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Influence of salinity and pH on bioconcentration of ionizable pharmaceuticals by the gulf killifish, *Fundulus grandis*

W. Casan Scott [a], Samuel P. Haddad [a], Gavin N. Saari [a], C. Kevin Chambliss [a, b], Jeremy L. Conkle [c], Cole W. Matson [a], Bryan W. Brooks [a, d, *]

[a] Department of Environmental Science, Center for Reservoir and Aquatic Systems Research, Baylor University, Waco, TX, USA
[b] Department of Chemistry and Biochemistry, Baylor University, Waco, TX, USA
[c] Department of Physical and Environmental Sciences, Texas A&M University, Corpus Christi, TX, USA
[d] School of Environment, Jinan University, Guangzhou, China

HIGHLIGHTS

- Urban coastal systems continuously receive effluent discharges of diverse quality.
- Bioaccumulation of ionizable contaminants is poorly understood in estuarine species.
- pH, but not salinity, altered uptake of ionizable pharmaceuticals by Gulf killifish.
- Inhalational exposure route is important, compared to drinking and intestinal uptake.
- Differential uptake of ionizables in freshwater and marine fish deserve future study.

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ABSTRACT

Estuaries routinely receive discharges of contaminants of emerging concern from urban regions. Within these dynamic estuarine systems, salinity and pH can vary across spatial and temporal scales. Our previous research identified bioaccumulation of the calcium channel blocker diltiazem and the antihistamine diphenhydramine in several species of fish residing in multiple urban estuaries along the Gulf of Mexico in Texas, where field-measured observations of diltiazem in fish plasma exceeded human therapeutic plasma doses. However, there remains a limited understanding of pharmaceutical bioaccumulation in estuarine environments. Here, we examined the influence of pH and salinity on bioconcentration of three pharmaceuticals in the Gulf killifish, *Fundulus grandis*. *F. grandis* were exposed to low levels of the ionizable pharmaceuticals carbamazepine, diltiazem, and diphenhydramine at two salinities (5 ppt, 20 ppt) and two pH levels (6.7, 8.3). pH influenced bioconcentration of select weak base pharmaceuticals, while salinity did not, suggesting that intestinal uptake via drinking does not appear to be a major exposure route of these pharmaceuticals in killifish. Compared to our previous pH dependent uptake observations with diphenhydramine in the fathead minnow model, killifish apparent volume of distribution values were markedly lower than fatheads, though killifish bioconcentration factors were similar at high pH and four fold higher at low pH than freshwater fish. Advancing an understanding of environmental gradient influences on pharmacokinetics among fish is necessary to improve bioaccumulation assessments and interpretation of toxicological observations for ionizable contaminants.

1. Introduction

Pharmaceuticals in the environment have been receiving global research because they are designed to be biologically active, and routinely accumulate in field-collected organisms (Daughton and Jones-Lepp, 2001; Daughton, 2004; Brooks et al., 2005; Kümmerer, 2010; Du et al., 2014, 2016; Scott et al., 2016; Haddad et al., 2017). Though pharmaceuticals are consistently detected in sewage, treated effluents, surface waters and aquatic organisms, particularly in regions influenced by urbanization, there remain

* Corresponding author. Department of Environmental Science, Baylor University, Waco, TX, USA.
E-mail address: Bryan_Brooks@baylor.edu (B.W. Brooks).

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important research questions regarding the accumulation and effects of these biologically active compounds (Boxall et al., 2012; Rudd et al., 2014). Because most pharmaceuticals are ionizable in surface waters, which influences bioavailability and toxicity to wildlife, a recent expert workshop identified, *How can the uptake of ionizable pharmaceuticals and personal care products (PPCPs) into aquatic and terrestrial organisms and through food chains be predicted?*, among the top research priorities necessary to understand risks of PPCPs in the environment (Boxall et al., 2012).

With urbanization and a growing and aging human population in some regions, chemical use, including pharmaceutical consumption, is continuing to increase and being concentrated in cities, which present challenges for sustainable water quality management (Brooks, 2018; Brooks and Conkle, 2019). In fact, almost half of human populations live within 100 miles of a coastline, and people are choosing to live in cities more than ever before (Li, 2003; Martínez et al., 2007; Small and Nicholls, 2003). Instream flows to estuaries in arid and semi-arid and other regions of the world are often influenced by, and dominated or even dependent on, reclaimed wastewater discharge (Brooks et al., 2006). In these effluent-dominated and dependent systems, effective exposure duration to consumer products are increased because of limited instream dilution, and continuous effluent introduction rates of down the drain chemicals routinely exceed instream degradation rates (Ankley et al., 2007). Effluent-dominated systems are now recognized as important watersheds for management, particularly in the face of climate change (Luthy et al., 2015). In addition to receiving discharges from urban areas, estuaries, an important interface among terrestrial, freshwater, and marine systems, experience substantial spatial and temporal fluctuations in physiochemical parameters, including salinity and pH (Beck and Bruland, 2000; Hubertz and Cahoon, 1999; Nelson et al., 1994; Pritchard, 1967; Scott et al., 2019).

Because of such inherent variability in water chemistry, combined with increasing concentration of chemical use and wastewater discharges, rapidly urbanizing estuaries represent unique opportunities to understand influences of physiochemical and urban gradients on emerging water quality challenges. In fact, we recently observed two ionizable base pharmaceuticals, the calcium channel blocker diltiazem and the antihistamine diphenhydramine, to accumulate in several species of fish residing in multiple urban estuaries along the Gulf of Mexico in Texas (Scott et al., 2016). In plasma of wild-caught fish, diphenhydramine levels approached, while diltiazem levels occasionally exceeded, human therapeutic plasma dosage levels (Scott et al., 2016). Such exceedances are recognized as an indicator of relatively high risk, where internal exposures to compounds with evolutionary conserved modes of action will likely result in adverse outcomes to aquatic life (Huggett et al., 2003; Brooks, 2014; Caldwell et al., 2014).

