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

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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). Results demonstrate that 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 of emerging concern.

Keywords: urbanization, bioaccumulation, pharmaceuticals, ionizable contaminants, environmental complexity

1. Introduction

Pharmaceuticals in the environment have been receiving global research for because they are designed to be biologically active, and routinely accumulate in field-collected organisms (Daughton and Jones-Lepp, 2001; Daughton, 2004; Kümmerer, 2010; Du et al., 2014; Du et al., 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 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

65 instream degradation rates (Ankley et al., 2007). Effluent-dominated systems are now recognized
66 as important watersheds for management, particularly in the face of climate change (Luthy et al.,
67 2015). In addition to receiving discharges from urban areas, estuaries, an important interface
68 among terrestrial, freshwater, and marine systems, experience substantial spatial and temporal
69 fluctuations in physiochemical parameters, including salinity and pH (Beck and Bruland, 2000;
70 Hubertz and Cahoon, 1999; Nelson et al., 1994; Pritchard, 1967; Scott et al., 2019).

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

83 Unfortunately, though bioaccumulation of pharmaceuticals has received increasing
84 attention since our initial reports of human pharmaceuticals accumulating in fish from an
85 effluent-dominated river (Brooks et al., 2005; Ramirez et al., 2007), there remains a poor
86 understanding of pharmaceutical bioaccumulation in estuarine environments (Daughton and
87 Brooks, 2011; Maruya et al., 2012; Alvarez et al., 2014; Gaw et al., 2014; Lazarus et al., 2015;

88 Du et al., 2016; Meador et al., 2016; Bean et al., 2018). In addition to considering pH influences
89 on bioavailability and bioaccumulation of ionizable contaminants in estuaries (Nichols et al.,
90 2015), salinity alters both the metabolic rate and the surface structure of the gill in fish, which
91 can potentially influence chemical uptake and elimination (Copeland, 1950; Laurent et al., 2006;
92 Nichols et al., 2015; Scott et al., 2004; Scott et al., 2004). Therefore, in the present study, we
93 examined the relative influence of pH and salinity on bioconcentration of three pharmaceuticals
94 in the Gulf killifish, *Fundulus grandis*. We specifically selected *F. grandis* for study because it is
95 common euryhaline teleost in estuaries of the Gulf of Mexico (Harrington and Harrington,
96 1982).

97

98 **2. Methods**

99 2.1 *Fundulus grandis*

100 Adult *F. grandis* were collected using minnow traps from a previously-recognized reference
101 population at Smith Point, near Galveston Bay, Texas (29º 32′ 37.26″ N, 94 º 47′ 08.12″ W;
102 Oziolor et al., 2016). These wild-caught fish were kept for over one month prior to the initiation
103 of experiments. Prior to experimental exposure, fish employed for the 20 ppt experiments (at
104 both pH 8.3 and 6.7) were reared at approximately 17 ppt for at least 2 weeks prior to initiating
105 exposure. Similarly, prior to initiating the 5 ppt uptake experiments, fish were then acclimated to
106 approximately 7 ppt salinity over a two week period. We then performed four discrete uptake
107 experiments in which high or low pH levels (8.3 and 6.7) were manipulated at one of two salinity
108 levels (20 ppt and 5 ppt). The pH levels were chosen based on the high and low mean salinities
109 observed in our previous studies of pharmaceutical accumulation and hazards in Texas Gulf

110 Coast estuaries (Scott et al., 2016; 2019). Because *F. grandis* has an isosmotic point of 12 ppt
111 (Fritz and Garside, 1974; Varsamos et al., 2005) and 5 ppt to 20 ppt represent an ideal salinity
112 range for survival and growth (Perschbacher et al., 1990; Patterson et al., 2012), we chose 20 ppt
113 and 5 ppt salinity treatment levels.

