FUTURE WATER QUALITY CHALLENGES TO AQUACULTURE AND INFLUENCES ON PRODUCT SAFETY

A Dissertation

by

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This dissertation meets the standards for scope and quality of
Texas A&M University-Corpus Christi and is hereby approved.

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ABSTRACT

Concerns about water quantity and quality are increasing due to climate change and population growth. Climate change is driving changes in evapotranspiration and precipitation patterns. This is exacerbated as population growth, particularly in arid and semi-arid regions, increases water extraction and consumption. After human consumption, water is treated and discharged to the environment, but generally at lower quality than what was originally extracted. This could cause trouble for consumers of surface waters. One such consumer is the aquaculture industry, which is growing to support human protein consumption demands, while depending on surface water.

Aquaculture is growing both globally and within the U.S. (worldwide 9.2% yr^{-1} 1990-2000 & 6.2% yr^{-1} 2000-2012). Freshwater aquaculture in the U.S. is largely dependent on surface water (80.78%) compared to ground water sources (19.32%). Surface water sources are increasingly dominated or dependent on treated wastewater effluent, potentially influencing downstream uses. Wastewater effluent generally contains trace levels of anthropogenic compounds, typically referred to as contaminants of emerging concern (CEC), for which our knowledge of their impacts is still evolving. Therefore, the introduction of CEC in aquaculture from surface waters influenced by wastewater effluent is a potential concern for cultured fish health as well as for humans when consuming farmed fish.

Studies were conducted to improve our understanding of future water resource quality and quantity in relation to the aquaculture industry and safety of farmed fish. Initially, wastewater effluent data was collected (e.g., USGS), consolidated, and analyzed (e.g., GIS) to understand its

influence on surface water quantity and quality, which was utilized to project potential future water quality and quantity scenarios in the USA and its potential effect on aquaculture. This was followed by laboratory-based studies to quantify the bioaccumulation and depuration in tilapia of diltiazem, an ionizable calcium channel blocker, and GenX, a perfluorinated compound, at environmentally relevant concentrations.

To broadly examine the extent of U.S. surface waters to dilute wastewater treatment plant (WWTP) effluent, data from wastewater discharge and surface flow from 2007-2017 was used to calculate a WWTP wastewater dilution factor (WWDF) within United Sates Geological Society (USGS) hydrologic unit code (HUC). A WWDF less than 10 indicates poor quality water when classified on <1 to >100 DF scale. The 4 HUCs with the lowest WWDF (i.e., <2) were located in the West or Southwest U.S. and were among the 10 HUCs with the highest proportional population growth from 2010-2016, with similar projections for the future. To identify the end water user impact, U.S. aquaculture farm area with WWDF < 2 was mapped. It was quantified at ~ 2.71% of total freshwater area, out of which 69% and 44% of the area was occupied by aquaculture farms with 100- and 1000-acre areas, respectively.

Water availability for the contiguous U.S. was estimated for each HUC during 2015 using a model developed from the earlier analysis of water quantity and quality in the U.S. The Mississippi River generally served as a dividing line for surface water availability, with five of the six HUC regions with very low water availability (<24,000 L/D/Km^2) residing in the west. These same areas also experience more drought as well as more severe droughts than regions in the east. In regions with lower surface water flows, water quality is more susceptible to the influence of wastewater effluent discharge, especially near large and growing population centers like San Antonio, Texas. A prediction model was established for this city, which found that from 2009-2017 wastewater effluent increased by 1.8%.

Diltiazem (DTZ) bioconcentration and depuration in tilapia was examined using a controlled time sequence (max time = 96 hr) exposure (1 µg L^{-1}) and non-exposure (max time = 96 hr) in freshwater. Fish carcass, blood plasma, liver, and muscle were analyzed in both exposure and depuration phases. Diltiazem bioconcentration was greatest in liver> plasma> carcass> muscle. Depuration rates were greatest for liver> carcass> plasma> muscle. The biological half-life ($t_{1/2}$) indicates that DTZ took the longest to depurate from muscle and least from the liver, which is similar for the stable bioconcentration factor (BCF_a) value order. The $t_{1/2}$ of DTZ in tilapia muscle was 18.8 hrs, indicating the compound is processed relatively quickly. Based on the 96 hr DTZ uptake by tilapia fingerlings in this study, human exposure to the highest DTZ muscle concentration would be ~6 orders of magnitude below the lowest daily human therapeutic (120 ppb) dose, resulting in very low human exposure.

GenX (ammonium 2,3,3,3-tetrafluoro-2- (heptafluoropropoxy)) bioconcentration and depuration in tilapia was examined using a controlled time sequence (max time = 96 hr) exposure (1 µg L^{-1}) and non-exposure (max time = 96 hr) in freshwater and brackish water (16 ppt). GenX bioconcentration (BCF_a) was greatest in plasma > liver > carcass > muscle, with higher distribution in liver compared to carcass and muscle. Bioconcentration in all tissues examined increased with increasing salinity, raising concern for euryhaline organisms. Muscle was found to have the highest $t_{1/2}$ followed by carcass, plasma, and liver. The rate of uptake and depuration was affected by salinity. Fish muscle (fillet) GenX concentration at 96 hrs at 0 ppt was 0.14 ppb whereas at 16 ppt it was 0.312 ppb. Therefore, a fillet serving size of ~3.5 oz (100 g) would contain 14.0 µg GenX from freshwater fish and 31.2 µg GenX from saltwater (16 ppt) fish. This would result in a single serving exposing a person to more than the subchronic oral reference dose of 0.2 ppb as recommended by U.S. EPA.

Water quality is a growing concern along with changing climate and increasing population. The projections and improved bioaccumulation models for farmed fish from this research will provide aquaculturists with knowledge to make pro-active management decisions regarding water quality in the future, while improving our general understanding of human exposure to CEC from nontraditional water use. It also helps to understand environmental exposure and ecological impacts of pharmaceuticals and other industrial chemicals for sustainable management of environmental quality, particularly in urbanizing ecosystems.

DEDICATION

This dissertation is dedicated to my husband, Muhammad Imran, family, friends and teachers who helped me make it a success.

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Abstract

Wastewater discharge and surface flow data from 2007-2017 was used to calculate wastewater dilution factors (WWDF) for U.S. Geological Society hydrologic unit codes (HUC) in the contiguous U.S. HUC 10-year average WWDF values generally increased from the east coast (HUC 1-3: WWDF range 125-466) as you move west to the Mississippi River (HUC 7, 8, & 10: 1435-1813) before further declining moving west (HUC 13-18: 7-908), particularly in California (HUC 18: 9) and the southwestern states (HUC 13-16: 7-351). Within HUCs, watersheds with higher population centers had lower WWDF values. This population effect on WWDF was greater in drier regions (e.g., Southwestern U.S.) or during drought. This is particularly pronounced in the regions of the Southwest and West where populations are growing in an already water limited region. Moderate WWDF improvement was observed and projected through 2022 in these regions. A few areas of the country where surface water is used for aquaculture overlap with areas of low (<2) WWDF, but it is not widespread for the period examined. With continued population growth and the intensification of climate change, the proportion of treated wastewater effluent in surface waters may grow and potentially influence users of those surface waters, although over the 10-year period examined WWDF values were relatively stable or improving for most regions of the contiguous U.S.

1. Introduction

Food security along with a supply of contaminant-free fresh water is a growing challenge for present and future human populations. Food security is a complex issue requiring political, economic, and social actions relating to consumer demand, supply, and nutrition (Grafton et al., 2015). According to the World Resource Institute (WRI), animal-based food demand is projected to increase 70% from 2010 to 2050 (Searchinger et al., 2018). As a food resource, fish consumption grew from 9 to 19 kg/capita/year between 1961 and 2011 (Food and Agriculture Organisation STAT, 2015.) with an expected increase to 22 kg/capita/year by 2024 (Food and Agriculture Organisation (FAO), 2015). This increased demand is already stressing wild fish stocks and driving aquaculture growth (Bell et al., 2011; Naylor et al., 2000). The expected growth in freshwater aquaculture will require more water extraction and consumption, adding another layer of stress on already tight water resources in some regions.

Fresh water constitutes only 2.5% of total water globally, out of which only about 1% is accessible for direct human use, making it scarce in many parts of the world (Liu et al., 2016). This is exacerbated when population growth occurs in arid and semi-arid regions, such as Texas, Southern California, and the U.S. southwest (U.S. Census Bureau, 2011). Limited water resources due to drought and climate change are only part of the problem. Everyday products used by people for their health, such as medicines, or lifestyle contain chemicals that may enter the environment through various pathways including treated wastewater effluent. Once in the environment, these chemicals at low concentrations are commonly referred to as "Contaminants of Emerging Concern" (CEC) (Field et al., 2006). CEC coupled with traditional pollutants (e.g., nutrients, pesticides, metals) and tighter water supplies are a concern for future water resources. According to the National Water Quality Inventory report of 2004, roughly 44% of stream miles, 64% of lake acres, and 30% of bay and estuary square miles in US waters are currently not safe for fishing and swimming (USEPA, 2009). Human population growth adds another layer of complexity to water resource management (Dias et al., 2015; Leite et al., 2011). Population growth may cause increased water withdrawals for various activities (United States Environmental Protection Agency (USEPA), 2011) and subsequent lower quality wastewater discharges. When coupled with climate change, this results in an increased annual mean discharge, flooding, drought, and many other environmentally related issues affecting water quality and quantity (Bosch and Hewlett, 1982; Brown et al., 2006; Chang, 2004; Hoyer and Chang, 2014; Kim et al., 2013; Montenegro and Ragab, 2012; Oki and Kanae, 2006; Tu, 2009; Wada et al., 2011). It has been determined that in response to increasing climate change, but without climate adaptations, per capita water demand will increase by 10.6% and 4.8% in suburban and urban areas, respectively, for the greater Portland, Oregon area by mid-21st century (i.e., 2035–2064; Parandvash and Chang, 2016).