Unfortunately, though bioaccumulation of pharmaceuticals has received increasing attention since our initial reports of human pharmaceuticals accumulating in fish from an effluent-dominated river (Brooks et al., 2005; Ramirez et al., 2007), there remains a poor understanding of pharmaceutical bioaccumulation in estuarine environments (Daughton and Brooks, 2011; Maruya et al., 2012; Alvarez et al., 2014; Gaw et al., 2014; Lazarus et al., 2015; Du et al., 2016; Meador et al., 2016; Bean et al., 2018). In addition to considering pH influences on bioavailability and bioaccumulation of ionizable contaminants (Nichols et al., 2015), salinity alters both the metabolic rate and the surface structure of the gill in fish, which can potentially influence chemical uptake and elimination (Copeland, 1950; Laurent et al., 2006; Nichols et al., 2015; Scott et al., 2004a,b). Therefore, in the present study, we examined the relative influence of pH and salinity on bioconcentration of three pharmaceuticals in the Gulf killifish, *Fundulus grandis*. We specifically selected *F. grandis* for study because it is common euryhaline teleost in estuaries of the Gulf of Mexico (Harrington and Harrington, 1982).

2. Methods

2.1. Fundulus grandis

Adult *F. grandis* were collected using minnow traps from a previously-recognized reference population at Smith Point, near Galveston Bay, Texas (29°32′37.26″N, 94° 47′08.12″W; Oziolor et al., 2016) These wild-caught fish were kept for over one month prior to initiation of experiments. Prior to experimental exposure, fish employed for the 20 ppt experiments (at both pH 8.3 and 6.7) were reared at approximately 17 ppt for at least 2 weeks. Similarly, prior to initiating the 5 ppt uptake experiments, fish were then acclimated to approximately 7 ppt salinity over a two week period. We then performed four discrete uptake experiments in which high or low pH levels (8.3 and 6.7) were manipulated at one of two salinity levels (20 ppt and 5 ppt). The pH levels were chosen based on the high and low mean salinities observed in our previous studies of pharmaceutical accumulation and hazards in Texas Gulf Coast estuaries (Scott et al., 2016, 2019). Because *F. grandis* has an isosmotic point of 12 ppt (Fritz and Garside, 1974; Varsamos et al., 2005) and 5 ppt–20 ppt represents an ideal salinity range for survival and growth (Perschbacher et al., 1990; Patterson et al., 2012), we chose 20 ppt and 5 ppt salinity treatment levels.

2.2. Fundulus grandis experiments

For this study, experimental methods generally followed those we have previously reported (Nichols et al., 2015). Adjustments to pH 6.7 and pH 8.3 were accomplished by titrating with hydrochloric acid and sodium hydroxide, respectively, following USEPA recommendations (US Enivornmental Protection Agency 1991). To initiate each experiment, a stock solution containing a mixture of carbamazepine, diltiazem, and diphenhydramine, was added to each treatment unit to achieve nominal concentrations of 10 μg/L, 1 μg/L, and 10 μg/L, respectively. Carbamazepine, diltiazem hydrochloride, and diphenhydramine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). We selected these substances because diltiazem and diphenhydramine are common contaminants in surface waters (Kristofco and Brooks, 2017; Saari et al., 2017) with pKa values (7.7 and 8.9, respectively; Bonferoni et al., 2000; Shaleva et al., 2008) indicating pH influences on ionization across estuarine conditions. The pKa is an integral aspect of drug research and development, because it influences rates of absorption through body compartments possessing different pH values (Manallack, 2007). Carbamazepine is also a common contaminant in urban systems that we anticipated would not be appreciably influenced by pH given its higher pKa (13.9). Glass aquaria were used as experimental units (e.g., 20 L aquaria) with a water volume of 15 L each. The positions of each experimental unit were randomized and maintained in an environmental chamber at 25 °C on a 16:8 light:dark cycle. Within the semi-flow through experimental system, water flow was adjusted to a constant flow without creating current, and a recirculating renewal rate of 2X per hour (30 L/h) was targeted for each experimental unit. Three water renewals were conducted daily (every 8 h) to maintain water quality, and consistency of the chemical exposure concentrations.

Four *F. grandis* ($n = 4$) were added to each of three replicate experimental units ($N = 3$), in each of the experimental systems. Within each experiment, exposures to a sublethal mixture of carbamazepine, diltiazem, and diphenhydramine were conducted with exposure durations of 1, 3, 6, 12, 24, and 48 h, with staggered

starting times due to experimental system setup. At each time point, triplicate experimental units (N = 3) were sampled such that all four fish from an experimental unit were removed, anesthetized/euthanized, and pooled for analytical measures (Nichols et al., 2015). Control fish were exposed for the entire 48 h. To ensure that fish were exposed to common concentrations, pharmaceutical levels in each aquaria were analytically verified at each time point in relation to water renewals according to previously published methods by our research group (Haddad et al., 2017; Du et al., 2014, 2016). Following an approved Institutional Animal Care and Use Committee protocol, fish were anesthetized with tricaine methane sulfonate (MS-222), weighed, and measured to obtain total length. Blood was then collected from the caudal artery using heparinized micro-hematocrit capillary tubes (StatSpin, Brea, CA, USA). Plasma and tissue from all fish within each aquarium were pooled, resulting in 3 pooled replicate samples (N = 3) for each exposure duration. Plasma was separated in a gel barrier microtube (StatSpin, Brea, CA, USA), centrifuged at 3000×*g* (4 °C) for 20 min, and stored immediately at −80 °C. Analytical methods for water, blood plasma, and tissue analyses followed recent experimental methods from our research team (Haddad et al., 2017; Du et al., 2014, 2016).

