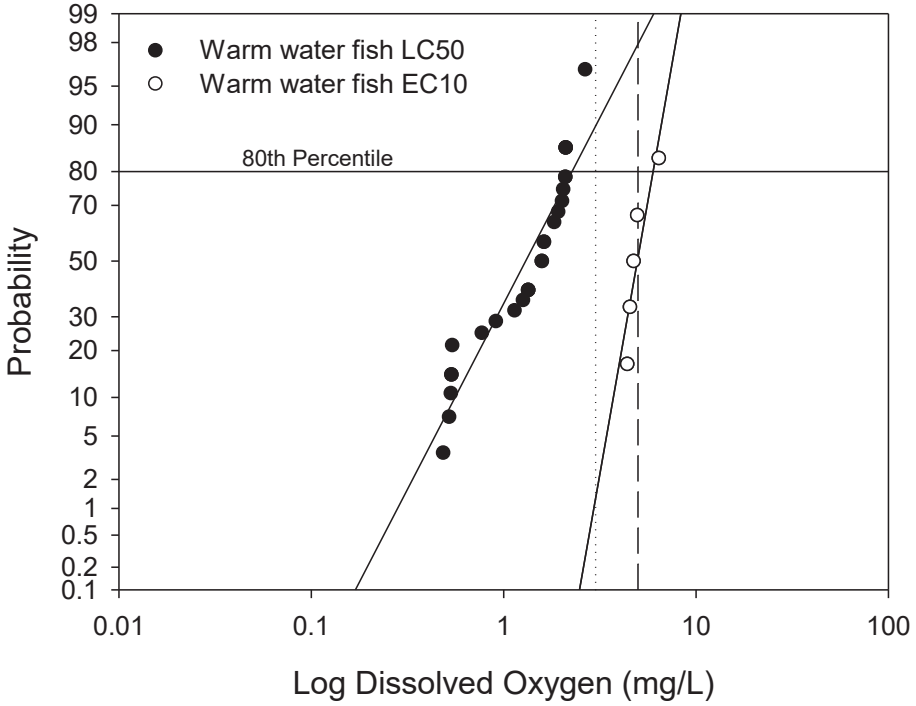
Supplemental: Figure: 20. Aquatic hazard assessment for dissolved oxygen with acute invertebrate data. The distribution represents all of the available acute invertebrate data compared to those newly published acute invertebrate data values within the dataset (red circles).



Supplemental: Figure: 21. Acute (2-96h) temperature-dependent dissolved oxygen (DO) toxicity relationships for A) invertebrates within the orders Ephemeroptera, Trichoptera, Megaloptera, and Odonata. Solid lines represent linear regression lines fitted for each species (*C. simile*, *H. betteri*, *S. fusca*; $p < 0.05$). B) The acute (2-96h) temperature-dependent dissolved oxygen (DO) toxicity SSD for five ephemeroptera species studied across the same three temperatures and C) the HC80 values of the five ephemeroptera species across three temperatures. Dose-response curves were plotted based on data availability when a species were studied across at least three different temperatures by the same researcher. All species inhabit either lotic (*Ephemerella mucronata, Hydropsyche betteni, Leptpohlebia marginata*), lentic (*Cloeon simile*), or both lotic and lentic (*Epeorus sylvicola, Ephemera vulgate, Sialis lutaria, Siphlonurus lacustris, Sympecma fusca*) habitats.



Supplemental: Figure: 22. Temperature-dependent chronic fish growth effect concentration (EC) data to illicit 10% (EC90) and 50% (EC50) decreased growth for *Oncorhynchus kisutch* and *Oncorhynchus tshawytscha*. Effect concentrations were calculated from log-linear normalized growth rates for each species across multiple temperatures (JRB Associates, 1984; Warren et al, 1973). Solid and long dashed lines represent *Oncorhynchus tshawytscha* fitted linear regressions for EC90 and EC50 values, respectively ($p < 0.05$). Short and long-dot-dot-long dashed lines represent *Oncorhynchus kisutch* fitted polynomial regressions describing the relationship between temperature and EC values.



Supplemental: Figure: 23. Aquatic hazard assessment for dissolved oxygen with acute invertebrate data. The distribution represents all of the available acute invertebrate data compared to those newly published acute invertebrate data values within the dataset (red circles).

APPENDIX B

Supplementary Information for Chapter Three

Supplementary: Table 17. Download additional supplemental data related to Chapter Three (214 KB spreadsheet) at https://doi.org/10.1016/j.chemosphere.2017.09.058

Supplementary: Table 18: Summary of studies evaluating adverse effects of calcium channel blockers in aquatic organisms.

Compound	Test organism	Response (mg/L)	Standard endpoint	Bioassay/Endpoint	Reference
Amlodipine	*Hydra vulgaris*	1	7 d LOEC	Mean number of Artemia ingested	Pascoe et al. 2003
	Hydra vulgaris	0.1	7 d LOEC	Clubbed tentacles, body slightly contracted	Pascoe et al. 2003
	Hydra vulgaris	0.01	17 d LOEC	Regeneration of digestive region (observed for 24-72 h)	Pascoe et al. 2003
	Brachionus calyciflorus	0.6	24 h LC50	Mortality	DellaGreca et al, 2007
	Thamnocephalus platyurus	2.6	24 h LC50	Mortality	DellaGreca et al, 2007
	Daphnia magna	26.4	24 h LC50	Mortality	DellaGreca et al, 2007
	Daphnia magna	17.9	24 h LC50	Mortality	DellaGreca et al, 2007
	Ceriodaphnia dubia	0.3	7 d EC50	Population growth inhibition	DellaGreca et al, 2007
Diltiazem	*Daphnia magna*	28.0	48 h EC50	Immobilization	Kim et al. 2007
	Daphnia magna	8.2	96 h EC50	Immobilization	Kim et al. 2007
	Oryzias latipes	15.0	48 h LC50	Mortality	Kim et al. 2007
	Oryzias latipes	25.6	96 h LC50	Mortality	Kim et al. 2007
	Thamnocephalus platyurus	10.2	1 h EC50	Food ingestion; Rapidtoxkit (2004)	Nalecz-Jawecki and Persoone 2006
	Thamnocephalus platyurus	73.0	24 h LC50	Mortality	Nalecz-Jawecki and Persoone 2006
	Vibrio fischeri	152.0	24 h	Bioluminescence inhibition; Chronic bioluminescence assay	Backhaus and Grimme 1999
	Vibrio fischeri	263.7	15 min	Alteration in light output; Microtox Toxicity Analyzer	Kim et al. 2007
	Vibrio fischeri	407.0	5 min	Alteration in light output; Microtox Toxicity Analyzer	Kim et al. 2007
Verapamil	*Artemia salina*	329.6	24 h LC50	Mortality	Calleja et al. 1994 (a)
	Artemia salina	1.3	24 h LC50	Mortality	Calleja et al. 1994 (b)
	Brachionus calyciflorus	0.6	24 h LC50	Mortality	Calleja et al. 1994 (b)
	Brachionus calyciflorus	10.1	24 h LC50	Mortality	Calleja et al. 1994 (a)
	Daphnia magna	302.3	24 h EC50	Immobilization	Lilius et al. 1994
	Daphnia magna	7.0	48 h LC50	Immobilization	Villegas-Navarro et al. 2003
	Daphnia magna	51.3	24 h EC50	Immobilization	Calleja et al. 1994 (a)