When there is a higher proportion of wastewater treatment plant (WWTP) effluent in surface waters than the baseflow, it is considered a low wastewater dilution (WWD) condition. This low WWD water may then be used for various purposes, such as drinking water supply, aquaculture, and agriculture. As a result, humans may be directly exposed to CEC in water supplies or indirectly through consumption of farmed fish and crops grown using this water (Boone et al., 2019; Goñi-Urriza et al., 2000; Han et al., 2006; Rice and Westerhoff, 2015). This raises a need to explore wastewater dilution ratios across the contiguous U.S. to create a risk assessment for policy makers. This was accomplished by calculating wastewater dilution factor (WWDF) from 2007-2017 as well as projections for the next 5 years (2018-2023) at the regional scale (i.e., United

States Geological Survey (USGS) Hydrologic Unit Code (HUC)) for the contiguous U.S. The results were then used to discuss the potential impacts of low WWDF and implications for end-users, such as aquaculture farms.

2. Material and Methods

2.1 *Modeling approach*

Effluent data from approximately 15,800 wastewater treatment plants (WWTPs) was collected from the USEPA ECHO (Environment and Compliance History Online) database (USECOEPA, 2018). This database excludes WWTPs that discharge into the ocean or groundwater. Streamflow data was downloaded from the USGS and National Hydrography Data set Plus (NHDPlus) (National Hydrography Dataset Plus (NHDPlus), 2018; USGS, 2018). All data utilized was at the county level for 2007 through 2017. The two datasets were combined, and 10-year mean streamflow was calculated in R (version 3.3).

WWTP and USGS gauge locations were joined with the spatial join tool in Arc GIS (version 10.4.1). Hydrography layers were obtained from NHDPlus Version 2. Coordinates for each WWTP and USGS gauge location were input into Arc GIS as a point vector layer and each WWTP outfall was spatially matched with a stream and verified by their Reach Code. Annual WWTP effluent data was consolidated with USGS stream data (monthly by county). This data was then used to calculate WWDF for all 18 HUCs within the contiguous U.S. using the calculator function in Arc GIS:

$$WWDF = \frac{Qp + Qr}{Qp} \times a$$

Where,

Qp = WWTP Annual Average Flow (cubic feet per second; CFS)

Qr = River Annual Average Flow (CFS)

a = 0.9 (Factor to reserve 10% of river's assimilation capacity for future use; USEPA, 2018)

HUC are USGS labels given to regions that represent the combined drainage area of a series of rivers or a major river. The 18 regions in the contiguous U.S. start with 1 in New England and move west with California being 18 (Seaber et al., 1987; USGS, 2018). Assimilative capacity is defined as the maximum daily amount of a contaminant that a water body can receive without a negative impact (Landis, 2008). Therefore, higher dilution lowers contaminant concentrations and reduces ecological effects (Rice and Westerhoff, 2017).

2.2 HUC WWDF and projections

HUC from 2007-2017 were sorted into 6 ranges based on WWDF from ≤1 to ≥100 based on similar, previously published research (Fig. (1)- 1A; Rice and Westerhoff, 2017). Using this data, WWDF projections were developed using the "ARIMA" model in R (version 3.6.1). The ARIMA model uses autocorrelation function (ACF) and partial autocorrelation (PACF) values and is verified through the Box-Ljung test to fit residue p-values higher than 0.05 for each model. This model is applicable and limited to time series data that are stationary (i.e., mean, variance, and autocorrelation should be approximately constant through time).

2.3 Precipitation data

Precipitation data (2007-2017) was downloaded from the PRISM Climate Group at Oregon State University (Climate Group, 2018) and imported into Arc GIS as a raster layer. The cell statistics tools from spatial analyst toolbox were used to calculate median rainfall over this 10-year period. Regions with the lowest WWDF (i.e., 10, HUC 13, 15, 16 and 18) were masked and a zonal calculator was used to extract data and calculate statistics of mean, median, and standard deviation for these regions.

2.4 Population assessment

U.S. population data from 1995-2015 was collected from the US Census Bureau (United States Census Bureau (USCB), 2018). The population data were examined for the last year available (2015-2016) as well as short-term (2010-2016) trends. Based on 2010-2016 U.S. population data by county, growth as percent change was calculated using the following formula:

$$\frac{Population(Present) - Population\ (Past)}{Population(Past)} \times 100$$

Contiguous U.S. population projections were taken from Foti et al. (2010).

2.4 Total and public supply water withdrawal

Water withdrawal analysis was performed in Arc GIS with USGS Water Use data (1995-2015) (USGS, 2018). Data were analyzed at the county level to examine total and public water withdrawals at 5-year intervals for the contiguous U.S.

2.5 Aquaculture

Aquaculture data was collected from the USGS Water Use data for the most recent year (i.e., 2015, USGS Science Base-Catalog) and most recent aquaculture census release (2013) from USDA (United Stated Department of Agriculture (USDA), 2018). Data used included total aquaculture surface freshwater withdrawal for the contiguous U.S. and the number of freshwater aquaculture farms and their area in each state. Data for the regions of interest (4 least diluted HUCs) was extracted and used to calculate the farm area percentage share compared to other regions in the contiguous U.S.

3. Results and Discussion

3.1 Decadal trends in wastewater dilution

The 10-year WWDF average for each HUC generally increases from east to west until the central U.S. near the mainstream of the Mississippi River (Fig. (1)- 1B). The subdivision ranges used to define WWDF were <1, 1-2, 2-10, 10-50, 50-100 and >100 as modified from Rice and Westerhoff (2017). WWDF are lower in west of the Mississippi River area, particularly in southern HUCs (predominantly WWDF <10). The exception to this is HUC 17 that covers the Pacific Northwest. Of the nine HUCs with the lowest 10-year average WWDF >50, six (HUC 2, 3, 12, 15, 16, & 18) exhibit an increasing WWDF trend (+5.5 – 9.2% annually), two (HUC 14 & 1) exhibit a slight WWDF decrease (~ -1.0% annually), and one (HUC 13) declined more dramatically. Despite this negative trend, HUC 13 exhibits a levelling off, thus indicating improvement after a multi-year drought in the first half of the 2010s (Fig. (1)- 2A). For HUCs with the highest 10-year average WWDF, six (HUC 4, 5, 6, 8, 10, & 17) had annual change trends from -2.0 to +2.2% indicating they are relatively stable (Fig. (1)- 2). However, HUC 7 and 11 exhibited more pronounced decreasing trends of -4.6% and -5.5%, respectively. For HUC 11, it was affected by the same multi-year drought of the early 2010s as HUC 13. In general, decadal trends at the HUC level indicate that regions with lower WWDF values, except HUC 13 are relatively stable to improving (i.e., greater WWDF values), while regions with higher WWDF values are stable.

When examining WWDF at the watershed scale (Fig. (1)- 1A), there is considerable variation within HUCs. In 2017, ~75% of the contiguous U.S. has a WWDF >100 (Fig. (1)- 1A), but lower values are found near population centers and regions with drier climates (Fig. (1)- 3). This is evidenced by small areas of WWDFs <10 (8% in year 2017) stretching up the coast from southeast

Virginia through New England, but also at major metropolitan areas of the Eastern and Midwest U.S. West of the Mississippi River, particularly from West Texas to California, there are large areas with WWDF <10. In the Southwest and West, the drier climate coupled with moderate (e.g. El Paso, TX and Albuquerque, NM) and large (e.g. Los Angeles, CA and Phoenix, AZ) population centers align with lower WWDF values over broader areas than those observed on the East Coast.

3.2 HUCs with low WWDF

HUCs with a 10-year average WWDF >10 and <100 were 13 > 16 > 18 > 15, which is in agreement with the analysis of Rice and Westerhoff (2017) for 2008 (Appendix A, Fig. S1). While there is annual variation, WWDF increased in HUC 15, 16 and 18 (Fig. (1)- 2). These gains were modest for HUC 15 and 18, increasing their WWDF from 5 to 9 and 2 to 17, respectively, over the 10-year period. The increase in HUC 16 was greater, going from 34 to 116. The overall improvement in HUC 15, 16, and 18, while seemingly small, is notable due to the growing populations in these regions and strain on existing water resources, particularly in HUC18, which covers most of California. Of these four HUCs, 13 exhibited the only decreasing trend (Fig. (1)- 2).

HUC 13, which is the Rio Grande catchment, covers the majority of New Mexico, a small portion of south-central Colorado, and parts of West Texas. Significant population centers in this region include Albuquerque, Santa Fe, Las Cruces, El Paso, Laredo, and the Rio Grande Valley of South Texas. This is one of the drier regions of the U.S., receiving between 400 to 700 mm of precipitation annually. This dry climate coupled with continued population growth (6% as of 2010-2016; Fig. (1)- 4), despite a relatively low population density (7.2 people per km^2 in 2016), places stress on the region's water supply and results in its low WWDF. The WWDF in HUC 13 declined from 2007 to 2013, before starting to improve through 2017 (Fig. (1)- 2). The decline is positively correlated with precipitation ($r = 0.38$, $p < 0.05$), which is demonstrated by a prolonged drought over most of the region from 2011 to 2014 (National Drought Mitigation Centre, 2018). As the drought passed, the WWDF trajectory improved. However, the prolonged drought of the early 2010s appears to heavily influence the short-term WWDF projections, which predict a continued decline in WWDF, despite improvement since 2014 (Fig. (1)- 2A). Water use is an additional variable that may influence WWDF, but it remained relatively stable over the period of study (Fig. (1)- 4A).

HUC 15 covers most of Arizona with some parts of Nevada (Fig. (1)- 1). Its WWDF range was 2 to 12.5 between 2007 and 2010, the lowest among all the HUCs (Fig. (1)- 2). The region has experienced a population growth rate of 8% from 2010 to 2016 (Fig. (1)- 5). Most of this region is the state of Arizona, which is the 14th most populated state (US Census Bureau, 2019), however public supply accounts for only 29% of water usage, with the majority (70%) used for irrigation (USGS, 2018). This is occurring in a region that averaged 441 mm of rainfall for the 10-year period. This region experienced drought from 2011-14 and abnormally dry in 2011-12 (National Drought Mitigation Centre, 2018). Despite a growing population and the prevalence of drought, this region exhibited an improved WWDF across the period of study, albeit at a moderate rate. Projections imply that this trend will continue, but that WWDF will not reach 20 prior to 2022 (Fig. (1)- 2B). The population is growing in HUC 15, but public water use (average 576 MGD) remains relatively flat, while total water use is decreasing over the last 20 years ($R^2 = 0.44$) indicating that water conservation is occurring (Fig. (1)- 4B). Fortunately, total water use in this HUC has decreased from 4,071 MGD in 1995 to 3,271 MGD in 2015. Despite this improvement, water management models predict that current supplies will not meet the increasing demand by 2024 and that an additional 2.46 × 108 m^3 per year of water will be needed annually to meet the growing demand (Ranatunga et al., 2014). To meet the growing water demand, agencies such as the Arizona Department of Water Resource (ADWR) are now utilizing reclaimed water or effluent water, which makes up 2% of their water source (ADWR, 2015).