2.3. BCFs, Blood:Water partitioning coefficients, and volume of distribution

For each experiment, mean data from 24 to 48 h were used to calculate bioconcentration factors (BCF), blood:water partition coefficients (P_{BW}), and apparent volumes of distribution (V_D) (Nichols et al., 2015). Tissue BCFs were calculated by dividing the mean whole-body tissue concentration by the water concentration for each target pharmaceutical (equation (1)). P_{BW} values were calculated by dividing the mean blood plasma concentration by the water concertation of each pharmaceutical (equation (2)). Apparent V_D values were calculated by dividing the steady-state whole-body tissue concentration by the steady-state blood plasma concentration for each pooled replicate sample (equation (3); Nichols et al., 2015). BCF and P_{BW} were calculated to identify drugs partitioning from water to fish tissue and blood plasma, respectively. V_D quantifies the distribution of these drugs between blood plasma and the rest of the body of each fish (Watkins et al., 2010).

$$BCF = \frac{\text{whole} - \text{body tissue concentration}}{\text{water concentration}} \quad (1)$$

$$P_{B:W} = \frac{\text{blood plasma concentration}}{\text{water concentration}} \quad (2)$$

$$\text{Apparent } V_D\ (\text{L/kg}) = \frac{\text{whole} - \text{body tissue concentration}\ (\mu\text{g/kg})}{\text{blood plasma concetration}\ (\mu\text{g/L})} \quad (3)$$

2.4. Statistical analysis

All statistical analyses were performed using SigmaPlot (Systat Software, San Jose, CA, USA). A two-way ANOVA was used to compare measured water, whole-body tissue, and blood plasma concentrations of the three pharmaceuticals, with two factors: salinity and pH. Mean concentrations in whole-body tissue and blood plasma from 24 to 48 h were used to calculate BCF, P_{BW}, and V_D. A two-way ANOVA was used to compare BCF, P_{BW}, and V_D with two factors: salinity and pH. The Sidak-Holm step-down test was used to make pairwise comparisons following the two-way ANOVA (Holm, 1979).

3. Results

3.1. Experimental conditions

Water chemistry parameters during this study were consistent with expectations, and no mortalities were observed over the entire study. Across all four experiments, mean (±SD) experimental temperature and dissolved oxygen was 25.04 ± 0.22 °C and 5.59 ± 0.57 mg/L, respectively. In the two experiments conducted at the higher pH, median measured pH was 8.29. Median pH in the two low-pH experiments was 6.78. Mean measured salinity for the high salinity experiments was 20.08 ± 0.34 ppt, and 5.05 ± 0.04 ppt for the low salinity experiments. Summary statistics for pH, dissolved oxygen (mg/L), salinity (ppt), and temperature (°C) for all studies are presented in Supporting Information.

Each treated experimental system was dosed with a mixture of carbamazepine, diltiazem, and diphenhydramine, at nominal target concentrations of 10 μg/L, 1 μg/L, and 10 μg/L, respectively. Consistent with previously reported pharmaceutical exposures at elevated salinities, measured exposure concentrations were lower than nominal (Blewett et al., 2013a,b). Mean (±SD) analytically verified concentrations of carbamazepine, diltiazem, and diphenhydramine across all four exposure scenarios were 4.11 ± 0.43, 0.85 ± 0.06, and 4.06 ± 0.49 μg/L, respectively. Despite the measured concentrations being slightly lower than nominal levels, mean exposure concentrations of the three pharmaceuticals (carbamazepine, diltiazem, and diphenhydramine) did not differ significantly across the four experiments ($p > 0.05$). Mean concentrations for each time point per experiment are provided in Supporting Information. Within the control experimental units, there were no detects of the three pharmaceuticals in water during any of the four studies.

3.2. Influence of pH and salinity on pharmaceutical uptake by fish

All three pharmaceuticals were detected in exposed fish whole-body tissue (Fig. 1) and blood plasma (Fig. 2), across all four experimental conditions (high salinity/high pH, high salinity/low pH, low salinity/high pH, and low salinity/low pH). For each of the four experimental combinations, mean steady-state whole-body tissue and blood plasma concentrations (pooled samples of fish collected at 24 and 48 h) of carbamazepine, diltiazem, and diphenhydramine are presented in Figs. 1 and 2. Across the four experiments, four of 48 individual fish from the controls contained low but detectable amounts of three pharmaceuticals in whole-body tissue and blood plasma. These limited observations may have resulted from processing of tissue samples, because no detections were observed in exposure water samples, as noted above.

Salinity did not significantly affect accumulation of the three pharmaceuticals by Gulf killifish ($p > 0.05$). Carbamazepine tissue concentrations were significantly higher at pH 6.7, compared to pH 8.3 ($p < 0.05$), but it did not significantly differ ($p > 0.05$) in blood plasma between pH treatment levels. Tissue and blood plasma concentrations of diltiazem and diphenhydramine were significantly higher at pH 8.3 compared to pH 6.7 ($p < 0.05$). Concentrations of each pharmaceutical appeared to reach steady-state in whole-body tissue and blood plasma by 24 h; therefore, BCF, P_{BW}, and apparent V_D values were calculated at 24 and 48 h (Table 1).