	Daphnia magna	0.9	24 h EC50	Immobility	Calleja et al. 1994 (b)
	Lemna minor	24.3	7 d EC50	Growth inhibition	Kaza et al. 2007
	Oncorhynchus mykiss (hepatocytes)	1704.8	3 h EC50	Extracellular radioactivity	Lilius et al. 1994
	Photobacterium phosphoreum	403.2	5 or 15 min EC50	Bioluminescence inhibition; Microtox test	Calleja et al. 1994 (a)
	Photobacterium phosphoreum	1.3	15 min EC50	Luminescence inhibition; Microtox test	Calleja et al. 1994 (b)
	Spirostomum ambiguum	9.8	24 h LC50	Morphological changes; Spirotox	Nalecz-Jawecki and Sawicki 2003
	Spirostomum ambiguum	7.8	24 h EC50	Spherical deformation and autolysis; Spirotox	Nalecz-Jawecki and Sawicki 2003
	Streptocephalus proboscideus	5.8 mg/L	24 h LC50	Mortality; Streptoxkit F (with slight modifications)	Calleja et al. 1994 (a)
	Streptocephalus proboscideus	0.5 mg/L	24 h LC50	Mortality; Streptoxkit F (with slight modifications)	Calleja et al. 1994 (b)
	Tetrahymena termophila	358.0 mg/L	24 h EC50	Growth inhibition; Protoxkit FTM	Nalecz-Jawecki and Sawicki 2003
	Thamnocephalus platyurus	9.3 mg/L	24 h LC50	Mortality	Nalecz-Jawecki and Persoone 2006
	Thamnocephalus platyurus	9.1 mg/L	1 h EC50	Food ingestion	Nalecz-Jawecki and Persoone 2006
	Pimephales promelas	0.6	28 d NOEC	Survival	Overturf et al, 2012
	Pimephales promelas	> 0.6	28 d LOEC	Survival	Overturf et al, 2012
	Pimephales promelas	> 0.6	28 d LC50	Lethality	Overturf et al, 2012
	Pimephales promelas	0.3	28 d NOEC	Growth	Overturf et al, 2012
	Pimephales promelas	0.6	28 d LOEC	Growth	Overturf et al, 2012
	Oncorhynchus mykiss	2.72	96 h LC50	Lethality	Li et al, 2010
	Oreochromis niloticus	2.29	96 h LC50	Lethality	Ajima et al, 2017
	Daphnia magna	21.0	96 h LC50	Lethality	Le et al, 2011
	Daphnia magna	15.0	96 h LC20	Lethality	Le et al, 2011
	Daphnia magna	11.0	96 h LC10	Lethality	Le et al, 2011
	Daphnia magna	8.2	96 h LC5	Lethality	Le et al, 2011
	Daphnia magna	2.1	21 d LOEC	Reproduction	Le et al, 2011
	Daphnia magna	1.1	21 d NOEC	Reproduction	Le et al, 2011
	Daphnia magna	4.2	21 d LOEC	Growth	Le et al, 2011
	Daphnia magna	2.1	21 d NOEC	Growth	Le et al, 2011
	Daphnia magna	4.2	21 d LOEC	Survival	Le et al, 2011

	Daphnia magna	2.1	21 d NOEC	Survival	Le et al, 2011
	Cyprinus carpio	16.4	96 h LC50	Lethality	Steinbach et al, 2013
	Cyprinus carpio	4.8	96 h LC50	Lethality	Steinbach et al, 2013

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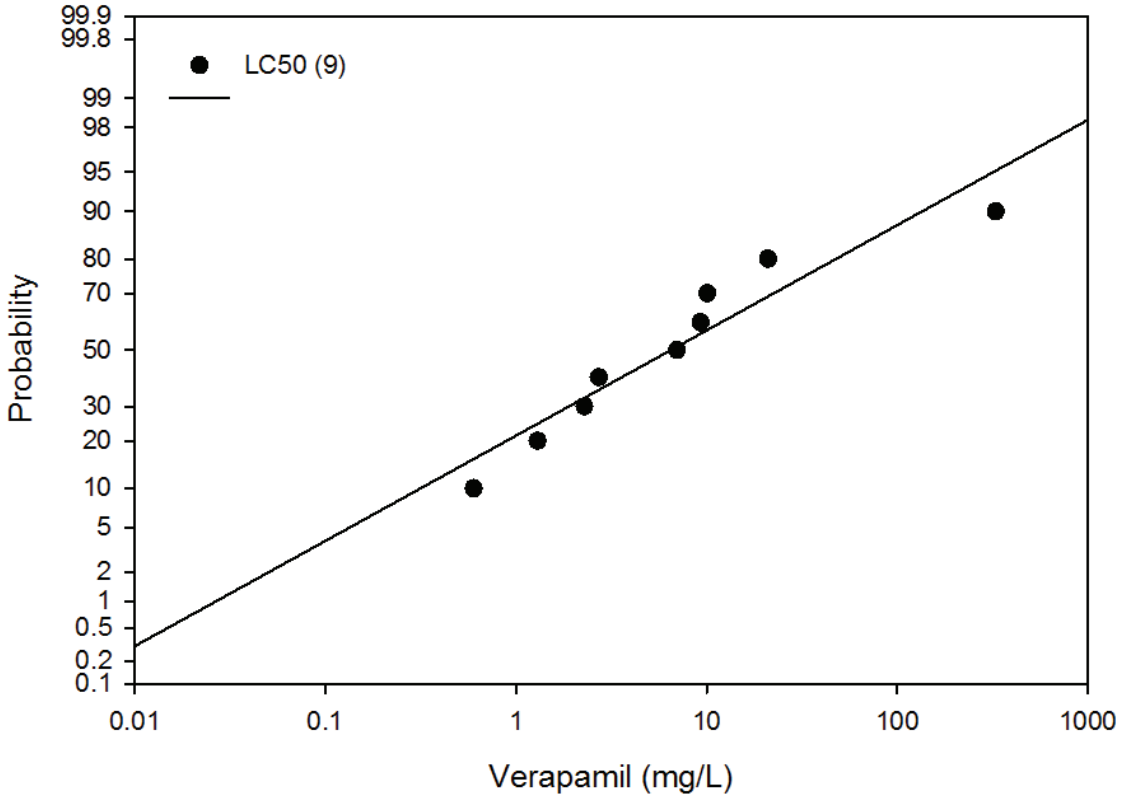