HUC 16, comprised mostly of Nevada and western Utah, has the third highest population growth rate among the 18 HUCs (Fig. (1)- 4). Although, the public supply only consumes 18% of the water in this region, this proportion increased 62% over the last 20 years (Fig. (1)- 4C). This is one of the drier regions of the U.S., with an average annual precipitation total of 259 mm during the 10-year study period, which makes this the lowest precipitation region within all HUCs of the U.S. (Fig. (1)- 3). Precipitation is likely a major driver of WWDF, which was <100 in this region for all but two years (2011 and 2017) during the period examined. The WWDF values at the start of this period were initially low due to prolonged dry and drought conditions that persisted from 2007 through 2010 (National Drought Mitigation Centre, 2018; US Census Bureau, 2019). This was followed by a non-drought year in 2011 and then additional abnormally dry and drought years through 2015 until precipitation improved from 2016-2017. The WWDF values for HUC 16 (Fig. (1)- 2C) follow this same trend as rainfall and are significantly correlated ($r = 0.43$, $p < 0.05$). The variation in WWDF (Fig. (1)- 2C) demonstrate that this region is susceptible to issues described below (next paragraph and section 3.3) associated with low wastewater dilution during periods of low rainfall. By 2065, water demand in HUC 16 is projected to increase by 85% (United States Environmetnal Protection Agency (USEPA), n.d.). The Colorado River with Lake Mead and other perennial rivers are major sources of surface water in this region and provide 70% of Nevada's total water supply. Due to a drought condition started in 2000 around the Colorado River watershed, water levels in Lake Mead dropped to 40% of storage capacity from 2012-2014 (United States Environmetnal Protection Agency (USEPA), n.d.). After which the Nevada Water Authority and USEPA took various conservation measures. As a result of the conservation efforts, the Las Vegas Valley helped reduce the region's Colorado River water consumption by 28 billion gallons annually between 2002 and 2017, even as the population increased by 660,000 residents (LVWD, 2018). Nevada and Utah were among the 10 fastest growing states at 12 and 11%, respectively, from 2007-2017. Growth coupled with frequent droughts, suggests that this region may have prolonged low WWDF despite the positive projections over the next 5 years (Fig. (1)- 2C).

HUC 18, which is mostly California, had an average WWDF of 9 from 2007-2017, which was the second lowest among HUC. The region's WWDF ranged from 2-18 over the decade, which correlated ($r = 0.38$, $P < 0.05$) with the widespread and prolonged drought from 2009-2014 (Californian Department of Water Resources (DWR), 2014; USGS, 2018). In particular, 2014 was the driest year on record in California, (National Drought Mitigation Centre, 2018) which is visible in Fig. (1)- 2D, where WWDF declines through 2015. Since this low point, this region's WWDF has improved as the drought receded, despite the 3rd highest total population increase (2.02 million) between 2010 and 2016 (Fig. (1)- 4). This may be due to water conservation efforts in this region that have resulted in a moderate decrease in public water usage, but a much larger drop in total water use (Fig. (1)- 4D). HUC 18 reduced its total daily water withdrawals (22,334 to 8,738 MGD between 1995 and 2015) by more than the total daily water used in HUC 13, 15, and 16 combined. Since the drought ended, California has instituted new statewide standards for water conservation to further decrease withdrawals (Californian Department of Water Resources (DWR), 2014). Even without accounting for these measures, the WWDF is projected to increase over the next 5 years (Fig. (1)- 2D).

3.3 WWDF and CEC

There is a direct link between CEC concentration and wastewater effluent in surface waters; as WWDF decrease, CEC concentrations increase (Rice and Westerhoff, 2017). The impacts of increasing CEC in aquatic systems is difficult to broadly determine due to the diverse nature of these chemicals and a wide array of environmental factors. However, there are many known effects of CEC that could be exacerbated due to low wastewater dilution. A nationwide study performed by USEPA in 2017 to identify CEC in U.S. drinking water systems and their source waters, where they sampled 29 drinking water treatment plants (DWTPs) from 24 states. Locations are anonymous, which limits the ability to compare their specific data with WWDFs in this work (Glassmeyer et al., 2017). However, in general at least 25 of the 331 target analytes were quantified at each location, demonstrating the presence of these chemicals in surface and drinking water systems. To demonstrate the relationship of CEC with WWDF, examples from the four least diluted HUCs are described below.

CEC are a broad class of chemicals and our understanding of their environmental impacts is still evolving, however, it is clear that CEC from wastewater effluent can negatively influence downstream environments. A few of the documented occurrences include estrogenic activity that induced oxidative stress in fathead minnows (*Pimephales promelas*), endocrine disruption in rainbow trout (*Oncorhynchus mykiss*), and decreased mate availability due to reduced reproductive fitness in male fathead minnow (Ekman et al., 2018; Lin and Reinhard, 2005; Martinović et al., 2007; Xie et al., 2004). While these effects are largely on aquatic organisms in the receiving waters, surface waters often have numerous human uses where poor water quality due to reduced wastewater dilution could have consequences.

The Santa Ana River in Southern California (HUC 18) receives WWTP effluent resulting in CEC contamination that impacted fish endocrine and reproductive systems (Lin and Reinhard, 2005; Williams, 2005; Xie et al., 2004). Examples of CEC in the Santa Ana River include alkylphenol polyethoxycarboxylates (APECs) and carboxylated APECs (CAPECs) ranging from 1.8–18.7 mg/L, as well as ibuprofen and its metabolites, tris-chloropropyl phosphate (TCPPs), and N-butyl benzenesulfonamide (NBBS) (<0.5 μg/L) (Gross et al., 2004). The Sacramento-San Joaquin Delta forms at the confluence of the Sacramento and San Joaquin Rivers and receives 80% of its water from the Sacramento River (Californian Department of Water Resources (DWR), 2014; Weston and Lydy, 2010). These two rivers receive an average of 22.3 cubic meters per second (cms) of WWTP effluent from roughly 5.2 million people living in the watershed. This wastewater and the runoff from urban and agricultural areas has contaminated the delta with pesticides and CEC (Weston and Lydy, 2010). Examples include pyrethroid insecticides, N-nitrosodimethylamine (NDMA) precursors and the artificial sweetener sucralose whose concentrations were higher downstream of WWTPs than upstream (Lee et al., 2015). This contamination raises concern towards the use of reclaimed water (Loraine and Pettigrove, 2006). These and other major freshwater resources in California are already exploited to their ecological and physical limits, which has resulted in legal mandates for water suppliers to evaluate desalination and recycling options to achieve their water resource management goals (Californian Department of Water Resources (DWR), 2014; Cooley et al., 2013; Gleick and Palaniappan, 2010).

Lake Mead located outside of Las Vegas, Nevada, is a drinking water source and receiving basin for treated wastewater. Together with the lower Colorado River it provides water to more than 30 million Americans (LaBounty and Burns, 2005). In 1996, the first report occurred of fish below wastewater outfalls in Lake Mead being contaminated with CEC (Bevans et al., 1996). Likewise, the disinfection by-product NDMA was found in the Las Vegas Wash (LVW), which is a hydrographic basin that drains the Las Vegas Valley (3998 km^2) in a 20 km channel that feeds into Lake Mead (Gautam et al., 2014). NDMA (range 300-350 ng/mL) was noted at seven sites (Woods and Dickenson, 2016). Goodbred et al. (2015) reported high concentrations of perchlorinated bisphenols (PCBs), dichlorodiphenyltrichloroethane (DDTs), polybrominated biphenol ethers (PBDEs), galaxolide, and methyl-triclosan in wild male largemouth bass (*Micropterus salmoides*) of Lake Mead at Las Vegas Bay (LVB) in 2007-2008. Additionally, they found fish with higher Fulton condition factor, hepatosomatic index, and hematocrit, and lower plasma 11-ketotestosterone concentration (KT). Evidence of endocrine disruption in common carp (*Cyprinus carpio*) sampled from both LVW and LVB suggested a presence of CEC in the WWTP effluents (Bevans et al., 1996; Patino et al., 2003).

3.4 Water user impacts

Treated wastewater is an alternate source of water and has been used directly or indirectly by various sectors globally for drinking water, agriculture, and aquaculture (Pedrero et al., 2010; Rice and Westerhoff, 2015; World Health Organisation (WHO), 2006a, 2006b). The California State Water Project, the Santa Ana River, and Lake Mead are systems where *de facto* wastewater reuse is a portion of the water supply for tens of millions of people. These water sources are in the HUCs with the lowest WWDF, but this is also occurring in less water-stressed regions. The

Quinnipiac River in CT, Wabash River in IL, Schuylkill River in Philadelphia, PA, the Occoquan River in Washington, DC and the Ohio River in Cincinnati, OH are systems that supply water for large population centers, while also containing a known portion of wastewater effluent (Wiener et al., 2016). In addition to the above major rivers, there are other surface waters in the U.S. where *de facto* reuse of wastewater is occurring and impacting drinking water treatment plants (Rice et al., 2013; Rice and Westerhoff, 2015). While CEC are not typically regulated, reduced surface water quality from CEC contamination can increase treatment costs for end users depending on their needs (Heberling et al., 2015). As climate change intensifies and populations grow, regions with lower WWDF will also experience these impacts, if they are not already.