Similar to tissue and plasma observations, the only significant differences in BCFs for carbamazepine were observed between pH 6.7 and 8.3 (BCFs at pH 6.7 > pH 8.3), at the 20 ppt salinity ($p < 0.05$). BCFs for carbamazepine did not differ significantly between pH levels at the lower salinity level of 5 ppt, nor did BCFs

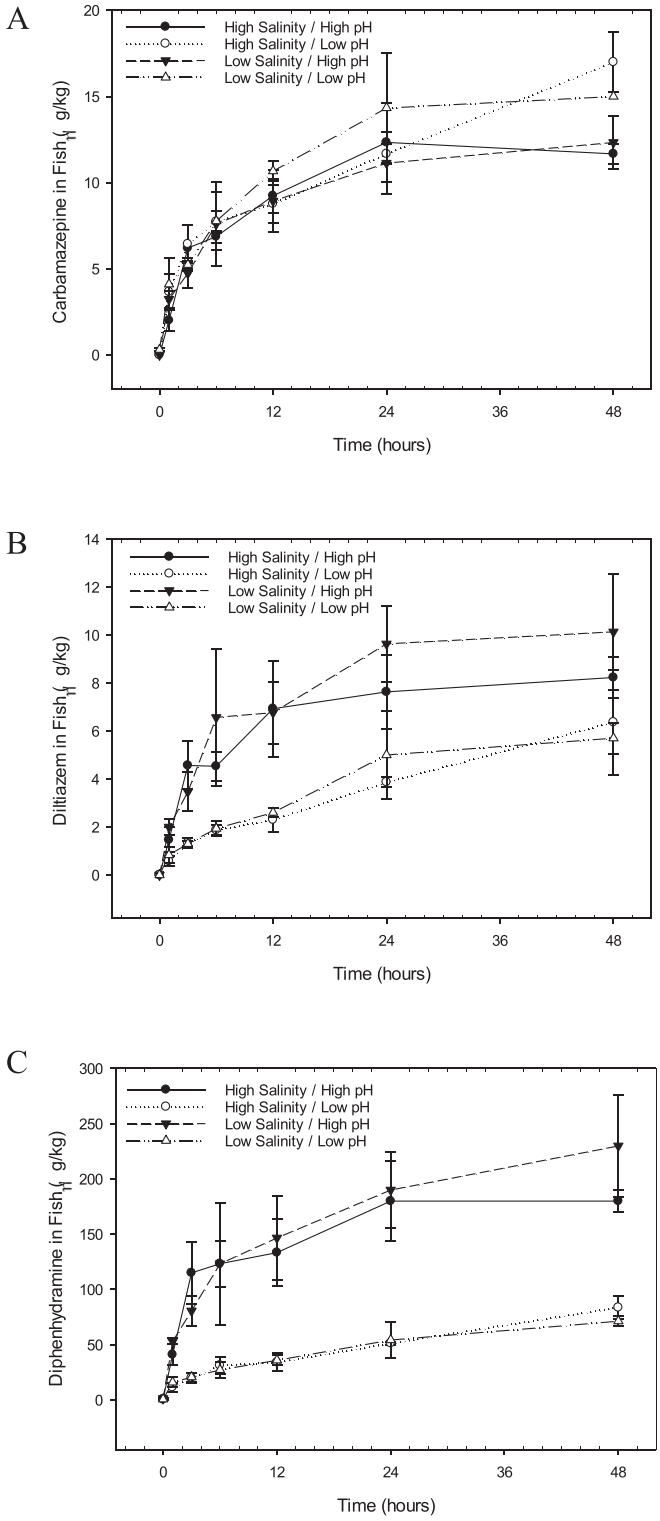

Fig. 1. Mean (±SD) whole-body tissue concentrations (N = 3) of carbamazepine (A), diltiazem (B), and diphenhydramine (C) in Gulf killifish (*Fundulus grandis*) over 48 h. Fish were exposed to one low concentration of each pharmaceutical at a combination of either high salinity (20 ppt) or low salinity (5 ppt), and high pH (8.3) or low pH (6.7). Different symbols denote four discrete experiments as follows: high salinity/high pH (•), high salinity/low pH (○), low salinity/high pH (▼), and low salinity/low pH (△).