114

115 2.2 *Fundulus grandis Experiments*

116 For this study, experimental methods generally followed those we have previously reported
117 (Nichols et al., 2015). Adjustments to pH 6.7 and pH 8.3 were accomplished by titrating with
118 hydrochloric acid and sodium hydroxide, respectively, following USEPA recommendations (US
119 EPA 1991). To initiate each experiment, a stock solution containing a mixture of carbamazepine,
120 diltiazem, and diphenhydramine, was added to each treatment unit to achieve nominal
121 concentrations of 10 µg/L, 1 µg/L, and 10 µg/L, respectively. Carbamazepine, diltiazem
122 hydrochloride, and diphenhydramine hydrochloride were purchased from Sigma-Aldrich (St.
123 Louis, MO, USA). We selected these substances because diltiazem and diphenhydramine are
124 common contaminants in surface waters (Kristofco et al., 2017; Saari et al., 2017) with pKa
125 values (7.7 and 8.9, respectively; Bonferoni et al., 2000; Shaleva et al., 2008) indicating pH
126 influences on ionization across estuarine conditions. The pKa is an integral aspect of drug
127 research and development, because it influences rates of absorption through body compartments
128 possessing different pH values (Manallack 2007). Carbamazepine is also a common contaminant
129 in urban systems we anticipated would not be appreciably influenced by pH given its higher pKa
130 (13.9). Glass aquaria were used as experimental units (e.g., 20 L aquaria) with a water volume of
131 15 L each. The positions of each experimental unit were randomized and maintained in an
132 environmental chamber at 25ºC on a 16:8 light:dark cycle. Within the semi-flow through

133 experimental system, water flow was adjusted to a constant flow without creating current, and an
134 hourly recirculating renewal rate of 2X per hour (30 L/h) was targeted for each experimental
135 unit. Three water renewals were conducted daily (every 8 hours) to maintain water quality, and
136 consistency of the chemical exposure concentration.

137 Four *F. grandis* (n=4) were added to each of three replicate experimental units (N=3), in
138 each of the experimental systems. Within each experiment, exposures to a sublethal mixture of
139 carbamazepine, diltiazem, and diphenhydramine were conducted with exposure durations of 1, 3,
140 6, 12, 24, and 48 hours, with staggered starting times due to experimental system setup. At each
141 time point, triplicate experimental units (N=3) were sampled such that all four fish from an
142 experimental unit were removed, anesthetized/euthanized, and pooled for analytical measures
143 (Nichols et al., 2015). Control fish were exposed for the entire 48 hours. To ensure that fish
144 were exposed to common concentrations, pharmaceutical levels in each aquaria were analytically
145 verified at each time point in relation to water renewals according to previously published
146 methods by our research group (Haddad et al., 2018; Du et al., 2016; Du et al., 2014). Following
147 an approved Institutional Animal Care and Use Committee protocol, fish were anesthetized with
148 tricaine methane sulfonate (MS-222), weighed, and measured to obtain total length. Blood was
149 then collected from the caudal artery using heparinized micro-hematocrit capillary tubes
150 (StatSpin, Brea, CA, USA). Plasma and tissue from all fish within each aquarium were pooled,
151 resulting in 3 pooled replicate samples (N=3) for each exposure duration. Plasma was separated
152 in a gel barrier microtube (StatSpin, Brea, CA, USA), centrifuged at 3000 x *g* (4°C) for 20 min,
153 and stored immediately at -80°C. Analytical methods for water, blood plasma, and tissue
154 analyses followed recent experimental methods from our research team (Haddad et al., 2018; Du
155 et al., 2016; Du et al., 2014).

156

157 2.3 *BCFs, Blood:Water Partitioning Coefficients, and Volume of Distribution*

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

169 Eq. 1 $$BCF = \frac{\text{whole-body tissue concentration}}{\text{water concentration}}$$

170 Eq. 2 $$P_{B:W} = \frac{\text{blood plasma concentration}}{\text{water concentration}}$$

171 Eq. 3 $$Apparent\ V_D\ (\text{L/kg}) = \frac{\text{whole-body tissue concentration (µg/kg)}}{\text{blood plasma concetration (µg/L)}}$$