Wastewater effluent in surface waters is already influencing U.S. drinking water systems (Rice and Westerhoff, 2015). Therefore, it is also likely entering aquaculture farms in the U.S. that rely on surface waters. But unlike drinking water systems, there is little if any water treatment before fish are exposed to the potential contaminants found in this diluted wastewater effluent. In the U.S., it is doubtful that regulations will allow, or the public will accept, fish to be cultured in untreated wastewater even if this is occurring in other countries. However, there are indirect water sources that may allow wastewater to impact the aquaculture industry. The U.S. aquaculture industry obtained 81% of their water from surface freshwater sources (Maupin et al., 2014). If the surface waters used contain CEC, which many do, the cultured stock will be exposed. This could potentially impact productivity or lead to the accumulation of contaminants in the final product. In 2013, there were 2,256 aquaculture farms in the U.S., occupying 249,274 acres of land (United Stated Department of Agriculture (USDA), 2018). The total surface water withdrawal in 2015 for U.S. aquaculture was 5,943 million gallons per day (MGD) with ~10% (589 MGD) occurring in the 4 HUCs with the lowest WWDF (Appendix A, Fig. S2). With variation occurring within each HUC, it is important to compare locations of aquaculture surface water usage with WWDF values. For example, Fig. (1)- 6 shows watersheds with WWDF < 2, while Fig. S2 (Appendix A) depicts aquaculture surface water use by county. Comparing these figures shows that parts of southern California and central Texas exhibit low WWDF in areas where aquaculture is also using surface waters. These two areas are more water limited than many other parts of the country where aquaculture is practiced. With growing populations and the acceleration of climate change, low WWDF is something to begin discussing and researching with regards to the aquaculture industry.

Previous studies have already shown that various anthropogenic chemicals accumulate in farmed fishes: e.g., PCBs and PBDEs in salmon (Hayward et al., 2007; Hites et al., 2004; Montory and Barra, 2006), PBDEs and dioxin in catfish (Minh et al., 2007), dioxin and PCB in turbot (Blanco et al., 2007), and PCBs in sea bass (Carubelli et al., 2007). Although these chemicals have been detected in farmed fishes, the levels vary and may not be considered harmful. For example, PCB concentrations were found to be similar in farmed and wild-caught shrimp in the U.S., but when compared by continental origin, North America sourced shrimp had the highest total PCB concentration in uncooked warm-water shrimp, although the estimated PCB intake for humans was far below the maximum daily dose noted by the USEPA (Fillos et al., 2012). PFOAs have been detected in aquacultured tilapia (0.22-0.61 ppm), salmon (0.15-0.45 ppm), mussel (0.33-1.5 ppm), and clams (0.70-1.9 ppm) sampled at a fish market in China (Yin et al., 2019). Farmed European sea bass, gilthead seabream, and Mediterranean mussels contained PCBs, PAHs, and other organochlorine pesticides in Italy (Cirillo et al., 2016; Fillos et al., 2012). In Spain, bogue fishes (*Boops boops*) captured near urban sewage outfalls had the highest levels of persistent (polychlorobiphenyls (PCBs) and organochlorine pesticides (OCPs)), semi-persistent (bromodiphenyl ethers (BDEs), polycyclic aromatic hydrocarbons (PAHs)), and emerging pollutants (e.g., organophosphate flame retardants (OPFRs) and UV-filters) (Henríquez-Hernández et al., 2017). The same fish species captured near aquaculture pens contained 1-10 ng/g (ppb) lipid weight dioxin-like PCBs (Henríquez-Hernández et al., 2017). Juvenile chinook salmon collected from hatcheries in Oregon were reported to contain 39-760 ng/g (ppb) lipid weight basis PCB in whole body tissue (Johnson et al., 2007), although the USEPA limit is 0.5 ppm PCBs per liter of drinking water and the U.S. Food and Drug Administration (FDA) range is 0.2-3 (ppm) of total PCBs for food (Agency for toxic substances and disease registry (ATSDR), 2000). At this time, the overall impact of CEC contaminated WWTP effluent to aquacultured organisms worldwide and in the U.S. is largely unknown and may be highly variable. CEC impact on ecosystems and aquacultured organisms will vary depending upon the CEC and its concentration in WWTP effluent. Therefore, there is a need for more research to describe CEC prevalence in waters used for aquaculture and their potential to accumulate in cultured food organisms, as well as their effects on aquatic organisms and their ecosystems.

4. Conclusion and Implications

Four USGS hydrological unit codes (HUCs 13, 15, 16 and 18) had the lowest average wastewater dilution factor (WWFD) over the last decade and were in the top 10 U.S. regions for population growth from 2010-2016; a trend that is projected to continue. This growth will further increase wastewater effluent discharge volumes, which will result in greater wastewater proportions in surface waters. Therefore, more CEC and higher concentrations of these compounds are likely to occur. Downstream users of this water, whether they perform treatment or not, may need to consider the presence of CEC and potential effects depending on the intended water use. These considerations will be most important during abnormally dry and drought conditions, when surface-water dilution of wastewater is limited. This may require monitoring CEC, particularly those that pose a risk to humans and aquatic organisms, and possibly targeted treatment based on risk assessments.

Today, when discharging into surface waters, regulations (e.g., National Pollutant Discharge Elimination System) require that wastewater is diluted by a specified factor within a specified distance from the discharge point in order to minimize negative environmental effects. Currently, treated wastewater is not accepted for direct reuse in U.S. aquaculture, but *de facto* reuse is occurring. Regions with the greatest temperature increases, precipitation declines, and population growth will experience increasing wastewater effluent loading, resulting in CEC concentrations that may require assessment of exposures and possible impacts to farm-raised fish and humans. The present study examined data to assess trends in wastewater dilution as well as short-term projections that can be used by policy makers as well as stakeholders for planning. It is imperative to make informed projections of future water quality and quantity and determine the potential for CEC to bioaccumulate in farmed aquatic organisms in order to ensure their continued health benefits for human consumers.

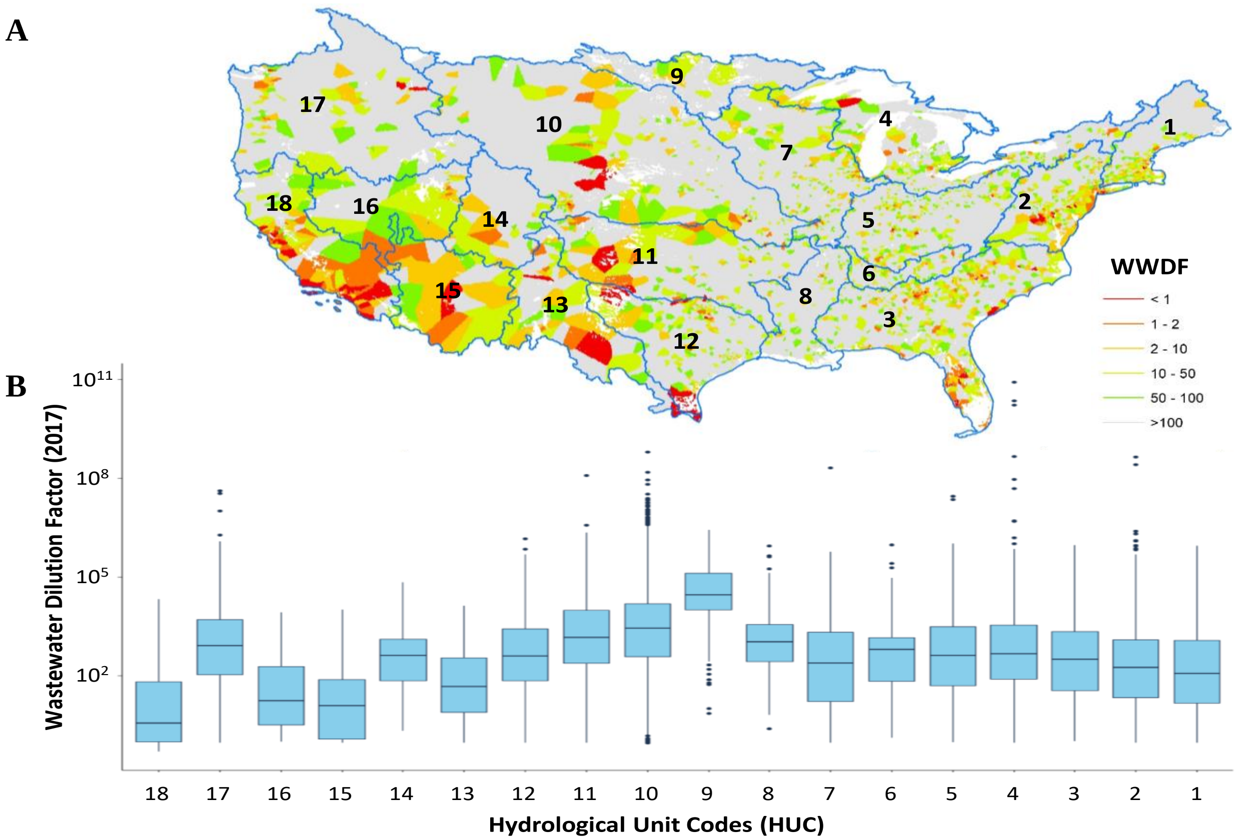


Figure (1)- 1: (A) 2017 wastewater dilution factors (WWDF) for watersheds within the contiguous U.S. hydrologic unit regions classified into six classes of WWDF from <1 to >100. Numbers inside the map represents respective HUC regions (B) The 2017 average WWDF for each HUC region. The top, middle and bottom lines of box represent the 75th, 50th and 25th percentile with points representing outliers beyond the 10th and 90th percentile. Top and bottom vertical lines of the boxes represent largest and smallest value within 1.5 times interquartile range above 75th percentile and below 25th percentile, respectively.