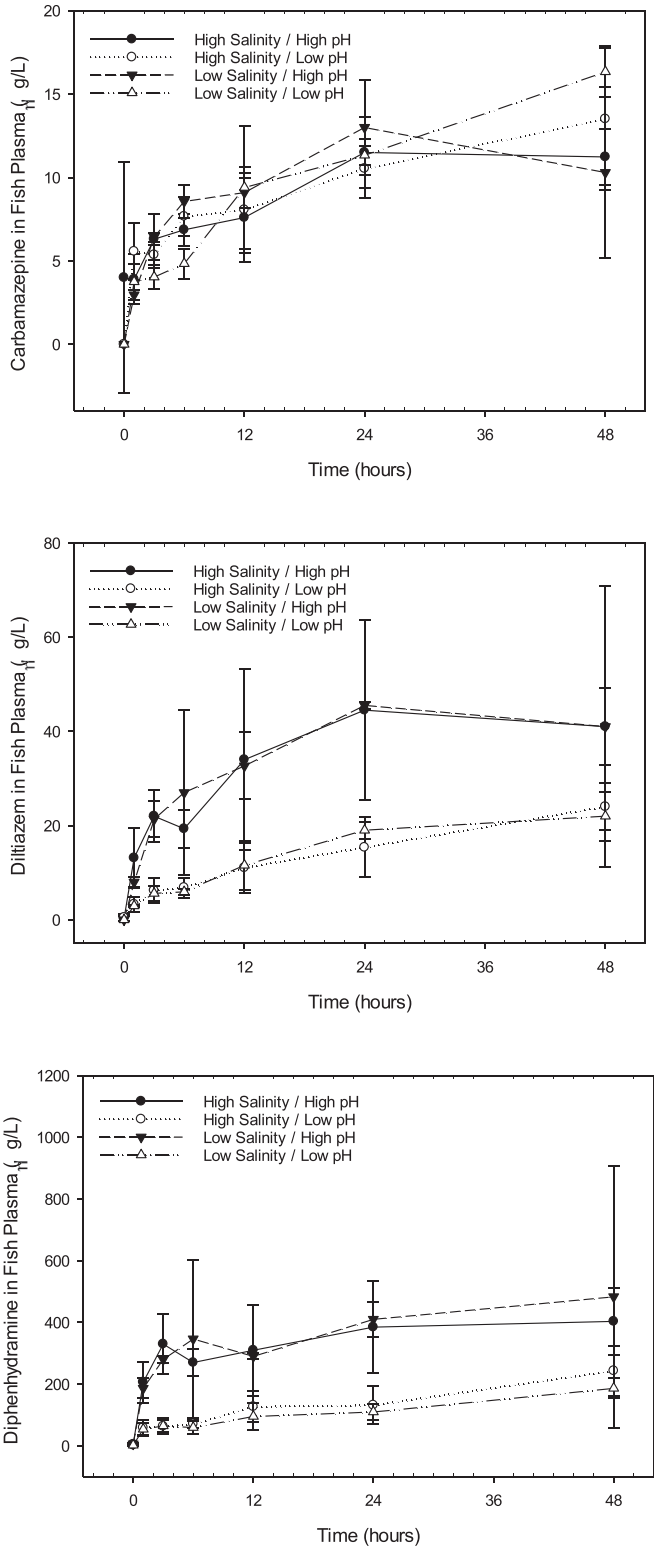

Fig. 2. Mean (±SD) plasma concentrations (N = 3) of carbamazepine (A), diltiazem (B), and diphenhydramine (C) in Gulf killifish (*Fundulus grandis*) over 48 h. Fish were exposed to one low concentration of each pharmaceutical at a combination of either high salinity (20 ppt) or low salinity (5 ppt), and high pH (8.3) or low pH (6.7). Different symbols denote four discrete experiments as follows: high salinity/high pH (•), high salinity/low pH (○), low salinity/high pH (▼), and low salinity/low pH (△).

Table 1
Mean (±SE) BCF (A), $P_{B:W}$ (B), and apparent volume of distribution values (V_D; C) for carbamazepine (CBZ), diltiazem (DTZ), and diphenhydramine (DPH) in Gulf killifish (*Fundulus grandis*). Four fish at 24 h and 48 h in each experiment were pooled for each experimental replicate (N = 3). Apparent volume of distribution (V_D) is derived from a steady state tissue concentration divided by a steady state blood plasma concentration.

A.	Salinity (ppt)	pH	Tissue BCF		
Treatment			CBZ	DTZ	DPH
High Salinity/High pH	20	8.3	2.61 (±0.42)	9.08 (±1.30)	41.65 (±6.47)
High Salinity/Low pH	20	6.7	3.66 (±0.99)	5.95 (±2.35)	16.88 (±5.38)
Low Salinity/High pH	5	8.3	2.59 (±0.34)	12.96 (±2.60)	51.00 (±8.52)
Low Salinity/Low pH	5	6.7	2.95 (±0.44)	7.03 (±1.67)	17.26 (±5.63)
B.	Salinity (ppt)	pH	$P_{B:W}$		
Treatment			CBZ	DTZ	DPH
High Salinity/High pH	20	8.3	2.49 (±0.40)	49.5 (±14.26)	93.13 (±26.11)
High Salinity/Low pH	20	6.7	3.14 (±0.99)	23.47 (±8.50)	48.21 (±24.06)
Low Salinity/High pH	5	8.3	2.57 (±0.99)	57.83 (±29.45)	111.55 (±71.04)
Low Salinity/Low pH	5	6.7	2.83 (±0.44)	27.62 (±4.94)	42.45 (±19.17)
C.	Salinity (ppt)	pH	VD (L/kg)		
Treatment			CBZ	DTZ	DPH
High Salinity/High pH	20	8.3	1.07 ± 0.24	0.20 ± 0.06	0.48 ± 0.18
High Salinity/Low pH	20	6.7	1.22 ± 0.38	0.28 ± 0.13	0.41 ± 0.20
Low Salinity/High pH	5	8.3	1.45 ± 0.72	0.19 ± 0.09	0.32 ± 0.14
Low Salinity/Low pH	5	6.7	1.07 ± 0.27	0.25 ± 0.06	0.42 ± 0.07

differ significantly with exposure salinity, when the influence of pH was accounted ($p > 0.05$). BCFs for diltiazem were significantly elevated by pH 8.3, and significantly higher at the lower salinity level of 5 ppt ($p < 0.05$). Diphenhydramine BCFs were significantly higher at pH 8.3, compared to pH 6.7, but BCFs exhibited no significant differences with salinity ($p < 0.05$). There were also no significant differences in P_{BW} values for carbamazepine across all four experimental conditions. Diltiazem and diphenhydramine P_{BW} values were significantly higher in pH 8.3 experiments, compared to pH 6.7 ($p < 0.05$), while salinity exhibited no influence on P_{BW} values for diltiazem or diphenhydramine ($p > 0.05$). Across all four experiments, mean apparent V_D values for carbamazepine, diltiazem, and diphenhydramine were 1.15 (±0.33), 0.26 (±0.1), and 0.48 (±0.2) L/kg, respectively (Table 1). Apparent V_D values for carbamazepine, diltiazem, and diphenhydramine did not differ significantly ($p > 0.05$) across the four experiments, confirming that waterborne exposure at these levels does not influence the internal distribution of these ionizable drugs (Nichols et al., 2015).