172

173

174

175 2.4 *Statistical Analysis*

176 All statistical analyses were performed using SigmaPlot (Systat Software, San Jose, CA, USA).
177 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 and 48 hours 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 ±0.22ºC and 5.59 ± 0.57 mg/L, respectively. In the two experiments conducted at the higher pH, mean measured pH was 8.27 ± 0.07, with a median of 8.29. Mean pH in the two low-pH experiments was 6.88 ± 0.22, with a median pH of 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 (ºC) 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,

201 respectively. Despite the measured concentrations being slightly lower than nominal levels,
202 mean exposure concentrations of the three pharmaceuticals (carbamazepine, diltiazem, and
203 diphenhydramine) did not differ significantly across the four experiments ($p > 0.05$). Mean
204 concentrations for each time point per experiment are provided in Supporting Information.
205 Within the control experimental units, there were no detects of the three pharmaceuticals in
206 water during any of the four studies.

207

208 3.2 *Influence of pH and Salinity on Pharmaceutical Uptake by Fish*

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

218 Salinity did not significantly affect accumulation of the three pharmaceuticals by Gulf
219 killifish ($p > 0.05$). Carbamazepine tissue concentrations were significantly higher at pH 6.7,
220 compared to pH 8.3 ($p < 0.05$), but it did not significantly differ ($p > 0.05$) in blood plasma
221 between pH treatment levels. Tissue and blood plasma concentrations of diltiazem and
222 diphenhydramine were significantly higher at pH 8.3 compared to pH 6.7 ($p < 0.05$).

223 Concentrations of each pharmaceutical appeared to reach steady-state in whole-body tissue and
224 blood plasma by 24 h; therefore, BCF, P_{BW}, and V_D values were calculated at 24 and 48 h (Table
225 1).

226 Similar to tissue and plasma observations, the only significant differences in BCFs for
227 carbamazepine were observed between pH 6.7 and 8.3 (BCFs at pH 6.7 > pH 8.3), at the 20 ppt
228 salinity ($p < 0.05$). BCFs for carbamazepine did not differ significantly between pH levels at the
229 lower salinity level of 5 ppt, nor did BCFs differ significantly with exposure salinity, when the
230 influence of pH was accounted for ($p > 0.05$). BCFs for diltiazem were significantly elevated by
231 pH 8.3, and significantly higher at the lower salinity level of 5 ppt ($p < 0.05$). Diphenhydramine
232 BCFs were significantly higher at pH 8.3, compared to pH 6.7, but BCFs exhibited no significant
233 differences with salinity ($p < 0.05$). There were no significant differences in P_{BW} values for
234 carbamazepine across all four experimental conditions. Diltiazem and diphenhydramine P_{BW}
235 values were significantly higher in pH 8.3 exposures, compared to pH 6.7 ($p < 0.05$), while
236 salinity exhibited no influence on P_{BW} values for diltiazem or diphenhydramine ($p > 0.05$).
237 Across all four experiments, mean V_D values for carbamazepine, diltiazem, and
238 diphenhydramine were 1.15 (± 0.33), 0.26 (± 0.1), and 0.48 (± 0.2) L/kg, respectively (Table
239 1).V_D values for carbamazepine, diltiazem, and diphenhydramine did not differ significantly ($p >$
240 0.05) across the four experiments, confirming that waterborne exposure at these levels does not
241 influence the internal distribution of these ionizable drugs (Nichols et al. 2015).

242

243 **4. Discussion**

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

255 *F. grandis* is an extremely euryhaline species, with populations existing in habitats
256 ranging from freshwater to tidal pools of 76 ppt salinity (Simpson and Gunter 1956; Tabb and
257 Manning 1961). Whereas fish in freshwater essentially only ingest water while feeding, fish in
258 saltwater environments will gulp copious amounts of water each day in order to maintain
259 osmotic balance (Copeland 1950; Fritz and Garside 1974; Marshall et al., 1999; Potts and Evans
260 1967; Scott et al., 2004a,b; Scott et al., 2006; Scott et al., 2008). With such differences in
261 drinking rate, euryhaline fish could be orally exposed to a greater extent to contaminants at
262 higher salinities. However, there remains little research exploring the influence of salinity on
263 pharmaceutical accumulation in estuarine fish. Blewett et al. (2013a,b) demonstrated that salinity
264 had a significant influence on uptake of the nonionizable contraceptive pharmaceutical 17-α-
265 ethinyl estradiol (EE2) by killifish. We anticipated rearing fish above and below the isosmotic
266 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 for diltiazem and