Supplementary: Figure: 24. Species sensitivity distribution of acute (≤ 96 h) LC50 toxicity values following exposure to verapamil. Nine different LC50 values were reported for verapamil from six different aquatic species (e.g., *Artemia salina, Brachionus calyciflorus, Daphnia magna, Oncorhynchus mykiss, Oreochromis niloticus, Thamnocephalus platyurus*).

APPENDIX C

Supplementary Information for Chapter Four

Supplementary: Table: 19. Mean (±SE) dissolved oxygen (DO; mg/L) and diltiazem (µg/L) concentrations measured and analytically verified in acute (48 h) and chronic (7 d) studies with *Pimephales promelas*. SE: standard error; Acute: 48 hour; Interaction: DO x diltiazem; Chronic: 7 day.

	Diltiazem (µg/L)			Dissolved Oxygen (mg/L)		
Study-#	Nominal	Analytically Verified (SD)	% Nominal	Nominal	Mean (±SE) Measured	% Nominal
[a]Acute-1	0	0.0	100	8.2	8.25±0.01	100.6
	0.15	0.094	62.8	5.0	4.82±0.07	96.4
	15000	12765	85.1	3.0	3.62±0.04	120.7
	30000	25059	83.5	2.0	2.08±0.09	104.0
	45000	39066	86.8	1.0	0.95±0.03	95.0
	60000	43008	71.7	0.5	0.58±0.01	116.0
[a]Acute-2	0	0.0	-	8.2	8.12±0.05	99.0
	0.15	0.099	66.1	5.0	5.70±0.03	114.0
	15000	11892.3	79.3	3.0	3.38±0.15	112.7
	30000	25804.0	86.0	2.0	2.09±0.08	104.5
	45000	39018.3	86.7	1.0	1.12±0.03	112.0
	60000	45603.9	76.0	0.5	0.65±0.03	130.0
[a]Acute-3	0	14.5	-*	8.2	8.12±0.04	99.0
	0.15	4.6	3044.9*	5.0	5.38±0.06	107.6
	15000	11341.8	75.6	3.0	3.06±0.07	102.0
	30000	22489.2	75.0	2.0	2.64±0.2	132.0
	45000	33145.1	73.7	1.0	1.28±0.03	128.0
	60000	40531.2	67.6	0.5	0.70±0.09	140.0
[a]Acute Interaction-1	0	0.0	-	8.2	8.6	104.9
	0.15	0.112	56.3	5.0	5.0	100.0
	15000	13000.2	83.4			
	30000	26296.7	68.1			
	45000	41297.0	83.9			

[a]Acute Interaction-1	0	0.00	-	8.2	8.5	103.7
	0.15	0.084	74.8	3.0	3.4	113.3
	15000	12505.8	86.7			
	30000	20417.9	87.7			
	45000	37776.2	91.8			
[b]Chronic-1	0	0.0 (0)	0.0	8.2	8.4±0.02	102.4
	0.03394	0.0 (0)	0.0	5.0	5.1±0.03	102.0
	0.3394	0.2 (0.02)	51.2	4.0	4.3±0.05	107.5
	3.394	2.5 (0.1)	69.5	3.0	3.2±0.04	106.7
	33.94	25.7 (0.5)	74.0	2.0	2.3±0.04	115.0
	339.4	277.8 (5.7)	80.2			
	3394	2355.7 (81.1)	66.7			
	33940	24883.2 (2070)	66.9			
[b]Chronic Interaction-1	0	0.0 (0)	-	8.2	8.3±0.02	101.2
	0.3394	0.1 (0.009)	17.4	5.0	5.4±0.03	108.0
	33.94	23.2 (2.0)	68.5			
	339.4	258.8 (19.0)	76.2			
	3394	2215.4 (75.6)	65.3			
[b]Chronic Interaction-1	0	0.0 (0)	-	8.2	8.3±0.01	101.2
	0.3394	0.1 (0.004)	21.6	3.0	3.2±0.04	106.7
	33.94	26.4 (0.7)	77.7			
	339.4	277.2 (13.6)	81.7			
	3394	2348.1 (70.0)	69.2			

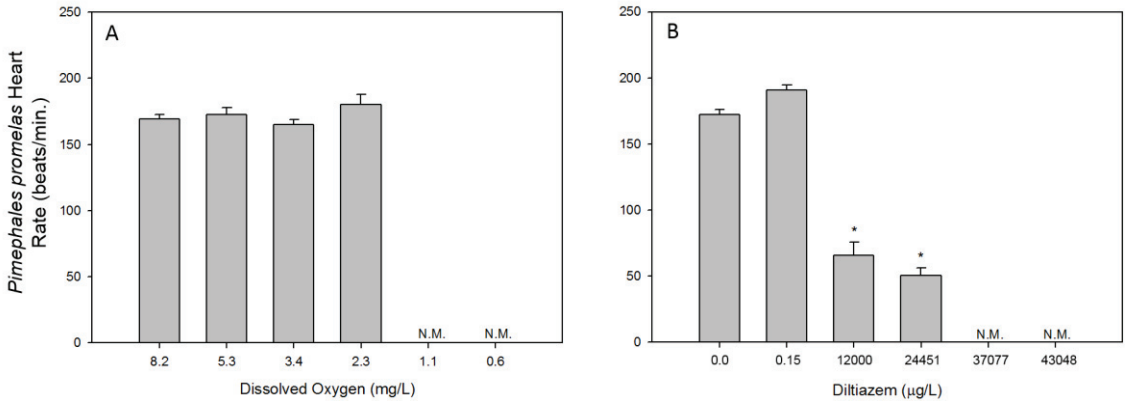
***** Analytical verification indicated 0 (control) and 0.15 µg/L treatments were contaminated; however, significant effects were not observed in *Pimephales promelas* at these levels.