4. Discussion

Here we examined influences of pH at two salinities representative of estuarine conditions on uptake of ionizable pharmaceuticals by the euryhaline species *F. grandis*. Similar to results reported by Nichols et al. (2015), we observed greater accumulation of diphenhydramine at higher pH. In fact, pH significantly ($p < 0.05$) influenced uptake of both diltiazem and diphenhydramine, while salinity did not. In both whole-body tissue and blood-plasma, diltiazem and diphenhydramine (weak bases with pKa values, 7.7 and 8.9 respectively, near the exposure pHs) concentrations were significantly elevated in high pH experiments. Additionally, diltiazem and diphenhydramine whole-body BCFs and P_{BW} were significantly higher in the high pH treatment level, compared to low pH. Carbamazepine, which was not expected to appreciably ionize between the two experimental pH levels, did display elevated levels in whole-body tissue at lower pH. However, this result was not observed in blood plasma.

F. grandis is an extremely euryhaline species, with populations existing in habitats ranging from freshwater to tidal pools of 76 ppt salinity (Simpson and Gunter, 1956; Tabb and Manning, 1961). Whereas fish in freshwater essentially only ingest water while feeding, fish in saltwater environments will gulp copious amounts of water each day in order to maintain osmotic balance (Copeland, 1950; Fritz and Garside, 1974; Marshall et al., 1999; Potts and Evans, 1967; Scott et al., 2004a,b; Scott et al., 2006; Scott et al., 2008). With such differences in drinking rate, euryhaline fish could be orally exposed to a greater extent to contaminants at higher salinities. However, there remains little research exploring the influence of salinity on pharmaceutical accumulation in estuarine fish. Blewett et al. (2013a,b) demonstrated that salinity had a significant influence on uptake of the nonionizable contraceptive pharmaceutical 17-α-ethinyl estradiol (EE2) by killifish. We anticipated rearing fish above and below the isosmotic point might alter osmoregulation processes, and thus change drinking rates of killifish.

Compared to the present study, Nichols et al. (2015) reported uptake observations after exposing fathead minnows (*Pimephales promelas*) to the same nominal concentration (10 μg/L) of diphenhydramine in freshwater at three pH levels: 6.7, 7.7, and 8.7. In the present study, steady-state concentrations of diphenhydramine in killifish whole-body tissue at pH 8.3 were considerably lower than the concentrations in fathead minnow exposed at pH 8.7 and 7.7 in the previous study by Nichols et al. (2015). Conversely, steady-state tissue concentrations of diphenhydramine in killifish exposed at pH 6.7 from the present study are approximately 1.5-fold higher than the fathead minnows exposed at pH 6.7 reported by Nichols et al. (2015). Interestingly, BCFs for killifish exposed at pH 8.3 were very similar to the fathead minnow BCFs pH 8.7 exposures reported by Nichols et al. (2015). Further, diphenhydramine BCFs at pH 6.7 were 4-fold higher in the present study compared to pH 6.7 as reported by Nichols et al. (2015). This may have resulted because in saltwater, compared to freshwater, more buffered conditions could decrease influences of excreted organic acids by gills on pH of the gill – water boundary. Our results also demonstrate that steady-state blood plasma concentrations of diphenhydramine in killifish at both pH 8.3 and 6.7 are higher than any values observed in fathead minnows (Nichols et al., 2015). Specifically, steady-state plasma concentrations at pH 8.3 and pH 6.7 were approximately 3-fold and 11-fold higher than those reported by Nichols et al. (2015). Collectively, these data suggest that diphenhydramine accumulates in gulf killifish whole-body tissue and blood plasma to a

greater extent than in the fathead minnow.

In addition to tissue and plasma concentrations, we also examined whether pH and salinity may influence the relative drug distribution in Gulf killifish. For all three drugs, we observed no pH or salinity influence on apparent V_D, which quantifies chemical distribution between blood plasma and the whole-body tissue of each fish. The mean V_D values for diltiazem and diphenhydramine were 0.26 ± 0.11, and 0.48 ± 0.21 L/kg, respectively, which demonstrates that these drugs are preferentially partitioning to blood plasma and other bodily fluids compared to other body compartments. As noted above, apparent V_D was not statistically different across all exposure scenarios for both diltiazem (0.19–0.28 L/kg) and diphenhydramine (0.32–0.48 L/kg). This consistency in V_D was also noted by Nichols et al. (2015), where, despite significant differences in diphenhydramine accumulation, V_D remained similar across all pH levels tested (Nichols et al., 2015). Interestingly, diphenhydramine V_D values for killifish in the present study (0.48 ± 0.2 L/kg) were nearly 10-fold lower than those for the fathead minnow reported by Nichols et al. (~3 L/kg reported by Nichols et al., 2015). This stark difference in V_D between these two species suggests that Gulf killifish are distributing diphenhydramine within intravascular fluid or blood to a much higher degree than fathead minnows under freshwater conditions.