290 diphenhydramine was 0.26 ± 0.11, and 0.48 ± 0.21 L/kg, respectively, which demonstrates that
291 these drugs are preferentially partitioning to blood plasma and other bodily fluids compared to
292 other body compartments. As noted above, apparent V_D was not statistically different across all
293 exposure scenarios for both diltiazem (0.19-0.28 L/kg) and diphenhydramine (0.32-0.48 L/kg).
294 This consistency in V_D was also noted by Nichols et al. (2015), where, despite significant
295 differences in diphenhydramine accumulation, V_D remained similar across all pH levels tested
296 (Nichols et al., 2015). Interestingly, diphenhydramine V_D values for killifish in the present study
297 (0.48 ± 0.2 L/kg) were nearly 10-fold lower than those for the fathead minnow reported by
298 Nichols et al. (~ 3 L/kg reported by Nichols et al., 2015). This stark difference in V_D between
299 these two species suggests that Gulf killifish are distributing diphenhydramine within
300 intravascular fluid or blood to a much higher degree than fathead minnows under freshwater
301 conditions.

302 While the internal distribution of neutral organic compounds is driven by passive
303 diffusion to lipids, phospholipid content and plasma protein content are thought to significantly
304 influence the distribution of ionizables (Armitage et al., 2017). Basic pharmaceuticals will
305 typically bind to proteins, specifically α1-acid glycoprotein in humans, which fish are known to
306 possess (Armitage et al., 2017). However, there is little information explaining plasma protein
307 binding of ionzable organics in fish blood plasma, and this knowledge gap could be a source of
308 variability in current fish plasma uptake models (Armitage et al., 2017). It is generally
309 recognized that the blood plasma protein content and the composition of blood changes within
310 the same species of fish as a function of many conditions, including season, stage of maturity,
311 spawning, food quantity and quality, and other factors (Lepkovsky 1930, Siddiqui 1976; Kalish
312 1991). However, the influence of salinity on intraspecies variability of plasma proteins is less

313 understood. Research by Peyghan et al. (2014) demonstrated no differences in total plasma
314 protein content between the same species of carp (*Ctenopharyngodon idella*) at salinities ranging
315 from freshwater to 12 ppt, but did observe freshwater-acclimated carp to exhibit differences in
316 specific types of plasma proteins compared to carp reared at salinities of 4, 8, and 12 ppt
317 (Peyghan et al., 2014). In the current study, gulf killifish were collected from the same reference
318 point, fed the same diet, and exposed to the same temperatures/light cycle, and no differences in
319 diphenhydramine V_D were observed across exposure salinity. As a result, our results suggest that
320 water chemistry within the current study did not change the blood plasma protein content or
321 plasma binding dynamics to such an extent to influence internal distribution of ionizable
322 pharmaceuticals in gulf killifish.

323 Considering the large differences in diphenhydramine V_D observed between the current
324 study with gulf killifish and our previous work with fathead minnows (Nichols et al. 2015),
325 plasma protein binding could represent a potential explanation of interspecies distributional
326 differences. Blewett et al. (2014) demonstrated that EE2 distribution varied between species,
327 including significant differences between killifish and fathead minnow. Specifically, killifish in
328 the study by Blewett et al. (2014) showed higher accumulation of EE2 in the liver and gall
329 bladder and less EE2 accumulation in the carcass compared to rainbow trout and fathead
330 minnow. These distribution differences were not correlated with the rate of EE2 uptake in fish,
331 and instead suggested that these interspecies differences could be driven by physiology and
332 metabolic processing, or lipid distribution within the body (Blewett et al., 2014). A study by
333 Nouws et al. (1988) demonstrated that V_D for the drug ciprofloxacin varied significantly between
334 three fish species (carp (*Cyprinus carpio*), African catfish (*Clarias gariepinus*), rainbow trout
335 (*Salmo gairdneri*)), and surmised that it most likely resulted from physiological differences