(a) Indicates water concentrations were analytically verified at time 0 h

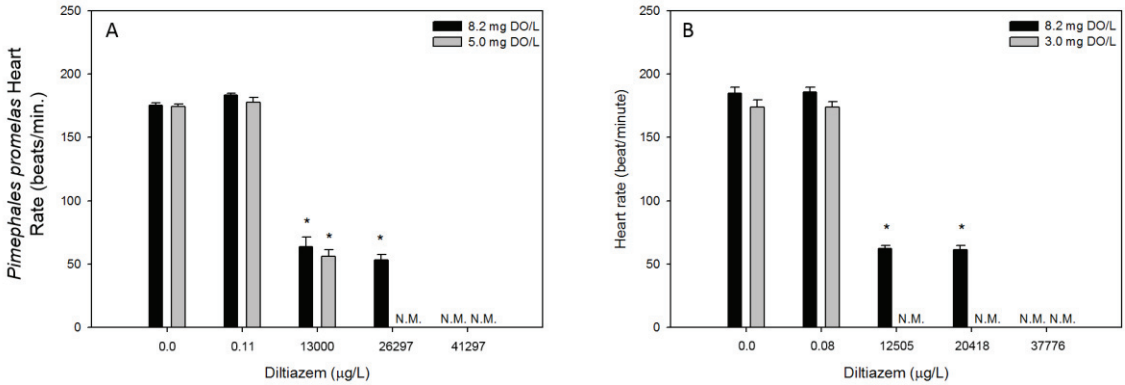
(b) Mean (±SD) water concentrations were analytically verified on days 0, 3, and 6.

Supplementary: Table: 20. Mean water chemistry measurements from acute (48h) and chronic (7 d) studies with *Pimephales promelas*. DO: dissolved oxygen; DZM: diltiazem; Acute: 48 hours; Chronic: 7 day

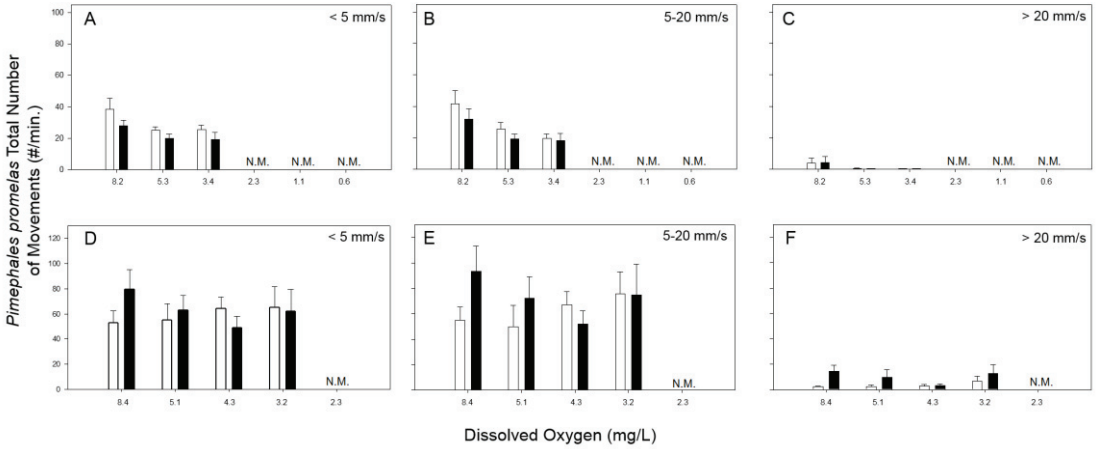
Study Type (N= # studies)	**Nominal DO (mg/L)**	**pH**	**Conductivity (µS)**	**Alkalinity (mg/L $CaCO_3$)**	**Hardness (mg/L $CaCO_3$)**
Acute DO (N=3)	8.2, 5.0, 3.0, 2.0, 1.0, 0.5	8.31	606.7	109.3	174.1
Acute DZM (N=3)	8.2	8.26	582.2	103.4	174.9
Acute DO x DZM (N=1)	8.2, 5.0	8.22	598.6	111.9	173.6
Acute DO x DZM (N=1)	8.2, 3.0	8.22	598.6	111.9	173.6
Chronic DO (N=1)	8.2, 5.0, 4.0, 3.0, 2.0	8.36	599.0	109.9	171.5
Chronic DZM (N=1)	8.2	8.19	620.8	107.9	173.6
Chronic DO x DZM (N=1)	8.2, 5.0	8.3	581.5	111.7	175.7
Chronic DO-Diltiazem (N=1)	8.2, 3.0	8.3	581.5	111.7	175.7



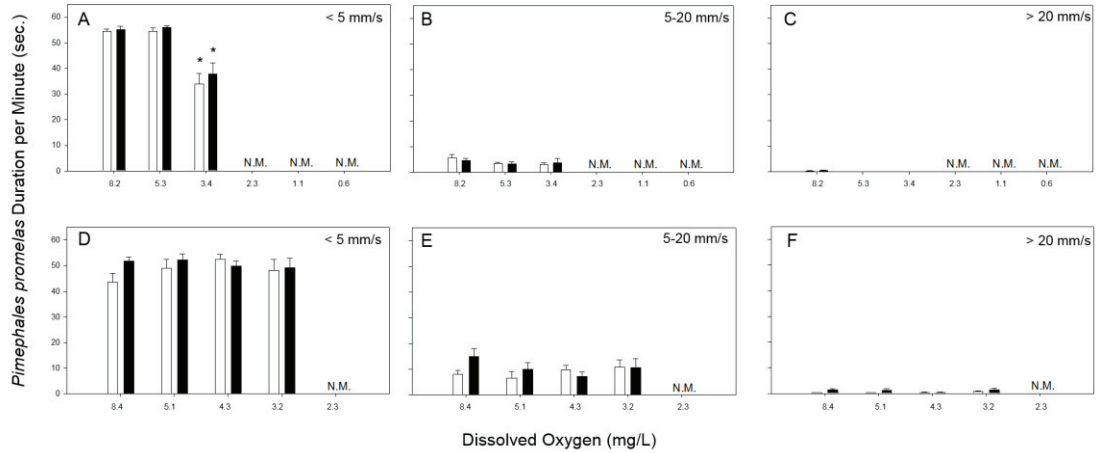
Supplementary: Figure: 25. Mean (±SE, N=3 studies) heart rate (beats/minute) by *Pimephales promeals* larvae following 48 h A) dissolved oxygen (DO) and B) diltiazem studies. Mean larval survival at 2.3 mg DO/L and 24451 µg/L diltiazem were 80 and 88%, respectively, while other experimental treatments with significant decreases in survival were not measured (N.M.: Not Measured). *: $p < 0.05$.