While the internal distribution of neutral organic compounds is driven by passive diffusion to lipids, phospholipid content and plasma protein content are thought to significantly influence the distribution of ionizables (Armitage et al., 2017). Basic pharmaceuticals will typically bind to proteins, specifically α1-acid glycoprotein in humans, which fish are known to possess (Armitage et al., 2017). However, there is little information explaining plasma protein binding of ionizable organics in fish blood plasma, and this knowledge gap could be a source of variability in current fish plasma uptake models (Armitage et al., 2017). It is generally recognized that the blood plasma protein content and the composition of blood changes within the same species of fish as a function of many conditions, including season, stage of maturity, spawning, food quantity and quality, and other factors (Lepkovsky, 1930; Siddiqui, 1977; Kalish, 1991). However, the influence of salinity on intraspecies variability of plasma proteins is less understood. Research by Peyghan et al. (2014) demonstrated no differences in total plasma protein content between the same species of carp (*Ctenopharyngodon idella*) at salinities ranging from freshwater to 12 ppt, but did observe freshwater-acclimated carp to exhibit differences in specific types of plasma proteins compared to carp reared at salinities of 4, 8, and 12 ppt (Peyghan et al., 2014). In the current study, gulf killifish were collected from the same reference point, fed the same diet, and exposed to the same temperatures/light cycle, and no differences in diphenhydramine V_D were observed across exposure salinity. As a result, our results suggest that water chemistry within the current study did not change the blood plasma protein content or plasma binding dynamics to such an extent to influence internal distribution of ionizable pharmaceuticals in gulf killifish.

Considering the large differences in diphenhydramine V_D observed between the current study with gulf killifish and our previous work with fathead minnows (Nichols et al., 2015), plasma protein binding could represent a potential explanation of interspecies distributional differences. Blewett et al. (2014) demonstrated that EE2 distribution varied between species, including significant differences between killifish and fathead minnow. Specifically, killifish in the study by Blewett et al. (2014) showed higher accumulation of EE2 in the liver and gall bladder and less EE2 accumulation in the carcass compared to rainbow trout and fathead minnow. These distribution differences were not correlated with the rate of EE2 uptake in fish, and instead suggested that these interspecies differences could be driven by physiology and metabolic processing, or lipid distribution within the body (Blewett et al., 2014). A study by Nouws et al. (1988) demonstrated that V_D for the drug ciprofloxacin varied significantly between three fish species (carp (*Cyprinus carpio*), African catfish (*Clarias gariepinus*), rainbow trout (*Salmo gairdneri*)), and surmised that it most likely resulted from physiological differences including vascularization, intercellular water content, tissue permeability, and tissue composition (i.e. muscle fibers). Tissues with higher phospholipid or plasma protein content are expected to exhibit higher concentrations of ionizable organic compounds, and interspecies variability in these potential depots for pharmaceuticals in fish deserve additional study (Armitage et al., 2017). Typically, lipid and protein binding is dominated by nonspecific partitioning interactions, while plasma protein binding of ionized compounds may be controlled by more specific interactions because of the relatively limited number of molecular binding sites (Nichols et al., 2015). Whether pharmaceutical protein binding differences exists between fathead minnows, killifish and other species is unknown. Future research is needed to determine the role of protein binding in ionizable bioaccumulation among fish.

In addition to studying two ionizable chemicals detected at elevated plasma levels in fish from urban estuaries (Scott et al., 2016), we studied carbamazepine (pKa of 13.9) because it would be an ionized compound at both pH levels examined here, thus negating the influence of the experimental pH treatments on ionization state and bioavailability. In the present study, at both of the tested pH levels of 8.3 and 6.7, carbamazepine was almost entirely ionized (>99.99%), which would offer an ideal opportunity to explore the influence of salinity and potentially drinking on uptake, in the absence of a pH effect. We hypothesized that if drinking was a significant route of exposure for ionizables, fish exposed at 20 ppt salinity (above the isosmotic point) would accumulate carbamazepine to a higher degree than fish exposed at 5 ppt (below the isosmotic point), due to an increased rate of drinking and transport of the drug across the gut. However, in our study, carbamazepine exhibited no differential uptake with salinity, and actually a small, but significant increase in steady-state tissue concentrations was observed at the lower pH. While Blewett et al. (2013a,b) did report that salinity markedly influences EE2 uptake in killifish, they concluded that drinking rate only negligibly affects EE2 uptake. Blewett et al. (2013a,b) further hypothesized that this salinity effect could instead depend upon the gill morphology at a given salinity. We exposed gulf killifish to pharmaceuticals at two different estuarine salinities (5 ppt and 20 ppt). However, it is possible that these fish did not fully adapt their gill function or structure to such a degree as to alter uptake kinetics during our study. Additional research is needed to determine whether specific structural changes to the gill may influence ionizable organic chemical uptake in fish.

One important consideration between gut and gill uptake is residence time, which spans hours in the gut and milliseconds at the gill surface (Armitage et al., 2017). Longer residence time in the gut could lead to increased metabolism and reductions in the uptake of the parent compound (Lo et al., 2015). Unless the relative proportion of neutral vs charged forms is very large, Armitage et al. (2017) suggested that the uptake efficiencies of these ionizable contaminants in the gut are not expected to be greatly reduced as a function of ionization state. Because we observed a significant pH influence but no salinity influence on accumulation of diphenhydramine and diltiazem, intestinal absorption following drinking, compared to inhalational uptake (Nichols et al., 2015) does not appear to be a major exposure route of the target parent compounds in Gulf killifish. However, additional research is needed to determine whether there is increased accumulation of ionizable

metabolites in fish exposed at higher salinities.