336 including vascularization, intercellular water content, tissue permeability, and tissue composition
337 (i.e. muscle fibers). Tissues with higher phospholipid or plasma protein content are expected to
338 exhibit higher concentrations of ionizable organic compounds, and interspecies variability in
339 these potential depots for pharmaceuticals in fish deserve additional study (Armitage et al.,
340 2017). Typically, lipid and protein binding is dominated by nonspecific partitioning interactions,
341 while plasma protein binding of ionized compounds may be controlled by more specific
342 interactions because of the relatively limited number of molecular binding sites (Nichols et al.,
343 2015). Whether pharmaceutical protein binding differences exists between fathead minnows,
344 killifish and other species is unknown. Future research is needed to determine the role of protein
345 binding in ionizable bioaccumulation among fish.

346 In addition to studying two ionizable chemicals detected at elevated plasma levels in fish
347 from urban estuaries (Scott et al., 2016), we studied carbamazepine (pKa of 13.9) because it
348 would be an ionized compound at both pH levels examined here, thus negating the influence of
349 the experimental pH treatments on ionization state and bioavailability. In the present study, at
350 both of the tested pH levels of 8.3 and 6.7, carbamazepine was almost entirely ionized
351 (>99.99%), which would offer an ideal opportunity to explore the influence of salinity and
352 potentially drinking on uptake, in the absence of a pH effect. We hypothesized that if drinking
353 was a significant route of exposure for ionizables, fish exposed at 20 ppt salinity (above the
354 isosmotic point) would accumulate carbamazepine to a higher degree than fish exposed at 5 ppt
355 (below the isosmotic point), due to an increased rate of drinking and transport of the drug across
356 the gut. However, in our study, carbamazepine exhibited no differential uptake with salinity, and
357 actually a small, but significant increase in steady-state tissue concentrations was observed at the
358 lower pH. While Blewett et al. (2013a,b) did report that salinity markedly influences EE2 uptake

359 in killifish, they concluded that drinking rate only negligibly affects EE2 uptake. Blewett et al.
360 (2013a,b) further hypothesized that this salinity effect could instead depend upon the gill
361 morphology at a given salinity. We exposed gulf killifish to pharmaceuticals at two different
362 estuarine salinities (5 ppt and 20 ppt). However, it is possible that these fish did not fully adapt
363 their gill function or structure to such a degree as to alter uptake kinetics during our study.
364 Additional research is needed to determine the specific effect that structural changes to the gill
365 have on ionizable organic chemical uptake in fish.

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

378 We observed no significant salinity effect on the uptake on ionizable pharmaceutical
379 accumulation in Gulf killifish. One limitation of our study is that we did not specifically
380 investigate pharmaceutical uptake as a function of salinity across a temporal scale. Unlike
381 anadromous fish (e.g., salmon) that only encounter different salinities a few times over an entire