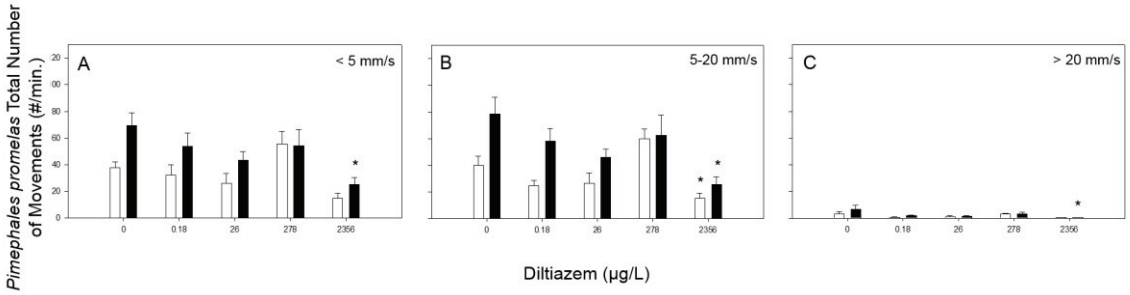
Supplementary: Figure: 26. Mean (±SE, N=4, n=4) heart rate (beats/minute) by *Pimephales promelas* larvae following 48 h A) normal and moderate dissolved oxygen (DO) x diltiazem and B) normal and low DO x diltiazem studies. In experimental treatments with significant decreases in survival, heart rates were not measured (N.M.: Not Measured). Normal DO: 8.2 mg DO/L; Moderate DO: 5.0 mg DO/L; Low DO: 3.0 mg DO/L. *: $p < 0.05$.



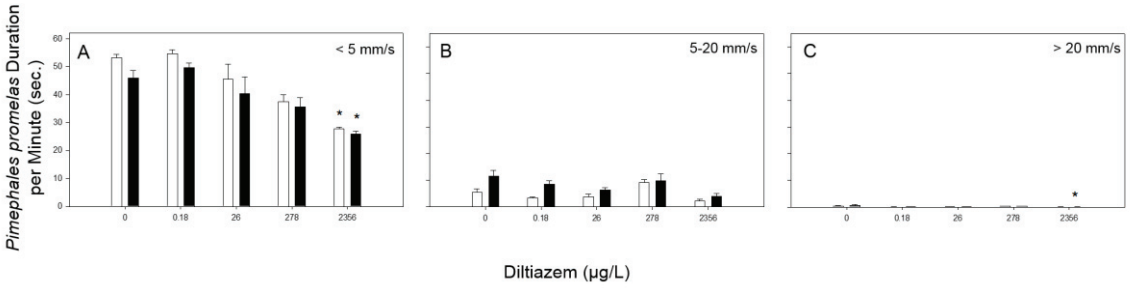
Supplementary: Figure: 27. Mean (±SE) total number of movements (counts) per minute by *Pimephales promelas* larvae following 48 h (A, B, C; N=3 studies) and 7 d (D, E, F; N=4, n=4-5) dissolved oxygen (DO) studies. Data presented as number of movements in speed (mm/s) categories for resting (< 5 mm/s; A,D), cruising (5-20 mm/s; B, E), and bursting (>20 mm/s; C, F). Number of movements were observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at DO concentrations causing significant mortality. *: $p < 0.05$.



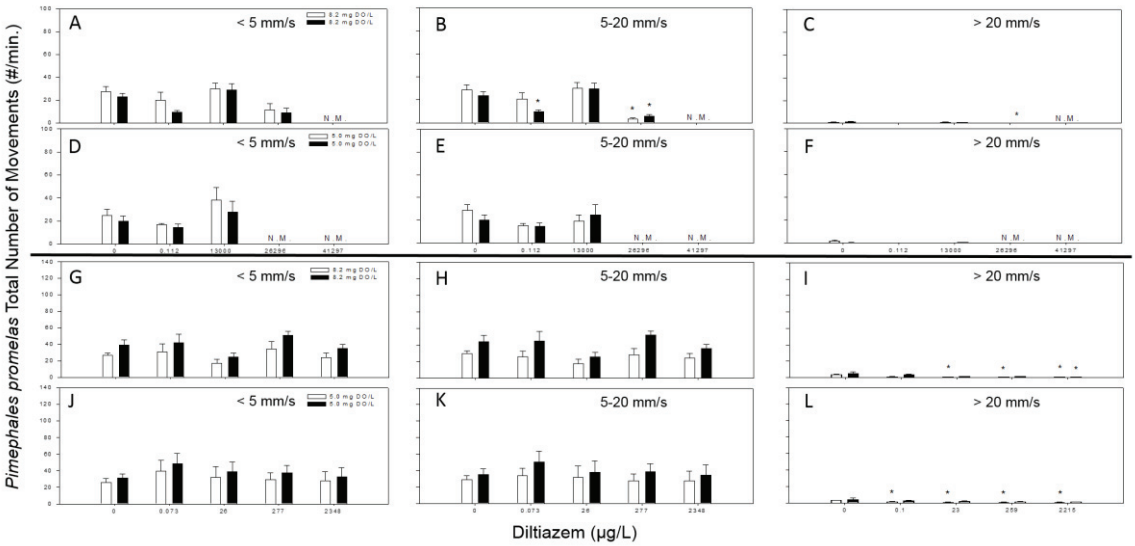
Supplementary: Figure: 28. Mean (±SE) duration per minute (60 sec.) by *Pimephales promelas* larvae following 48 h (A, B, C; N=3 studies) and 7 d (D, E, F; N=4, n=4-5) dissolved oxygen (DO) studies. Data presented as duration in speed (mm/s) categories for resting (< 5 mm/s; A, D), cruising (5-20 mm/s; B, E), and bursting (>20 mm/s; C, F). Duration was observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at DO concentrations causing significant mortality. *: $p < 0.05$.



