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Assessment of Mosquitofish (*Gambusia affinis*) Health Indicators in Relation to Domestic Wastewater Discharges in Suburbs of Houston, USA

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Abstract Personal care products, pharmaceuticals, and other contaminants of emerging concern (CECs) in domestic wastewater treatment plant (WWTP) effluents can impact aquatic organisms. Health indicators were compared for mosquitofish (*Gambusia affinis*) collected above and below WWTP discharges from five streams in suburban areas of the Houston metropolitan area, Texas, USA. Specimens were evaluated for reproductive, morphological, and histological indicators. Several indicators revealed significant spatial and temporal variation; however, possibly because of their mobility, fish collected upstream and downstream of wastewater treatment plants did not reveal consistent trends based on the endpoints examined. CEC concentrations in water samples from stream reaches below WWTP discharges were quantified for the first time in the Houston Metropolitan area. The 18 CECs detected in stream water had concentrations lower than values currently reported to impact fish. Future research should examine caged fish at each site and fish collected over longer stream reaches that receive successive discharges from WWTP and stronger CEC gradients.

Keywords Caffeine · Sucralose · Contaminants of emerging concern · Personal care products · Pharmaceuticals

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Effluents released from domestic wastewater treatment plants (WWTPs) are recognized as a source of chemicals of emerging concern (CECs) that include pharmaceuticals and compounds derived from personal care products (Kolpin et al. 2002). Wastewater treatment plants in urban centers can release significant concentrations of CECs (Elorriaga et al. 2013); among which caffeine, diphenhydramine and carbamezapine, have been reported in fish tissues (Ramirez et al. 2009; Wang and Gardinali 2012). Some substances of CECs have an endocrine disrupting potential that has been linked to masculinization of females, altered sex ratios, and changes in reproductive behavior (Toft et al. 2003; Kidd et al. 2007).

Mosquitofish (*Gambusia affinis*) are common in aquatic habitats throughout most of the Houston area. This fish species has been used as an indicator of chemical pollution and effects on biota (Batty and Lim 1999; Angus et al. 2005; Doyle and Lim 2005). The Houston metropolitan area is the 6th largest in the US (6.18 million) and has more than 400 WWTPs that discharge into local streams. The aim of this study was to determine if indicators of health and reproduction in mosquitofish are influenced by location upstream or downstream of domestic WWTP outfalls

within suburbs of Houston, Texas. In addition, a preliminary evaluation of pharmaceuticals and other CECs was done for water at four of the five downstream and one upstream sampling points.

Materials and Methods

Headwater reaches within Buffalo Bayou, Greens Bayou, and White Oak Bayou draining suburbs of the Houston metropolitan area were sampled over 5 days during both May and August 2010. Mosquitofish were collected at locations downstream and upstream from domestic WWTP discharges at five locations. Collection sites were chosen based on absence of any WWTPs in the upstream reach. Downstream sites were 50–200 m below a WWTP discharge; upstream sites were approximately 250 m above a discharge point. Fish were collected using a seine, with a goal of capturing at least 15 adult male and 15 adult female specimens at each site during both sampling occasions. Fish were anesthetized using tricaine methanesulfonate (MS222), then preserved in 10 % formalin, and later transferred to 70 % ethanol for storage.

Standard length (SL) of each specimen was measured to the nearest 0.5 mm. Following dissection, gonads, livers and eviscerated carcasses of both males and females were weighed for calculation of hepatosomatic (HSI) and gonadosomatic (GSI) indices. Material was placed in a drying oven for 2 days at 60°C until constant weight. The gonopodium length/body length ratio (GL) of mature males with fully-developed hooks on the gonopodium terminus was recorded. Embryo developmental stages within ovaries of pregnant females were classified, and numbers of middle- and late-eyed stage embryos were recorded.

Eighteen mature male and female mosquitofish from sites below WWTPs were randomly chosen for histological analysis. Maturity in females was based on the presence of a distinct gravid spot (dark pigmentation) near the base of the anal fin. Gonad, brain, liver, intestine, and other tissues from each specimen were fixed in 10 % formalin, embedded in paraffin wax, sectioned at 4 μm, and stained with hematoxylin and eosin for examination under a microscope. Gonad cross sections were examined for abnormalities. Tissues also were examined for parasites.