382 lifetime, estuarine fish like killifish will inhabit habitats that endure salinity fluctuations daily as
383 a result of tidal movement or precipitation events (Marshall and Grosell 2006; Marshall et al.,
384 1999). Potts and Evans (1967) reported that *F. heteroclitus* exhibit a significant decrease in
385 drinking rate when transferred to freshwater, but do not significantly change drinking rate
386 between seawater (~32 ppt) and brackish water (~13 ppt) (Potts and Evans, 1967). Scott et al.
387 (2006) demonstrated that freshwater-acclimated killifish drank less than fish fully acclimated to
388 brackish water over 7 days, but that the initial fall in drinking rate appeared to recover slightly
389 over time. These studies demonstrate the variability in drinking rate as a function of salinity
390 change, and highlight the potential need for higher temporal resolution for water chemistry
391 considerations in bioaccumulation modeling efforts. Research suggests that chloride cells are
392 either absent or dormant in freshwater killifish, but appear at salinities of 10% (3–4 ppt) or
393 higher (Laurent 1984). Killifish appear to retain more chloride cells while living in freshwater as
394 an adaptation to their estuarine existence, and while those chloride cells cannot be stimulated
395 immediately to secrete chloride in freshwater adapted fish, chloride secretion can be induced
396 within 24-48 h after transfer into seawater conditions (Marshall et al., 1999). Aside from the
397 molecular and stress responses caused by abrupt salinity changes, there is evidence that sudden
398 and smaller salinity perturbations may change the rate of drinking in estuarine fish to a greater
399 extent than the gradual change from marine to freshwater conditions (Chen et al., 2017; Scott et
400 al., 2006). For killifish residing in areas that experience daily fluctuations in salinity, including
401 the urban estuaries where we recently observed pharmaceutical bioaccumulation (Scott et al.,
402 2016), it is plausible that short-term transformations of pre-existing cells would be the
403 energetically most feasible adaptation, and perhaps was the adaptation employed by the fish in

404 our study. Nonetheless, our results emphasize the need for further study exploring how temporal
405 variability in salinity affects the uptake of pharmaceuticals in fish.

406 There remains little information on how salinity influences excretion and metabolism of
407 organic contaminants of emerging concern, including ionizable pharmaceuticals and chemicals.
408 Several studies have demonstrated that organic chemical excretion rates are slower in freshwater
409 fish compared to estuarine or marine fish (Feng et al., 2008; Ishida 1992; Tachikawa and
410 Sawamura 1994; Tachikawa et al., 1991). However, diphenhydramine uptake by killifish in the
411 present study was 11-fold higher than uptake by fathead minnow as reported by Nichols et al.
412 (2015). A study by Connors et al. (2013) demonstrated that neither diltiazem nor
413 diphenhydramine is transformed *in vitro* by rainbow trout. However, there are no data describing
414 the *in vitro* metabolism of these drugs in *Fundulus*, which limits our understanding of
415 pharmacokinetics in this and other estuarine species. Research is needed to better understand the
416 influence of salinity on pharmaceutical metabolism and excretion, including kidney function,
417 among fish species and exposure conditions, in addition to implications of such interspecies
418 variability in ionizable pharmaceutical uptake and resulting toxicological responses.

419 In our study, measured concentrations of pharmaceuticals were 40-85% of the targeted
420 nominal concentrations. There is evidence that salinity can elicit a "salting-out" effect on organic
421 contaminants, in which aqueous solubility decreases with increasing salt concentration (Chen et
422 al., 2017; Jonker and Muijs 2010). The "salting-out" of emerging contaminants in saline water
423 has been documented recently, and could influence bioaccumulation models if the effect on
424 solubility was sufficiently significant (Blewett et al., 2013a,b; Chen et al., 2017). Bioavailability
425 estimates and bioaccumulation models for ionizable organics could be particularly susceptible to
426 complexation at elevated salinities. At a higher salinity, more ion–counterion complexes will be

427 present when compared with freshwater because of the higher ionic strength (Wezel 1998). Chen
428 et al. (2017) reported that high salinity decreased sulfamethoxazole bioaccumulation in
429 zebrafish, and further demonstrated that more sulfamethoxazole was found adsorbed on sediment
430 in high salinity water, leading to reduced bioavailability and reduced body burdens in zebrafish.
431 In the present study, measured concentrations of carbamazepine, diltiazem, and
432 diphenhydramine were lower than nominal concentrations of 10, 1, and 10 µg/L, respectively.
433 This could have resulted from simple experimental implementation, though these lower
434 percentages were similar to those observed by Blewett et al. (2013a,b), where despite adding
435 radiolabeled EE2 to achieve a target nominal concentration of 100 ng/L, there was a large initial
436 loss of EE2 prior to the initial (0 minute) sample collection resulting in mean measured
437 concentrations ranging from 43 to 65 ng/L (or 43% and 65% of the nominal). Blewett et al.
438 (2013a,b) further observed that the concentrations of EE2 declined slightly over time. Results
439 from the present study, in addition to observations by Chen et al. (2017) and Blewett et al. (2013
440 a,b), identify the potential impacts of "salting out" on contaminants of emerging concern
441 bioaccumulation and BCF modeling. Future studies are needed to better understand the "salting-
442 out" effect on ionizable pharmaceuticals and other contaminants across salinity gradients.