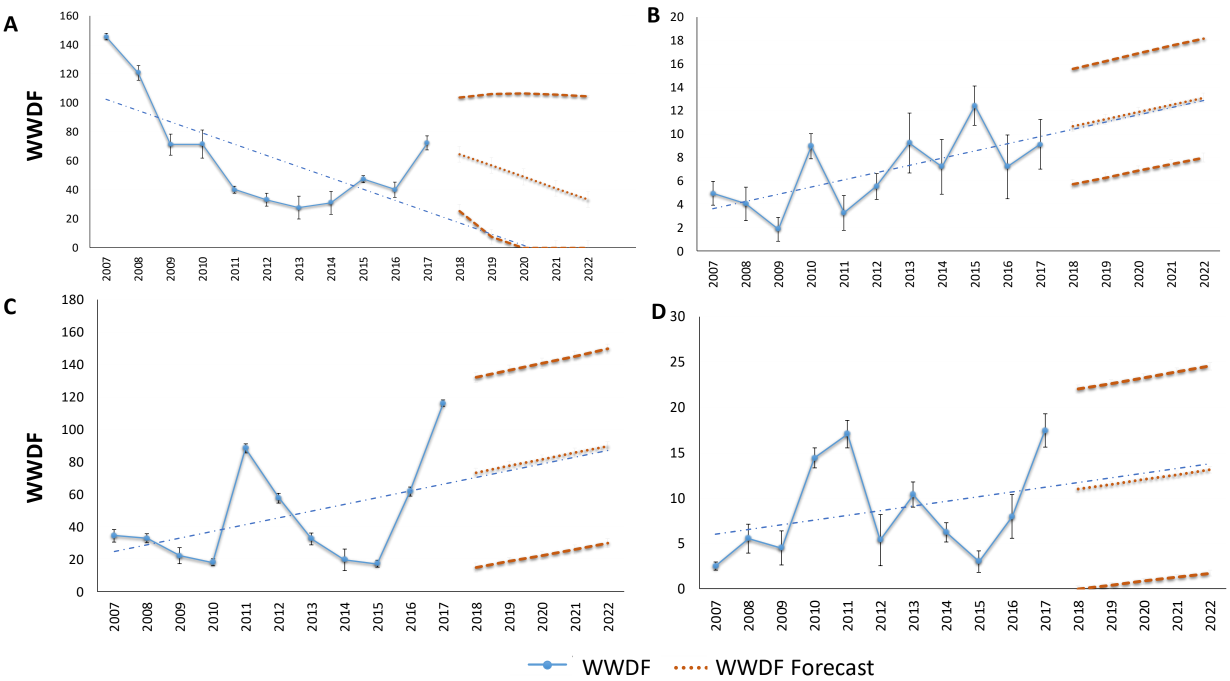


Figure (1)- 2: Wastewater dilution factor (WWDF) from 2007 to 2017 with 5 year forecast from 2018 to 2022 with 95% confidence interval for (A) Hydrologic unit Code (HUC) 13, (B) HUC 15, (C) HUC 16, and (D) HUC 18.

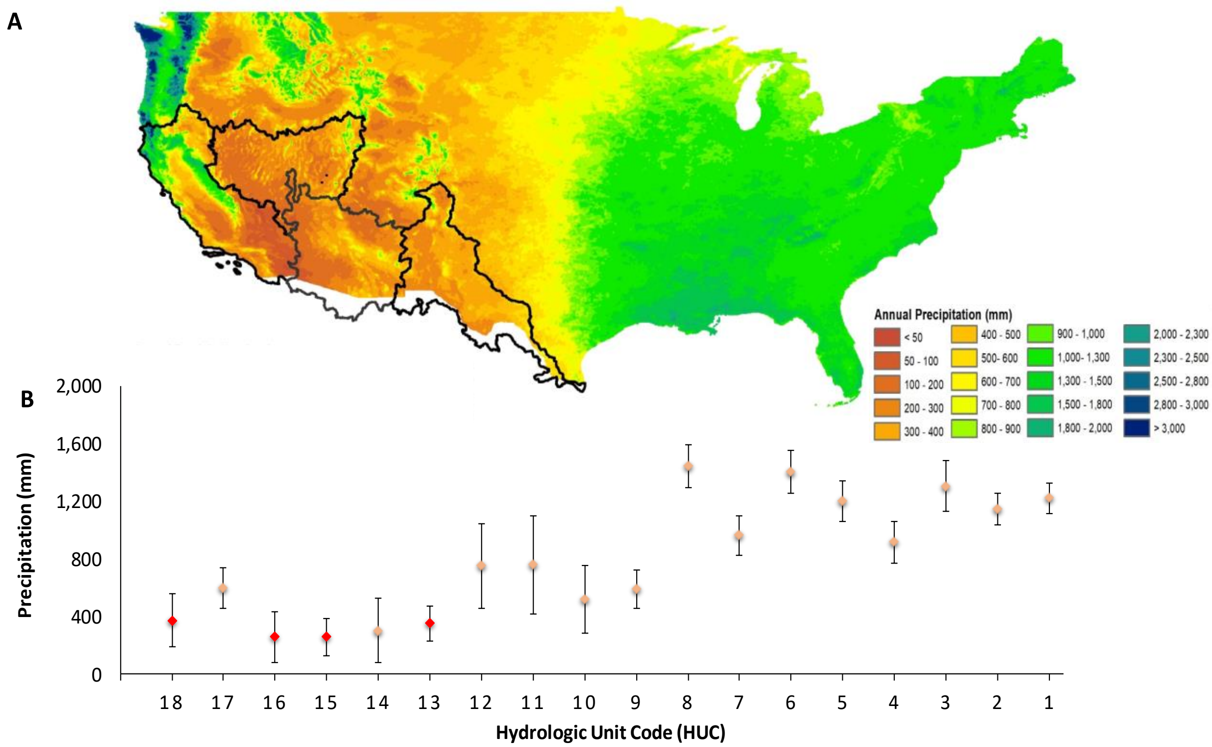


Figure (1)- 3: (A) Colour coded map representing average annual precipitation (mm) over the 10-year period from 2007-2017 in contiguous U.S. (B) Scatter plot representing 10 years annual precipitation with standard error in hydrological units (HUC) across contiguous U.S. ** Red colour bars in scatter plot represents the HUC of interest.

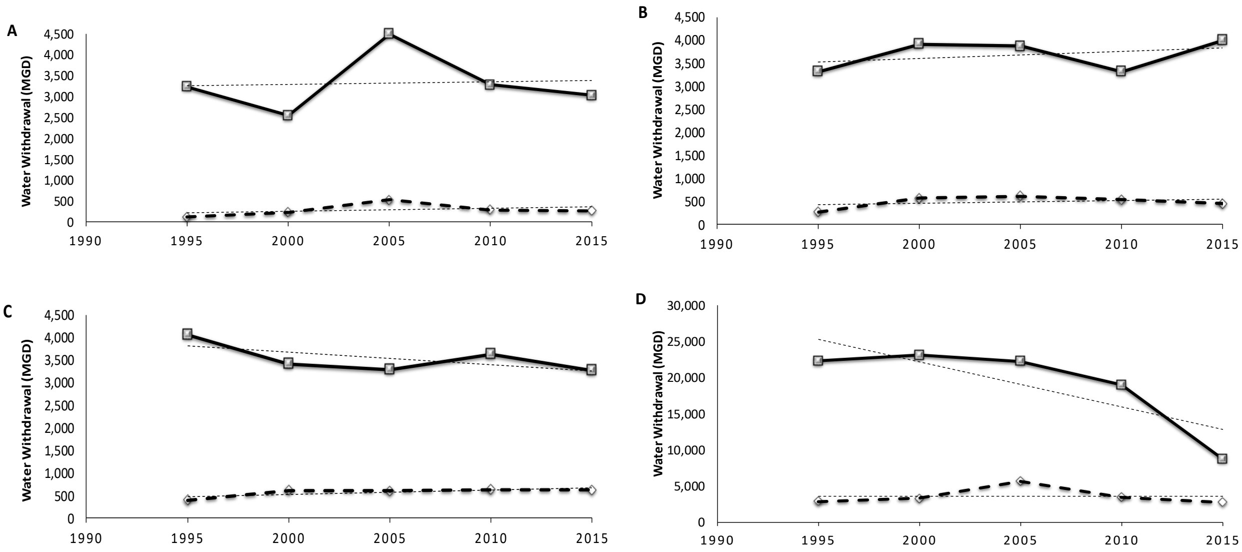


Figure (1)- 4: Total surface freshwater (dashed line) and public supply withdrawal (solid line) with trend line (dotted line) from 1995 to 2015 in (A) Hydrologic unit Code (HUC) 13, (B) HUC 15, (C) HUC 16 and (D) HUC 18.

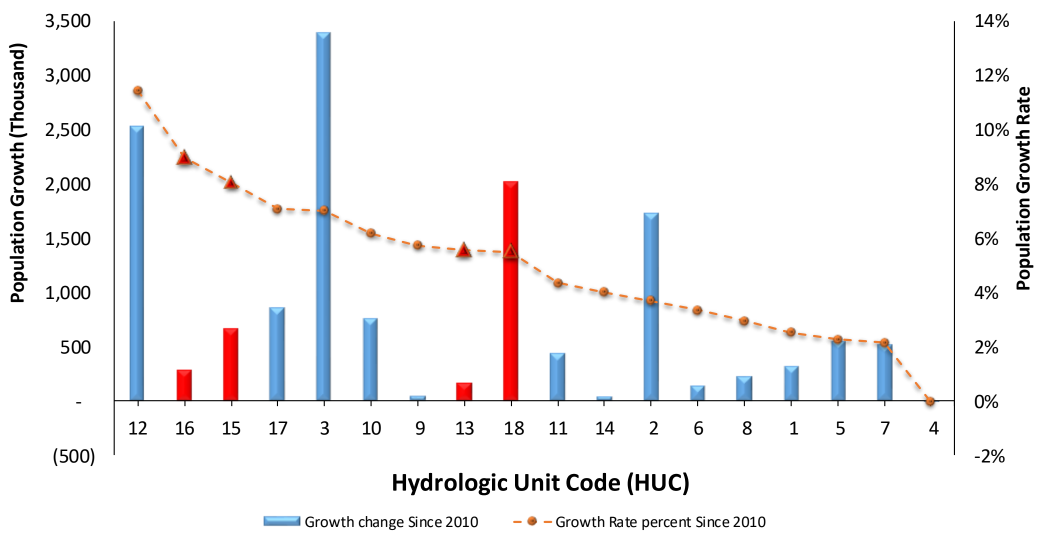


Figure (1)- 5: U.S. population growth change and percent by Hydrologic unit Code (HUC) from 2010 to 2016. Red columns and markers are representing HUC of interest (HUC 13, 15, 16, and 18).