Duplicate water samples were obtained from four downstream sites and one upstream site during August 2011. Chemical analysis was performed to screen for presence of pharmaceuticals and other CECs, and due to budgetary constraints, sampling emphasized locations downstream from WWTPs. Samples were collected in acetone-washed, amber glass bottles and stored on ice for transport to Baylor University for analysis within 24 h following established methods (Du et al. 2014). Briefly, sample extraction followed the protocol of Vanderford and Snyder (2006); 450–500 mL of each sample was loaded onto a preconditioned HLB solid phase extraction cartridge (Waters Corp., Milford, Massachusetts, US). The loaded cartridge was air-dried and eluted with 5 mL methanol followed by 5 mL 10:90 (v/v) methanol–methyl tertiary butyl ether. The combined eluate was evaporated to dryness under a stream of nitrogen and reconstituted in 1 mL of the initial chromatographic mobile phase (i.e., 5:95 (v/v) methanol-0.1 % (v/v) aqueous formic acid). Prior to analysis, samples were sonicated for 1 min and filtered using Pall Acrodisc hydrophobic Teflon membrane syringe filters (13-mm diameter; 0.2-μm pore size). Samples were screened for 19 target analytes (i.e., those reported in Table 3 plus ivermectin which was not detected in any sample) using reverse-phase liquid chromatography-tandem mass spectrometry (LC–MS/MS) with electrospray ionization (ESI). Details pertaining to LC–MS/MS analysis, including the ionization mode (ESI+ or ESI−) and precursor-to-product ion transitions monitored for most analytes, are reported elsewhere (Du et al. 2012). The ionization mode and monitored transitions for target analytes and isotopically labeled internal standards not utilized in the previous study were as follows: Positive ESI – methylphenidate $234 > 84$; methylphenidate-d9 $243 > 93$; erythromycin $716 > 558$; erythromycin-13Cd3 $720 > 562$; warfarin $309 > 163$; warfarin-d5 $314 > 163$; celecoxib $382 > 362$; celecoxib-d4 $386 > 366$; ivermectin $892 > 569$; abamectin (internal standard for ivermectin) $890 > 567$. Negative ESI – diclofenac $294 > 250$; diclofenac-d4 $298 > 254$; sucralose $395 > 359$; sucralose-d6 $401 > 365$.

Statistical analyses of biological data were performed using SAS (v. 9.3, SAS Systems Inc, Cary, North Carolina, USA). Standard length, CW, GL, GSI, HSI, and number of embryos carried by pregnant females were compared, separately for males and females, for sites upstream and downstream of individual WWTPs separately during each period. Normality of data distributions was confirmed using the Shapiro–Wilks test. Significance of mean differences of SL, CW, GSI, and HSI between upstream and downstream samples was evaluated using the t test, with significance set at $p < 0.05$. Between-location differences in male gonopodium length and number of embryos carried by pregnant females was tested using analysis of covariance, with SL as the covariate (Fig. 1).

Results and Discussion

Morphological and reproductive indices revealed relatively few statistically significant differences between samples from upstream and downstream WWTPs, and trends were inconsistent among the five stream reaches and, in some cases, between seasons within a stream reach (Table 1).