We observed no significant salinity effect on the uptake on ionizable pharmaceutical accumulation in Gulf killifish. One limitation of our study is that we did not specifically investigate pharmaceutical uptake as a function of salinity across a temporal scale. Unlike anadromous fish (e.g., salmon) that only encounter different salinities a few times over an entire lifetime, estuarine fish like killifish will inhabit habitats that endure salinity fluctuations daily as a result of tidal movement or precipitation events (Marshall and Grosell, 2006; Marshall et al., 1999). Potts and Evans (1967) reported that *F. heteroclitus* exhibit a significant decrease in drinking rate when transferred to freshwater, but do not significantly change drinking rate between seawater (~32 ppt) and brackish water (~13 ppt) (Potts and Evans, 1967). Scott et al. (2006) demonstrated that freshwater-acclimated killifish drank less than fish fully acclimated to brackish water over 7 days, but that the initial fall in drinking rate appeared to recover slightly over time. These studies demonstrate the variability in drinking rate as a function of salinity change, and highlight the potential need for higher temporal resolution for water chemistry considerations in bioaccumulation studies and modeling efforts. Research suggests that chloride cells are either absent or dormant in freshwater killifish, but appear at salinities of 10% (3–4 ppt) or higher (Laurent, 1984). Killifish appear to retain more chloride cells while living in freshwater as an adaptation to their estuarine existence, and while those chloride cells cannot be stimulated immediately to secrete chloride in freshwater adapted fish, chloride secretion can be induced within 24–48 h after transfer into seawater conditions (Marshall et al., 1999). Aside from molecular and stress responses caused by abrupt salinity changes, there is evidence that sudden and smaller salinity perturbations may change the rate of drinking in estuarine fish to a greater extent than the gradual change from marine to freshwater conditions (Chen et al., 2017; Scott et al., 2006). For killifish residing in areas that experience daily fluctuations in salinity, including the urban estuaries where we recently observed pharmaceutical bioaccumulation (Scott et al., 2016), it is plausible that short-term transformations of pre-existing cells would be the energetically most feasible adaptation, and perhaps was an adaptation employed by the fish in our study. Nonetheless, our results emphasize the need for further study exploring how temporal variability in salinity affects the uptake of pharmaceuticals in fish.

There remains little information on how salinity influences excretion and metabolism of organic contaminants of emerging concern, including ionizable pharmaceuticals and other chemicals. Several studies have demonstrated that organic chemical excretion rates are slower in freshwater fish compared to estuarine or marine fish (Feng et al., 2008; Ishida, 1992; Tachikawa and Sawamura, 1994; Tachikawa et al., 1991). However, diphenhydramine uptake by killifish in the present study was 11-fold higher than uptake by fathead minnow as reported by Nichols et al. (2015). A study by Connors et al. (2013) demonstrated that neither diltiazem nor diphenhydramine is transformed *in vitro* by rainbow trout. However, there are no data describing the *in vitro* metabolism of these drugs in *Fundulus*, which limits our understanding of pharmacokinetics in this and other estuarine species. Research is needed to better understand the influence of salinity on pharmaceutical metabolism and excretion, including kidney function, among fish species and exposure conditions, in addition to implications of such interspecies variability in ionizable pharmaceutical uptake and resulting toxicological responses.

In our study, measured concentrations of pharmaceuticals were 40–85% of the targeted nominal concentrations. There is evidence that salinity can elicit a "salting-out" effect on organic contaminants, in which aqueous solubility decreases with increasing salt concentration (Chen et al., 2017; Jonker and Muijs, 2010). The "salting-out" of emerging contaminants in saline water has been documented recently, and could influence bioaccumulation models if the effect on solubility was sufficiently significant (Blewett et al., 2013a,b; Chen et al., 2017). Bioavailability estimates and bioaccumulation models for ionizable organics could be particularly susceptible to complexation at elevated salinities. At a higher salinity, more ion–counterion complexes will be present when compared with freshwater because of the higher ionic strength (Wezel, 1998). Chen et al. (2017) reported that high salinity decreased sulfamethoxazole bioaccumulation in zebrafish, and further demonstrated that more sulfamethoxazole was found adsorbed on sediment in high salinity water, leading to reduced bioavailability and reduced body burdens in zebrafish. In the present study, measured concentrations of carbamazepine, diltiazem, and diphenhydramine were lower than nominal concentrations of 10, 1, and 10 μg/L, respectively. This could have resulted from simple experimental implementation, though these lower percentages were similar to those observed by Blewett et al. (2013a,b), where despite adding radiolabeled EE2 to achieve a target nominal concentration of 100 ng/L, there was a large initial loss of EE2 prior to the initial (0 min) sample collection resulting in mean measured concentrations ranging from 43 to 65 ng/L (or 43% and 65% of the nominal). Blewett et al. (2013a,b) further observed that the concentrations of EE2 declined slightly over time. Results from the present study, in addition to observations by Chen et al. (2017) and Blewett et al. (2013a,b), identify the potential impacts of "salting out" on contaminants of emerging concern bioaccumulation and BCF modeling. Future studies are needed to better understand the "salting-out" effect on ionizable pharmaceuticals and other contaminants across salinity gradients.

5. Conclusions

In a common euryhaline estuarine fish, we observed pH, but not salinity, to influence bioconcentration of select weak base pharmaceuticals, suggesting that gut uptake via drinking, in contrast to inhalational uptake (Nichols et al., 2015), does not appear to be a major exposure route of these pharmaceuticals in Gulf killifish. We also observed Gulf killifish to accumulate diphenhydramine in whole-body tissue and blood plasma to a greater extent than previous observations with the fathead minnow. Though water chemistry did not influence apparent V_D for any of the tested pharmaceuticals, V_D values were lower in killifish than the fathead minnow, which suggests that killifish are preferentially distributing diltiazem and diphenhydramine within intravascular fluid and plasma compared to whole-body tissue. Clearly, additional research is warranted to better understand bioaccumulation of ionizable contaminants in urban estuaries. Future research is also needed to elucidate the extent to which protein binding, metabolism, and the "salting out" effect influences bioaccumulation of pharmaceuticals and other contaminants across salinity gradients.

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Appendix A. Supplementary data

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