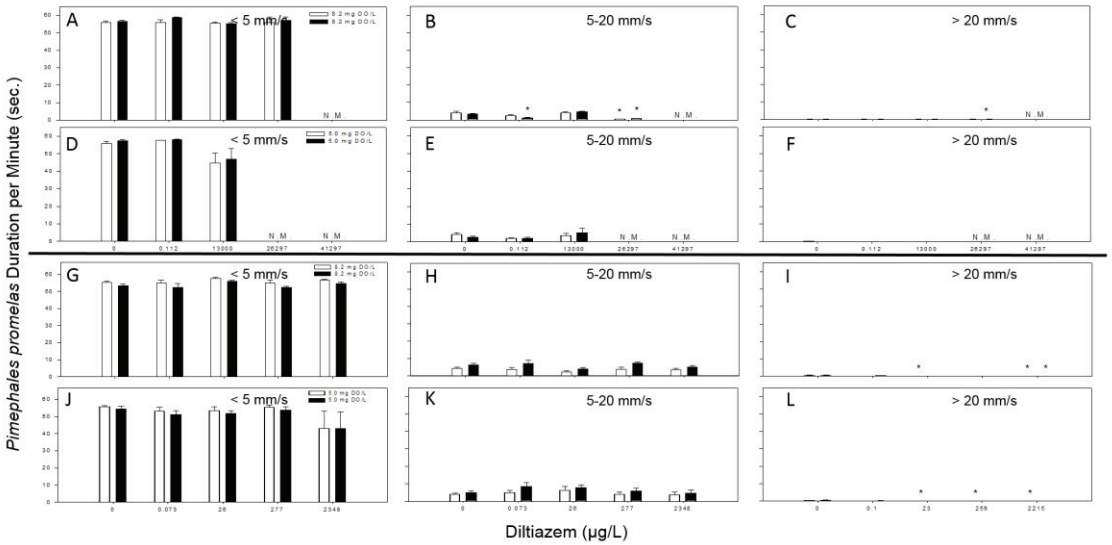
Supplementary: Figure: 29. Mean (±SE) total number of movements (counts) per minute by *Pimephales promelas* larvae following and 7 d (A, B, C; N=4, n=4-5) diltiazem studies. Data presented as number of movements in speed (mm/s) categories for resting (< 5 mm/s; A), cruising (5-20 mm/s; B), and bursting (>20 mm/s; C). Number of movements were observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at DO concentrations causing significant mortality. *: $p < 0.05$.



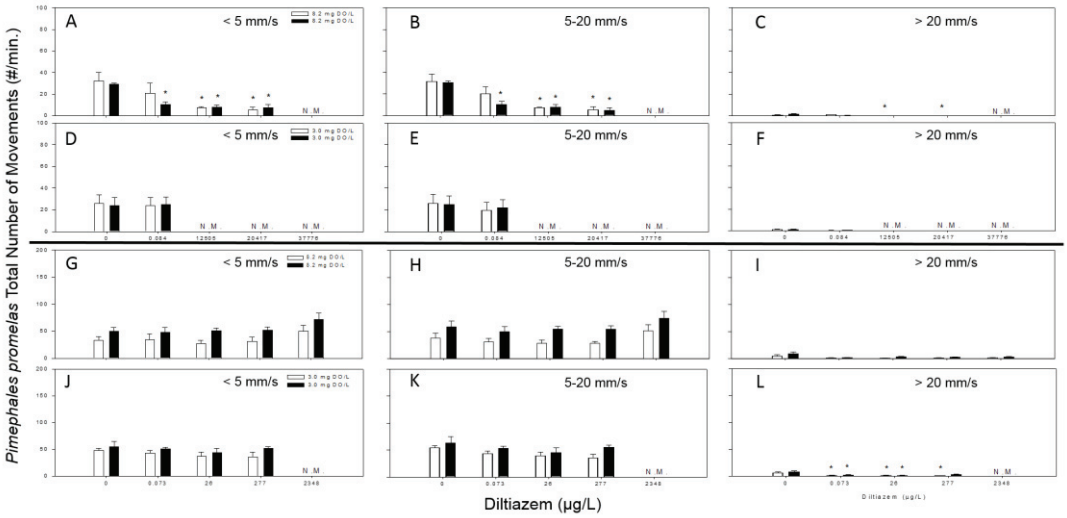
Supplementary: Figure: 30. Mean (±SE) duration per minute (60 sec.) by *Pimephales promelas* larvae following 7 d (A, B, C; N=4, n=4-5) diltiazem studies. Data presented as duration in speed (mm/s) categories for resting (< 5 mm/s; A, D), cruising (5-20 mm/s; B, E), and bursting (>20 mm/s; C, F). Duration was observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at DO concentrations causing significant mortality. *: $p < 0.05$.



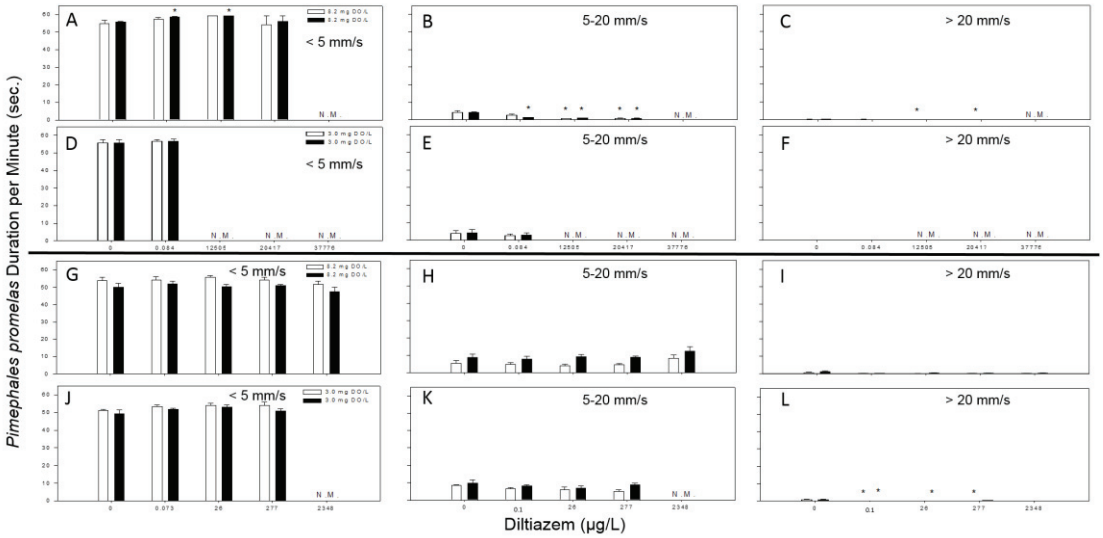
Supplementary: Figure: 31. Mean (±SE; N=4, n=4-5) total number of movements (counts) per minute by *Pimephales promelas* larvae following 48 h (panels A, B, C, D, E, F) or 7 d (panels G, H, I, J, K, L) studies across normal and moderate dissolved oxygen (DO) x diltiazem interaction treatments. Data presented as number of movements in speed (mm/s) categories for resting (< 5 mm/s; panels A, D, G, J), cruising (5-20 mm/s; B, E, H, K), and bursting (>20 mm/s; C, F, I, L) behaviors. Number of movements were observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at concentrations causing significant mortality. *: $p < 0.05$. Normal DO: 8.2 mg DO/L; Moderate DO: 5.0 mg DO/L.