443

444

445

446 **5. Conclusions**

447 In a common euryhaline estuarine fish, we observed pH, but not salinity, to influence
448 bioconcentration of select weak base pharmaceuticals, suggesting that gut uptake via drinking, in
449 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 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 across salinity gradients.

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Figure Captions

Figure 1: Mean (±SD) whole-body tissue concentrations (N=3) of carbamazepine (A), diltiazem (B), and diphenhydramine (C) in Gulf killifish (*Fundulus grandis*) over 48 hours. 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 (△).

Figure 2: Mean (±SD) plasma concentrations (N=3) of carbamazepine (A), diltiazem (B), and diphenhydramine (C) in Gulf killifish (*Fundulus grandis*) over 48 hours. 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 hours and 48 hours 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.

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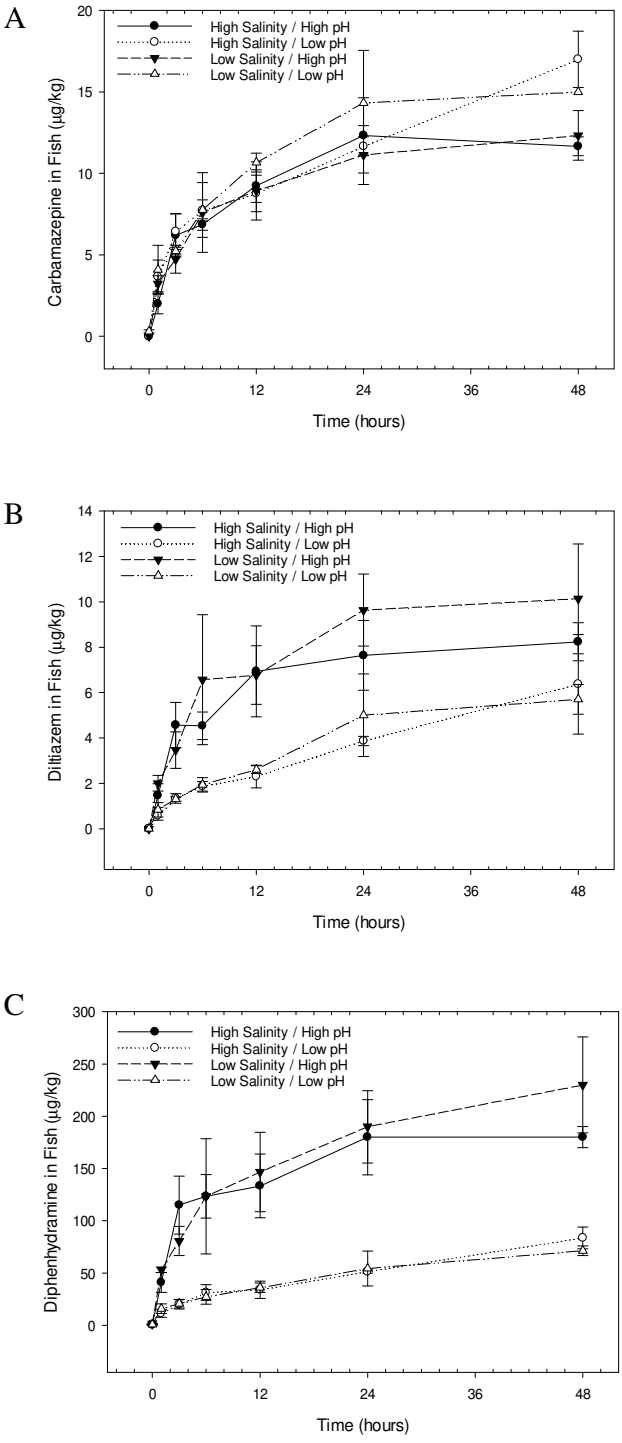
A
High Salinity / High pH
High Salinity / Low pH
Low Salinity / High pH
Low Salinity / Low pH
Carbamazepine in Fish (µg/kg)
Time (hours)
B
Diltiazem in Fish (µg/kg)
C
Diphenhydramine in Fish (µg/kg)