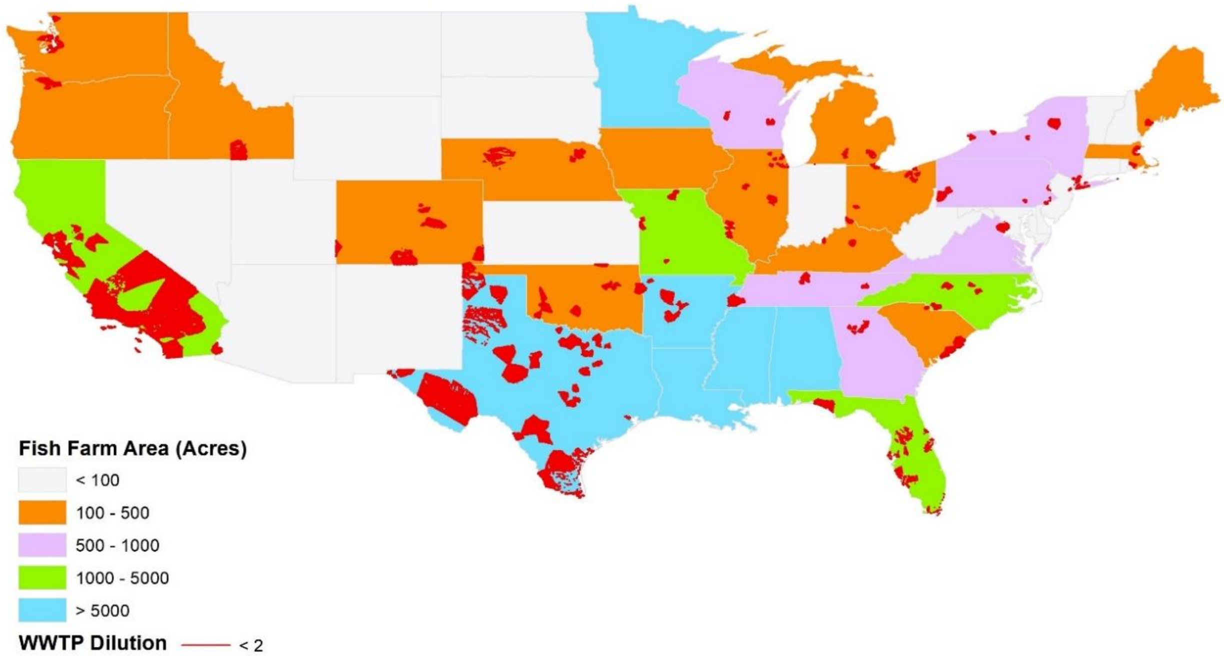


Figure (1)- 6: Map of the contiguous U.S. showing total aquaculture surface freshwater fish farm area (acres) in each state overlaid with areas where the WWDF is <2 for year 2015.

CHAPTER II: CONTIGUOUS U.S. SURFACE WATER AVAILABILITY AND WASTEWATER EFFLUENT FLOWS

Abstract

Surface water is a vital and sometimes stressed resource in the U.S. The quantity of this resource is threatened by population shifts and growth concurrently with climate change intensification. Additionally, growing population centers can impact water quality by discharging treated wastewater effluent, which is typically of lower quality than its receiving surface waters. Depending on baseflow and environmental factors, this could decrease water quality. Water availability and wastewater effluent ratio has been examined for this study by using statistical approaches. The Mississippi River generally served as a dividing line for surface water availability, with five of the six hydrological unit codes (HUC) regions with very low water availability (<24,000 L/D/Km^2) residing in the west. These same HUC regions also experience more drought as well as more severe droughts than regions in the east. In regions with lower surface water flows, their water quality is more susceptible to the influence of wastewater effluent discharges, especially near large and growing population centers like San Antonio, Texas. A prediction model was established for this city, which found that from 2009-2017 wastewater effluent increased by 1.8%. As cities grow, especially in the Southwest and Western U.S. together with intensified climate change, surface water quantity and quality become more crucial to sustainability. This study indicates where surface water availability is already an issue and provides a model to estimate, as well as project, wastewater effluent flows into surface water bodies.

1. Introduction

Freshwater constitutes only 2.5% of all water globally, out of which only ~1% is accessible for direct human use, making it scarce in many parts of the world (Gleick, 1996). Water scarcity in the U.S. is most pronounced in arid and semi-arid areas that include the Southwest as well as parts of Texas and California. Parts of these areas are also experiencing some of the highest rates of population growth in the U.S (U.S. Census Bureau, 2011). California's drought from 2011-14 provides an example where increasing populations demand more water and food resources, which can decimate local water resources, particularly under drought conditions (Larsen et al., 2014). Tighter water resources are only part of the problem as our lifestyles and health depend on regular use of a wide array of chemicals (referred to hereafter as contaminants of emerging concern; CECs), a portion of which enters surface waters, largely from wastewater treatment plant (WWTP) effluent. The impacts of these chemicals are being studied, but as temperatures rise due to climate change and populations shift and grow, anticipated lower surface water baseflows will provide less dilution to WWTP effluent. In the long-term, this will result in higher concentrations of CECs as well as generally lower water quality (Duan et al., 2019; Jackson et al., 2005; Portner et al., 2014). Therefore, not only will water resources be more limited in the future, but the quality of those resources may be lower.

Per capita domestic water use in the U.S. was 302-378 L/person/day from 2000-2015 with total withdrawals expected to increase regionally due to population growth, but more broadly because of climate change (Brown et al., 2013; U.S. Geological Survey (USGS), 2016). Changing climate has increased temperature 2 ˚F over the last 50 years in the U.S. with another 10 ˚F increase possible by 2100 (NOAA, 2018). Increasing temperatures and shifting precipitation patterns are projected to increase annual water withdrawals 5% and 3.5%, respectively, by 2090 (Brown et al., 2013). However, the largest driver of water withdrawals will be higher rates of evapotranspiration, which will result in a 23% increase in water withdrawal over the same time (Brown et al., 2013; U.S. Global Change Research Program (USGCRP), 2009). Together, these three factors will increase water withdrawal ~30% by 2090 when compared to scenarios with no climate change. Additionally, precipitation has increased ~5% globally and >6% in the contiguous U.S., with the heaviest rain downpours increasing ~20% since 1901 (NOAA's National Climatic Data Center, 2008). These precipitation trends are expected to continue over most of the U.S., although in the Southwest precipitation is projected to decline 0-15% by the end of this century (Christensen et al., 2007; Cook et al., 2008). Over time, this will also cause changes in humidity and wind speed patterns. These factors, as well as population and precipitation collectively affect WWTP effluent volumes and its influence in surface waters.

As of January 2012, >14,000 WWTPs served 238.2 million people in the US (76% of the total population) and 85% of those facilities discharge their treated effluents directly into the surface water (Clean Watersheds Needs Survey, 2008; USEPA, 2016). Wastewater effluent typically contains elevated nutrient concentrations and potentially hundreds of CECs (Dickenson et al., 2011; Glassmeyer et al., 2005). A study by USEPA and USGS targeting 110 CECs at 11 WWTPs, found up to 68 per effluent sample, while at least 34 CECs were found in half of the effluent samples (Glassmeyer et al., 2005). Once in surface waters, CECs flow downstream where they could affect organismal health (Daughton and Ternes, 1999; Malaj et al., 2014) or be captured by the next community's drinking water treatment system (Rice and Westerhoff, 2015). WWTP effluent must be sufficiently diluted and assimilated into surface waters to preserve water quality, ecosystem health, and reduce drinking water treatment costs on downstream communities. Continuing from a previous model on WWTP dilution for contiguous USA (Siddiqui et al., 2020), this study assesses current and future surface water availability as well as its influence by WWTP effluent on existing water resources that are stressed by climate change and population growth. This was addressed by quantifying surface water availability for regions within the contiguous U.S. from existing data and developing a statistical model to predict WWTP effluent flows into surface waters of a large and rapidly growing city with relatively low precipitation (i.e., San Antonio, TX).

2. Material and Methodology

2.1 Data collection

Data was obtained from U.S. government agencies as follows. The 30-year temperature and precipitation average (1981-2010) was collected from the PRISM Climate Group at Oregon State University and used for map preparation and water availability calculations (Climate Group, 2018). Evapotranspiration data was collected from Dewes et al., (2017) and NOAA (2018). Data from 2000-2015 was averaged annually for model calculations. All WWTP effluent data was obtained from the USEPA's Enforcement and Compliance History Online (ECHO) (USECOEPA, 2018) database for publicly owned treatment plants (hereafter referred to as wastewater treatment plants or WWTPs) that were not classified based on treatment levels. River discharge volumes were obtained from the USGS (2018), population data from the US Census Bureau (2019), and weather data (i.e., air temperature, humidity, wind speed, and precipitation) from NOAA (2018). These datasets were then consolidated based on geographic location within the contiguous U.S. and further delineated by USGS Hydrologic Unit Codes (HUCs).

2.2 Surface water availability

Surface water availability was calculated for the 10-year period (2005-2015) in the contiguous U.S. for each HUC, which are delineated by watersheds with USEPA's National Hydrography Dataset NHDPlus map in Arc GIS using the calculator tool. Data were divided into HUCs identified by USGS and then statistically tested for the differences. The formula used to calculate values was:

$$\frac{\left[(E + Ww) + \left(S_f + R\right)\right]}{A}$$

Where E is evaporation (mm), W_w is water withdrawal (MGD), S_f is surface water flow (CFS), R is rainfall (mm), and A is the area (acres) of each HUC.