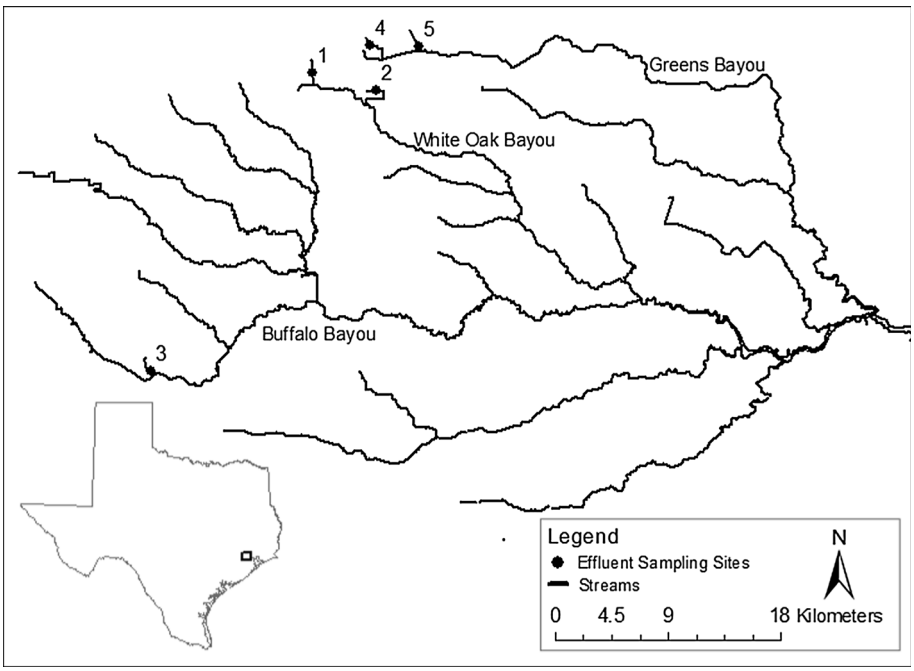

Fig. 1 Map of Harris County Waterways, Harris County, Texas. The *numbers* represent the locations of the five streams sampled (site 1 – 29.938248°N, 95.620934°W; site 2 – 29.923719°N, 95.568718°W; site 3 – 29.728731°N, 95.762833°W; site 4 – 29.955528°N, 95.572128°W; site 5 – 29.953371°N, 95.531822°W)

Gonopodium length, male GSI, and female HSI revealed no statistically significant mean differences (data not shown). At site 1, males and females were significantly larger upstream WWTPs during May, but with one exception, not significantly different during August (Table 1). Females at the location upstream the WWTP at site 1 had significantly greater numbers of embryos during May, but GSI and number of embryos were lower at the upstream location during August. At site 2, female SL and number of embryos were significantly greater downstream the WWTP during May, and female GSI was significantly greater at the upstream location during August. Females at sites 3 and 4 revealed no significant differences upstream and downstream from WWTPs with regard to any of the indices. Male SL and CW were significantly greater upstream the WWTP at site 4 during August. Male SL but not CW also was significantly greater upstream the WWTP at site 4 during May. Females at site 5 had significantly greater SL and number of embryos at locations upstream WWTPs during both May and August, greater GSI at the upstream location during May, and greater CW at the upstream location during August.

Except for one individual, males showed histological evidence of active spermatogenesis. Diverse stages of follicular development were observed in ovaries, which is expected during summer. No evidence of intersex (ovarian and testicular tissue in the same individual) was encountered. Acute inflammation and degeneration of skeletal muscle was observed in two specimens. The most apparent anomaly was the presence of parasites in gill, stomach, intestine, muscle and cartilage tissues from 9 of 18 specimens (Table 2). Exposure to WWTP effluents has been associated with higher parasite infection rates in trout (*Salmo trutta*); however, the incidence of parasite infections in mosquitofish in the present study is similar to that reported for mosquitofish from the spring-fed and relatively unpolluted headwaters of the San Marcos River in central Texas (Davis and Huffman 1977).

Analysis of stream water samples revealed 18 compounds (Table 3). Caffeine and sucralose, commonly used as domestic wastewater effluent tracers (Soh et al. 2011), were present in all samples, and erythromycin and celecoxib were the least prevalent. Half of the compounds identified in samples taken below WWTPs were not detected in the single upstream sample, and concentrations of all but two of the other chemicals were lower at the upstream site than the downstream sites. However, it is important to point out there was only one upstream site analyzed relative to four downstream sites. Contrary to expectations, acetaminophen and caffeine were about three times higher at the upstream site 5 than the downstream sites (1–4), except for site 1, where caffeine was about seven times higher (Table 3). CEC analyte concentrations were an order of magnitude less than those currently reported to impact fish health (Fick et al. 2010). Streams were surveyed in a suburban region within the Houston Metropolitan Area that has lower population density (ca. 237/km^2) than the city center (1,505/km^2); therefore, concentrations of pharmaceuticals and other CECs should be higher in downstream reaches where population densities and accumulations of WWTP effluents are higher.