Figure 1

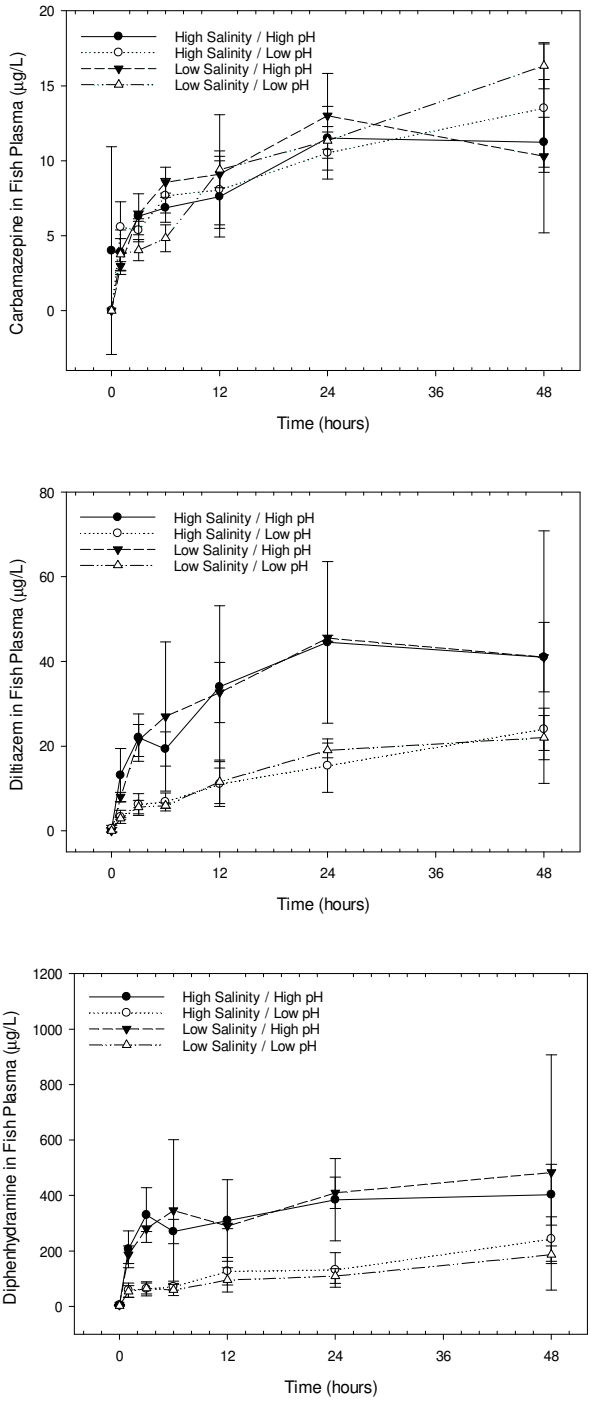
High Salinity / High pH
High Salinity / Low pH
Low Salinity / High pH
Low Salinity / Low pH
Carbamazepine in Fish Plasma (µg/L)
Diltiazem in Fish Plasma (µg/L)
Diphenhydramine in Fish Plasma (µg/L)
Time (hours)
0
12
24
36
48

Figure 2

Table 1

A.

Treatment	Salinity (ppt)	pH	Tissue BCF 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.

Treatment	Salinity (ppt)	pH	$P_{B:W}$ 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.

Treatment	Salinity (ppt)	pH	V_D (L/kg) 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