2.3 Model preparation

A statistical prediction model was prepared for San Antonio, TX due to its characteristics as a fast-growing region with precipitation <800 mm/yr, and with an assumption that an increasing population will add more WWTP effluent in surface waters. The model was developed using existing data from 2010-2015 (Fig. (2)- 1). Predictors for the model were river discharge, precipitation, air temperature, humidity, and wind speed. The predictand is WWTP outfall flow, hereafter referred to as WWTP effluent. Data was consolidated and analyzed using R. Box plots and scatter plots were prepared to describe the central tendency and data distribution of the variables used. A correlation matrix with frequency distribution and basic trends was also prepared to assess the relationship between each variable to that of predicted. Based on the correlation matrix assessment, population, temperature, humidity, wind speed, discharge, and precipitation were used in the model. A statistical prediction model was prepared using the Artificial Neural

Net (ANN) tool in R. Raw data were normalized for the ANN and partitioned into training and validation data set in a 80:20 ratio. The data output was taken as a linear function with three hidden nodes, after which prediction for training and validation data were performed (Appendix B, Fig. S1). The output data was denormalized to the original form to make the output readable. The model performed 506 steps with 0.0486 ANN step error and run 100 times to identify the best fit for this situation, which may improve in the future with the availability of more data.

3. Results and Discussion

3.1 Surface water availability

Surface water availability is a growing concern as the world's population increases and climate change accelerates resulting in higher temperatures, sea-level rise, salt-water intrusion, and droughts (Jones and van Vliet, 2018). Therefore, it is necessary to quantify existing freshwater resources and attempt to understand their ability to support future generations. By 2070, 18 % of U.S. land area, which supports 24 % of the U.S. population will be water-stressed (Duan et al., 2019). Depending upon consumer needs, water quality requirements may vary and water availability can be allocated based on its use (i.e., public, industrial, or agricultural) (van Vliet et al., 2017). In our study, surface water availability was defined as the volume of surface water available for public use in a given area per day (L/D/Km^2). Estimates for surface water availability within each HUC during 2015 (Fig. (2)- 3) were grouped into four categories: very low surface water availability (VLWA; <24,000 L/D/Km^2), low surface water availability (LWA; 24,000 – 50,000 L/D/Km^2), medium surface water availability (MWA; 50,000 – 89,000 L/D/Km^2) and high surface water availability (HWA; >89,000 L/D/Km^2). Six HUCs were categorized as VLWA, with five of the six (13, 15-18) located in the Southwest and Western U.S. where precipitation rates are

<800 mm/yr in much of that area. Three of these HUCs (13, 15 and some parts of 18) also have annual average temperatures >15 ^{o}C. Additionally, much of HUC 13, 15, 16 and 18 are within the top 10 regions for population growth over the last 5 years (U.S. Census Bureau, 2011). Despite this growth, HUCs in the western U.S. with VLWA also have some of the lowest population densities (24 – 92 people/Km^2; Fig. (2)- 3), although they also contain cities with some of the highest population densities in the U.S., particularly HUCs 17 and 18. This presents additional challenges with the distribution of water and its transport from areas of relative abundance to areas of need. Surprisingly, HUC 01 in the northeast U.S. also has VLWA. Parts of this region were previously identified as an area of concern for surface water availability (Tidwell et al., 2018). With a population density of 1,513 people/Km^2 that is largely concentrated in the southern half of the region, low surface water availability is a potential issue. However, HUC 01 is not generally considered water limited as its precipitation ranges from 1,000-2,000 mm/yr. Furthermore, in the rural northern part of HUC 02, 40% of the population uses private groundwater wells for their household water supply (Maine Department of Agriculture Forestry and Conservation, n.d.). Therefore, in HUC 01, as opposed to the VLWA HUCs in the west, precipitation and groundwater availability reduces potential stress due to very low surface water availability.

Water use is driven by per capita water consumption in agriculture, industrial, urban, domestic as well as with population growth. With population growth projections, decreasing water availability can lead to higher water demand and stress (Alcamo et al., 2007). From 1981-2010 high-water demand was reported in western and central U.S. (HUC 10-18) where water supplies are expected to decrease in the future (Duan et al., 2019). More broadly, the U.S. General Accounting Office (GAO) found that 40 states expected water shortages, whether at local, regional or state level from 2014-2023 (Government Accountability Office Report (GAO), 2014). This includes 24 states with regional shortages and 15 states expecting local shortages. Montana was the only state expecting a state-wide shortage. The Mississippi River appears to be a dividing line, with most states expecting regional shortages being west of the river, while most local shortages were east of the river (Government Accountability Office Report (GAO), 2014). One driver of this water stress is population growth. The U.S. Census Bureau predicts that from 2000-2030 the west will grow 45.8% followed by South (42.9%), Midwest (9.5%) and Northeast (7.6%). Overall the U.S. population is expected to increase 29.2% by 2030 (US Census Bureau, 2019). This growth, coupled with drought prediction estimates, indicate that the southwest U.S. will become drier with earlier snowmelt causing earlier runoff (Barnett et al., 2008). Moreover, from 2005-2090, most of the U.S. can expect at least a 13% water withdrawal increase (Brown et al., 2013). Similar results were reported in the National Climate Assessment Report (Thomas et al., 2009) according to which western and the southeastern U.S. may experience decreased water availability due to increasing temperature and changing precipitation pattern.

3.2 Wastewater effluent flow modelling

A model was developed to assess variables that influence wastewater effluent flows near San Antonio, Texas. WWTP effluent and river discharge volumes together with precipitation increased over the study period from 2011 to 2016. During this time frame, WWTP effluent flow was positively correlated with river discharge > precipitation > humidity > population. It was negatively correlated with wind speed > temperature. Each of these variables were incorporated into the model (Appendix B, Fig. S1). The resultant model has a root mean square error of 0.000176 and 0.0278 for the training and validation data, respectively, with Gaussian distribution (Appendix B, Fig. S2). The R^2 value was 0.235 and 0.246 for the training data set and validation data set, respectively. The confusion matrix resulted in 32 data points within range and 16 incorrect predictions. In contrast there were 6 correct against 4 incorrect predictions for the training dataset and validation dataset, respectively. This model was then used to back predict data from 2009-2011 (Fig. (2)- 4). The actual and back predicted data from 2009-2017 show that wastewater effluent flows increased 1.13%, with the trend continuing in the forecasted data for 2017-2020 ($R^2 \geq 0.05$; Appendix B Fig. S3). The San Antonio region's continued growth will result in the consumption of an additional 69,000 acre-feet of water annually by 2070 over the 255,000 acre-feet expected annually in 2020 (San Antonio Water System, 2017). This indicates that wastewater effluent volumes may continue to increase depending on this city's implementation of wastewater recycling. Currently, San Antonio has the nation's largest water recycling system, capable of distributing 29 million gallons per day (MGD) for irrigation as well as commercial and industrial uses (San Antonio Water System, 2017). With additional data inputs, the model can be applied to additional areas within the U.S. to improve prediction of WWTP effluent locally, regionally or nationally.

3.3 Prevalence and impacts of high wastewater effluent flow

In the U.S. ~19,137 MGD WWTP effluent is released into freshwater systems (Clean Watersheds Needs Survey, 2008). The ability of a receiving water body to assimilate this effluent and minimize changes in water quality is tied to wastewater treatment levels and in-stream dilution (i.e., river base flows). To better understand trends in average daily WWTP effluent flow, values from 2007-2017 were analyzed within the contiguous U.S. and divided into three categories <0.01, 0.01-0.1, and >0.1 cubic feet per second (CFS) (Fig.(2)- 5), which ranged from 20-38%, 10-23% and 36-58%, respectively. Overall total WWTP effluent discharge in the contiguous U.S. decreased 5% despite an 8.13% increase in total population from 2007-2017, indicating that variables such as precipitation and water recycling or reuse also influence effluent volumes.

However, the number of discharges exceeding 0.1 CFS increased 50% over the study period. These individual sources, where daily discharge is increasing, may result in a higher proportion of treated effluent to surface water base flows that decrease water quality (Rice and Westerhoff, 2015).

Wastewater effluent flows can also dominate surface waters due to drought (Brooks et al., 2006). Droughts occurred in each of the 18 HUC regions from 2000 to 2019, with all experiencing at least a moderate drought over 70% of their area for two weeks during this period. Additionally, HUC 10, 4 and 2 experienced moderate, severe and extreme drought over 100% of their area for at least one week. However, the average severity of drought experienced within the HUCs is generally greater for regions west of the Mississippi River (HUC 9-18; Fig. (2)- 6). These regions, with the exception of HUC 11, are also LWA and VLWA regions. This indicates that most HUC regions where surface water availability is already low or very low are also prone to more intense droughts, which could lead to a greater influence of wastewater effluent in those areas. This has already been observed due to the drought experienced within the Colorado River system since 2000 (The National Drought Mitigation Centre, 2018). Warmer temperature due to climate change has reduced the Colorado River flow by ~ 6% and is projected to be reduced ~20% by 2050 and up to 35% by 2100 (Udall and Overpeck, 2017). Both Lake Mead and Lake Powell lost half of their storage due to low flow in River Colorado, which supplies water to over 30 million people in the Southwest including parts of Los Angeles, Phoenix, Las Vegas and Denver (U.S. Global Change Research Program (USGCRP), 2009). Lake Mead also receives wastewater effluent from the regions three treatment plants through the Las Vegas Wash (Benotti et al., 2010). During 2003-2007 there was a statistically significant increase in source water conductivity, nitrate, and CEC concentrations due to the decline in Lake Mead storage associated with drought (Benotti et al., 2010).

When wastewater effluent becomes a higher proportion of streamflow, less dilution of contaminants in the effluent occurs, which could stress aquatic organisms and ecosystem health (Brodin et al., 2017; Saari et al., 2018). Increased wastewater flow in surface waters may cause water quality concerns as demonstrated in other studies (Rice and Westerhoff, 2017; Siddiqui et al., 2020). For downstream communities, it may also increase drinking water treatment costs in the long run (Keiser et al., 2019) and lead to contaminants in tap water (Bradley et al., 2018; Rice et al., 2013). While the specific impacts are not well known, this *de facto* and unplanned water reuse may impact 50% of drinking water treatment plants by 2056 (Rice and Westerhoff, 2015). Under average streamflow the extent of *de facto* reuse is minimal (<1%), but under low streamflow, this value jumps in some areas to 50% (Rice and Westerhoff, 2015). Despite the projections of low influence on drinking water supplies in the future, U.S. tap water already contains various CECs that are likely present due to treated wastewater effluent discharges upstream. The USGS sampled tap water at 25 locations in 11 U.S. states and identified 482 organic and 19 inorganic contaminants (Bradley et al., 2018). The effects of increasing contamination in drinking water and its supplies and what, if anything, should be done to regulate this pollution is a work in progress.