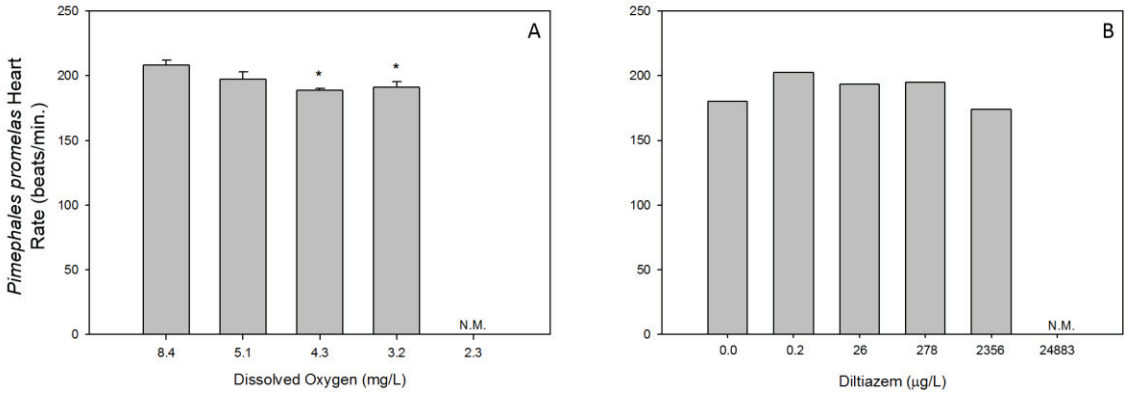
Supplementary: Figure: 32. Mean (±SE; N=4, n=4-5) duration per minute by *Pimephales promelas* larvae following 48 h (panels A, B, C, D, E, F) or 7 d (panels G, H, I, J, K, L) studies across normal and moderate dissolved oxygen (DO) x diltiazem interaction treatments. Data presented as duration in speed (mm/s) categories for resting (< 5 mm/s; panels A, D, G, J), cruising (5-20 mm/s; B, E, H, K), and bursting (>20 mm/s; C, F, I, L) behaviors. Duration was observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at DO concentrations causing significant mortality. *: $p < 0.05$. Normal DO: 8.2 mg DO/L; Moderate DO: 5.0 mg DO/L.



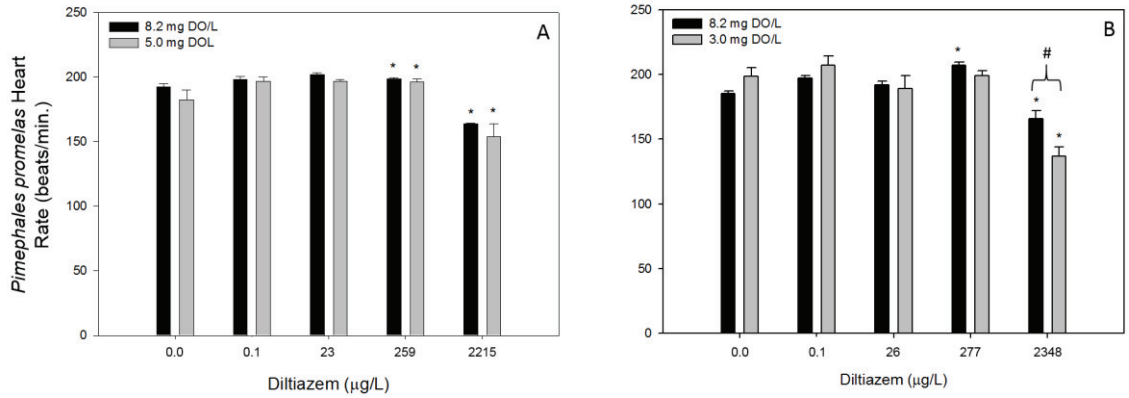
Supplementary: Figure: 33. Mean (±SE; N=4, n=4-5) total number of movements (counts) per minute by *Pimephales promelas* larvae following 48 h (panels A, B, C, D, E, F) or 7 d (panels G, H, I, J, K, L) studies across normal and low dissolved oxygen (DO) x diltiazem interaction treatments. Data presented as number of movements in speed (mm/s) categories for resting (< 5 mm/s; panels A, D, G, J), cruising (5-20 mm/s; B, E, H, K), and bursting (>20 mm/s; C, F, I, L) behaviors. Number of movements were observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at concentrations causing significant mortality. *: $p < 0.05$. Normal DO: 8.2 mg DO/L; Low DO: 3.0 mg DO/L.