Table 1 Morphological and reproductive values (Mean ± SD) for mosquitofish collected during May and August, 2010, from stream locations upstream and downstream from wastewater treatment plants Houston Metropolitan Area

Sex	Variable	Site	May				August			
			n	Upstream	n	Downstream	n	Upstream	n	Downstream
Males	Standard	1	10	22.1 ± 1.4 A[a]	7	19.9 ± 1.8 B	4	17.8 ± 1.9	10	19.8 ± 1.9
	Length (mm)	2	10	20.6 ± 0.8	8	20.9 ± 1.6	3	17.1 ± 0.5	9	18.5 ± 1.0
		3	10	19.0 ± 2.4	10	19.0 ± 2.7	10	18.7 ± 1.0	10	18.0 ± 1.7
		4	10	19.7 ± 0.6 A	10	18.8 ± 0.9 B	10	20.7 ± 1.5 A	6	19.1 ± 1.0 B
		5	10	19.0 ± 1.3	10	19.2 ± 1.6	5	17.5 ± 1.7	3	17.8 ± 1.9
	Carcass	1		0.04 ± 0.01 A		0.03 ± 0.01 B		0.02 ± 0.01		0.03 ± 0.01
	Weight (g)	2		0.03 ± 0.01		0.03 ± 0.01		0.02 ± 0.0		0.02 ± 0.0
		3		0.02 ± 0.01		0.02 ± 0.01		0.02 ± 0.0		0.02 ± 0.01
		4		0.03 ± 0.0		0.02 ± 0.0		0.03 ± 0.01 A		0.02 ± 0.01 B
		5		0.02 ± 0.0		0.02 ± 0.01		0.02 ± 0.0		0.02 ± 0.01
	HSI (%)	1		0.9 ± 0.7		2.0 ± 2.1		1.4 ± 1.2		2.0 ± 1.3
		2		0.8 ± 0.8		1.1 ± 1.0		3.4 ± 1.3 A		1.3 ± 0.5 B
		3		2.0 ± 1.0		0.8 ± 0.9		2.6 ± 1.4		3.4 ± 1.2
		4		0.7 ± 0.8		0.9 ± 0.8		1.0 ± 0.6		2.5 ± 2.2
		5		1.6 ± 1.8		1.4 ± 1.2		2.7 ± 2.4		3.4 ± 1.5
Females	Standard	1	10	32.0 ± 2.6 A	10	24.9 ± 3.1 B	10	25.2 ± 1.7 B	10	29.5 ± 1.9 A
	Length (mm)	2	10	27.6 ± 2.2 B	10	31.6 ± 4.1 A	10	24.0 ± 3.1	10	26.3 ± 3.4
		3	10	23.7 ± 4.3	10	26.2 ± 4.2	10	24.1 ± 2.8	10	25.1 ± 2.2
		4	10	27.6 ± 3.2	10	27.6 ± 4.7	10	26.3 ± 2.6	10	27.0 ± 1.6
		5	10	30.6 ± 3.9 A	10	27.2 ± 2.2 B	10	20.1 ± 2.6	9	17.3 ± 1.4
	# Embryos	1		44.4 ± 19 A		22.9 ± 13.3 B		7.5 ± 1.1 B		14.3 ± 6.3 A
		2		12.9 ± 4.7 B		23.0 ± 12.0 A		7.2 ± 3.8		6.3 ± 2.7
		3		27.1 ± 18.2		20.3 ± 9.9		8.7 ± 3.6		12.4 ± 4.6
		4		16.0 ± 6.6		17.8 ± 7.9		8.1 ± 0.3		8.6 ± 1.9
		5		24.9 ± 9.6 A		16.4 ± 6.2 B		5.6 ± 2.6		4.9 ± 3.1
	Carcass	1		0.12 ± 0.02 A		0.06 ± 0.03 B		0.07 ± 0.01		0.12 ± 0.03
	Weight (g)	2		0.09 ± 0.02		0.13 ± 0.05		0.06 ± 0.03		0.08 ± 0.03
		3		0.06 ± 0.03		0.08 ± 0.04		0.06 ± 0.02		0.07 ± 0.03
		4		0.09 ± 0.03		0.05 ± 0.05		0.07 ± 0.02		0.08 ± 0.02
		5		0.11 ± 0.05		0.08 ± 0.02		0.03 ± 0.01 A		0.02 ± 0.01 B
	GSI (%)	1		29.5 ± 5.3		25.7 ± 8.3		5.2 ± 5.1 B		13.4 ± 4.7 A
		2		14.0 ± 6.7		17.0 ± 10.0		15.5 ± 5.5 A		7.9 ± 3.2 B
		3		22.0 ± 21.4		17.9 ± 9.4		12.0 ± 9.5		11.2 ± 5.6
		4		5.3 ± 6.5		7.7 ± 10.7		47.4 ± 53.7		11.5 ± 6.6
		5		19.8 ± 9.0 A		10.2 ± 8.6 B		9.0 ± 4.4		4.2 ± 4.0