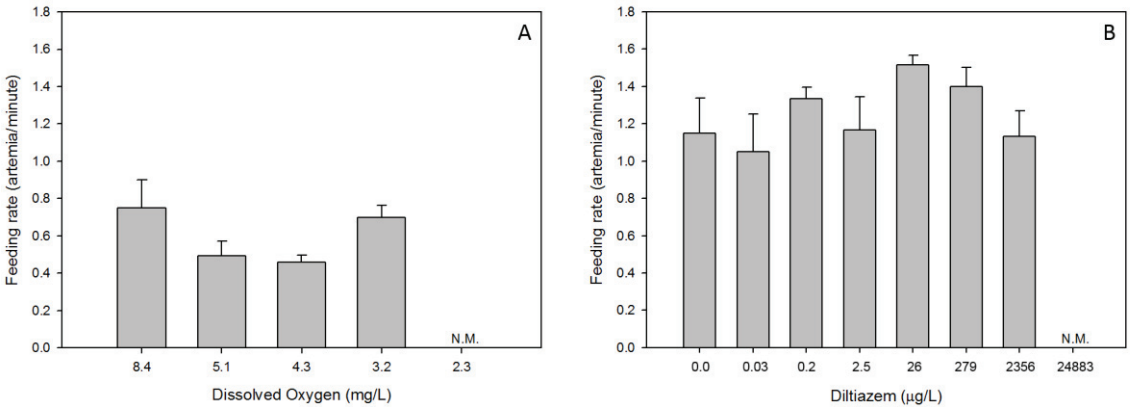
Supplementary: Figure: 34. Mean (±SE; N=4, n=4-5) duration per minute by *Pimephales promelas* larvae following 48 h (panels A, B, C, D, E, F) or 7 d (panels G, H, I, J, K, L) studies across normal and low dissolved oxygen (DO) x diltiazem interaction treatments. Data presented as duration in speed (mm/s) categories for resting (< 5 mm/s; panels A, D, G, J), cruising (5-20 mm/s; B, E, H, K), and bursting (>20 mm/s; C, F, I, L) behaviors. Duration was observed over two alternating 10 minute periods of light (white bars) and dark (black bars) conditions. No behavioral observations were recorded (N.M.: Not Measured) at DO concentrations causing significant mortality. *: $p < 0.05$. N.M.: Not Measured. Normal DO: 8.2 mg DO/L; Low DO: 3.0 mg DO/L.



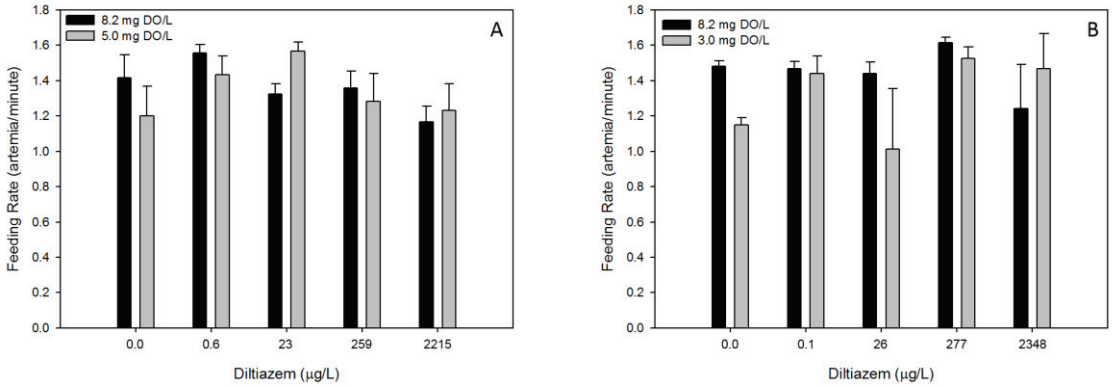
Supplementary: Figure: 35. Mean heart rate (beats/minute) by *Pimephales promelas* larvae following 7 d A) dissolved oxygen (DO; ±SE N=4, n=4) and B) diltiazem (N=2, n=4) studies. In experimental treatments with significant decreases in survival, heart rates were not measured (N.M.: Not Measured). *: $p < 0.05$.



Supplementary: Figure: 36. Mean (±SE, N=4, n=4) heart rate (beats/minute) by *Pimephales promelas* larvae following 7 d A) normal and moderate dissolved oxygen (DO) x diltiazem and B) normal and low DO x diltiazem studies. Low DO x 2348 µg/L diltiazem decreased survival but were measured to demonstrate concomitant DO x diltiazem reduced heart rates. Normal DO: 8.2 mg DO/L; Moderate DO: 5.0 mg DO/L; Low DO: 3.0 mg DO/L. *: $p < 0.05$. #: $p < 0.05$, significant influence of DO across diltiazem concentrations.



Supplementary: Figure: 37. Mean (±SE, N=4, n=2) feeding rate (artemia/minute) by *Pimephales promelas* larvae following 7 d A) dissolved oxygen (DO) and B) diltiazem studies. In experimental treatments with significant decreases in survival, feeding rates were not measured (N.M.: Not Measured). *: $p < 0.05$.



Supplementary: Figure: 38. Mean (±SE, N=4, n=6-8) dry weight (growth) by *Pimephales promelas* larvae following 7 d experiments across A) normal and moderate dissolved oxygen x diltiazem and B) normal and low DO x diltiazem studies. No growth measurements were observed (N.M.: Not Measured) at DO or diltiazem concentrations causing significant mortality. *: $p < 0.05$. Normal DO: 8.2 mg DO/L; Moderate DO: 5.0 mg DO/L; Low DO: 3.0 mg DO/L.

APPENDIX D

Supplementary Information for Chapter Five

Supplementary: Table 21: Mean (±SE, N=3-4) water chemistry measurements from normal and low dissolved oxygen (DO) x diltiazem (DZM) bioconcentration (96 h) and burst swim performance (24 h) studies with adult male *Pimephales promelas*. Normal DO: 8.2 mg DO/L. Low DO: 3.0 mg DO/L. Temp: Temperature. Cond: Conductivity. Cl_2: Chlorine. NH_3: ammonia. NM: not measured.

Study Type	[a]Measured DZM (µg/L)	Temp (^{0}C)	pH	Cond (µS)	Alkalinity (mg/L $CaCO_3$)	Hardness (mg/L $CaCO_3$)	Free Cl_2 (mg/L)	Total NH_3 (mg/L)	NO2 (mg/L)
Bioconcentration Studies									
8.2 mg DO/L x DZM	0.84	24.3±0.04	8.1±0.02	370±0.1	[b]110	[b]140	[b]0	[b]0.07	NM
3.0 mg DO/L x DZM	0.83	25.5±0.2	8.4±0.01	371±1.6	[b]85	[b]116	[b]0	[b]0.07	NM
Burst Swim Performances Studies									
8.2 mg DO/L x DZM	0, 3.2, 36.5	24.2±0.1	8.3±0.00	381±2.2	109±2.2	140±4.0	0.02±0.0	0.03±0.01	0.03±0.0
3.0 mg DO/L x DZM	0, 3.1, 33.5	24.9±0.1	8.5±0.02	368±4.6	103±2.7	141±5.5	0.02±0.0	0.02±0.01	0.02±0.0

(a) Exact mean (±SE) measured diltiazem concentrations are listed in Table 2. (b) Alkalinity, hardness, free chlorine, total ammonia measured at 96 h sampling time point only.

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