The n values for sites 1–5 are the same for all the variables

[a] Letters indicate statistically significant mean differences (A > B) between upstream and downstream locations at the respective site

Although mosquitofish reproductive and growth parameters have been shown to be sensitive to diverse chemical pollutants (Batty and Lim 1999); lack of detection of responses by mosquitofish to WWTP effluents in this study also could have been influenced by various other factors, including dispersal. These small fish are capable of moving distances of 250–450 m across series of pools and riffles in small streams during their short life span (Rehage and Sih 2004), which would confound exposure in relation to stream location. Also, mosquitofish reproductive indices are influenced not only by pollutants, but also by natural environmental variation and successive broods during the summer reproductive season may contain fewer embryos (Hughes 1985). Finally, downstream reaches located near the city center probably have higher CEC concentrations and could yield different results. Future research should

Table 2 Summary of histological observations (number and percent of total examined)

Tissue	Observation	Male (n)	Female (n)	Immat. (n)	% total	Additional observations
Brain	Heterophils (ventral, rostral)	3	4	1	44.4	
Gills	One single-celled parasite	2	5		38.9	one male with multiple
Stomach	Cestode		1		5.6	
Intestine	Cestode		1		5.6	
Swim bladder	Partially collapsed by parasites		1		5.6	
Spleen	Heamosiderosis	1	2	1	22.2	
Pancreas	Large histiocytic nodule		1		5.6	
Pancreas	Increased heterophils			1	5.6	
Liver	Glycogen present	5	8	2	83.3	5 indiv. with intracellular parasites
Ovary	Very large to small follicles		8		44.4	
Testis	Active spermatogenesis	5			27.8	
Muscle	Extensive degeneration and heterophil infiltration	1	1	1	16.7	2 samples with a single parasite in muscle cavity
Bone	Possible parasites in cartilage		1		5.6	
Skin	Thickening and increased heterophils	1	3		22.2	
Caudal fin	Fin damaged		7		38.9	

n = 18 (ten females, six males, two immature)

Table 3 Concentrations of contaminants of emergent concern detected in water samples collected from suburban Houston streams at locations below (sites 1–4) and above (site 5) WWTP discharges

Analyte	Use	LOD[a] (ng/L)	MDL[a] (ng/L)	Downstream				Upstream
				Site 1	Site 2	Site 3	Site 4	Site 5
Diphenhydramine	Allergy drugs	0.11	0.22	7.7	46	58	5.7	0.60
Codeine	Analgesic	3.5	8.3	94	<MDL	–	–	–
Carbamazepine	Analgesic/ antiepileptic	0.14	0.53	–	220	140	160	11
Acetaminophen	Analgesic/ antiinflammatory	1.0	2.9	–	–	10	13	31
Diclofenac	Analgesic/ antiinflammatory	0.96	2.8	43	17	37	–	–
Erythromycin	Antibiotic	2.7	8.6	–	–	32	–	–
Sulfamethoxazole	Antibiotic	0.50	1.3	910	870	1,400	18	4.8
Trimethoprim	Antibiotic	0.35	1.3	43	18	46	–	–
Warfarin	Anticoagulant	0.26	0.78	13	0.96	1.4	–	–
Celecoxib	Antiinflammatory	5.17	11	140	–	–	–	–
Propranolol	Antihypertensive	0.90	1.8	–	20	22	–	–
Atenolol	Antihypertensive	1.4	4.3	45	100	140	–	3.8
Diltiazem	Antihypertensive	0.07	0.24	1.9	16	36	4.1	0.82
Sucralose	Artificial sweetener	8.2	36	630	890	1,000	830	220
Methylphenidate	CNS stimulant	0.25	0.30	–	0.56	1.0	–	–
Gemfibrozil	Lipid regulator	0.78	2.1	–	140	48	7.2	2.2
Diazepam	Psychiatric drug	1.7	4.6	–	–			–
Caffeine	Stimulant	1.6	4.5	1,100	42	47	40	140

Values are means of two samples reported as ng/L

[a] LODs and MDLs are from Du et al. (2014)

compare fish caged on site for a given period of time and fish collected over longer stream gradients that encompass a succession of WWTP discharges and stronger gradients of CEC concentrations.

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