4. Conclusion and Implications

Surface water availability depends upon a variety of factors. Changing climate and population affect surface water availability especially in high water demand areas. During 2015, the Southwest and Western U.S. (10 HUCs), where populations are growing and there is low precipitation, contained 5 of the 6 VLWA and 4 of the 5 LWA regions. Population growth may increase surface water withdrawals, which can reduce river baseflows, but also at least partially recharge those flows due to increasing volumes of lower quality WWTP effluent. This may result in the overall water quality of a system declining, which will be most evident in rivers that are permanently or periodically effluent-dominated or dependent. In the early 2000's, 23% of regulated U.S. effluent releases into streams received less than a 10-fold dilution; under low-flow conditions it may go up to 60% (Brooks et al., 2006). For example, 285 of 582 regulated discharges in Texas, Oklahoma, New Mexico, Arkansas, and Louisiana enter surface water bodies in which effluent accounts for >90% of the instream flow (Brooks et al., 2006). As climate change accelerates and populations grow, what is considered low flow today may become the norm in parts of the U.S., increasing the number of effluent-dominated or dependent rivers and streams.

The present study provides an accounting of surface water availability at the regional scale for the contiguous U.S., while also establishing a statistical model to estimate wastewater effluent flow for one of the largest and fastest growing U.S. cities. Surface water availability concerns grow in hot, dry regions of the west and southwest. However, the northeast U.S. also had very low surface water availability in 2015. While the northeast U.S. can make up for low surface water availability, other regions with low availability must explore alternative sources of water or use existing supplies more efficiently to account for the impacts of climate change and the needs of a growing population. These growing populations have the potential to increase wastewater effluent discharges, which may also lead to declines in water quality for downstream communities. A wastewater effluent prediction model (prepared to estimate discharges as a function of environmental factors) for San Antonio, TX demonstrated an increase of 1.13% over an 8-year period. Surface water resources are stressed in expected and unexpected areas of the U.S. With populations growing and climate change intensifying, it is important to delineate these regions today and explore the potential growing influence of wastewater effluent on water quality and river base flows.

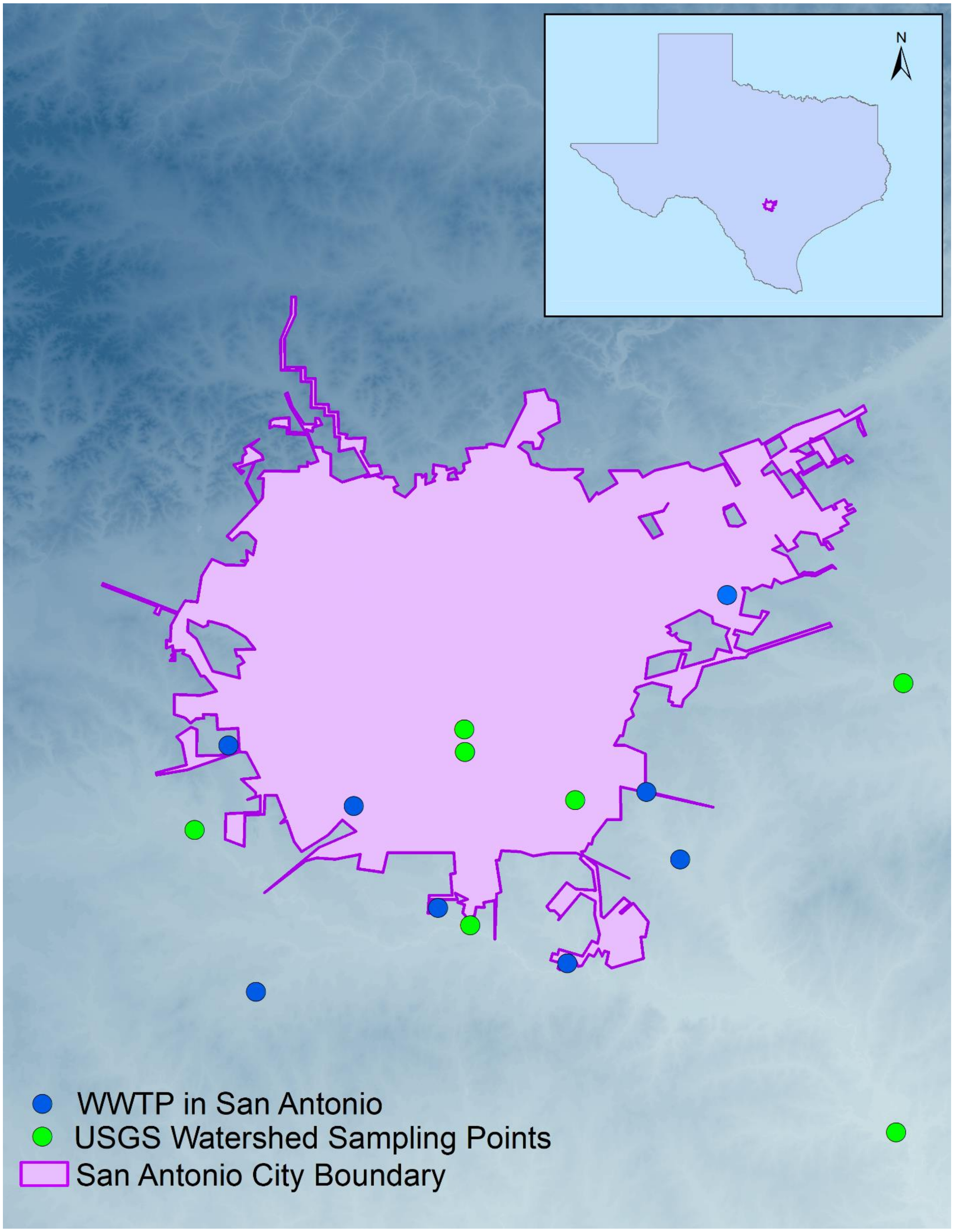


Figure (2)- 1: Map of wastewater treatment plant (WWTP) locations and USGS water-sampling points within San Antonio, Texas region.

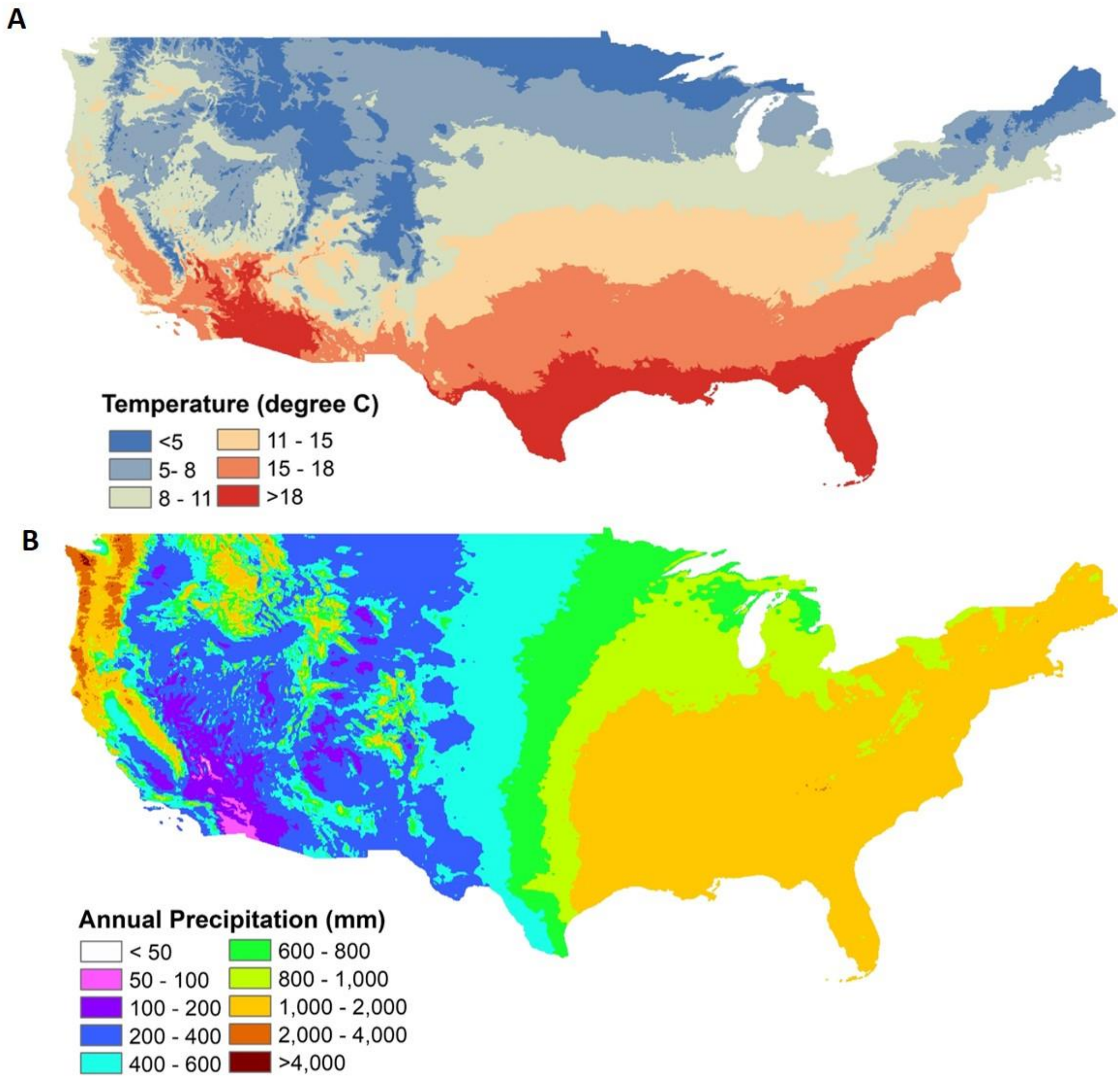


Figure (2)- 2: Maps representing 30-year annual average (1981-2010) (A) temperature distribution (°C) and (B) precipitation (mm) distribution for the contiguous U.S.

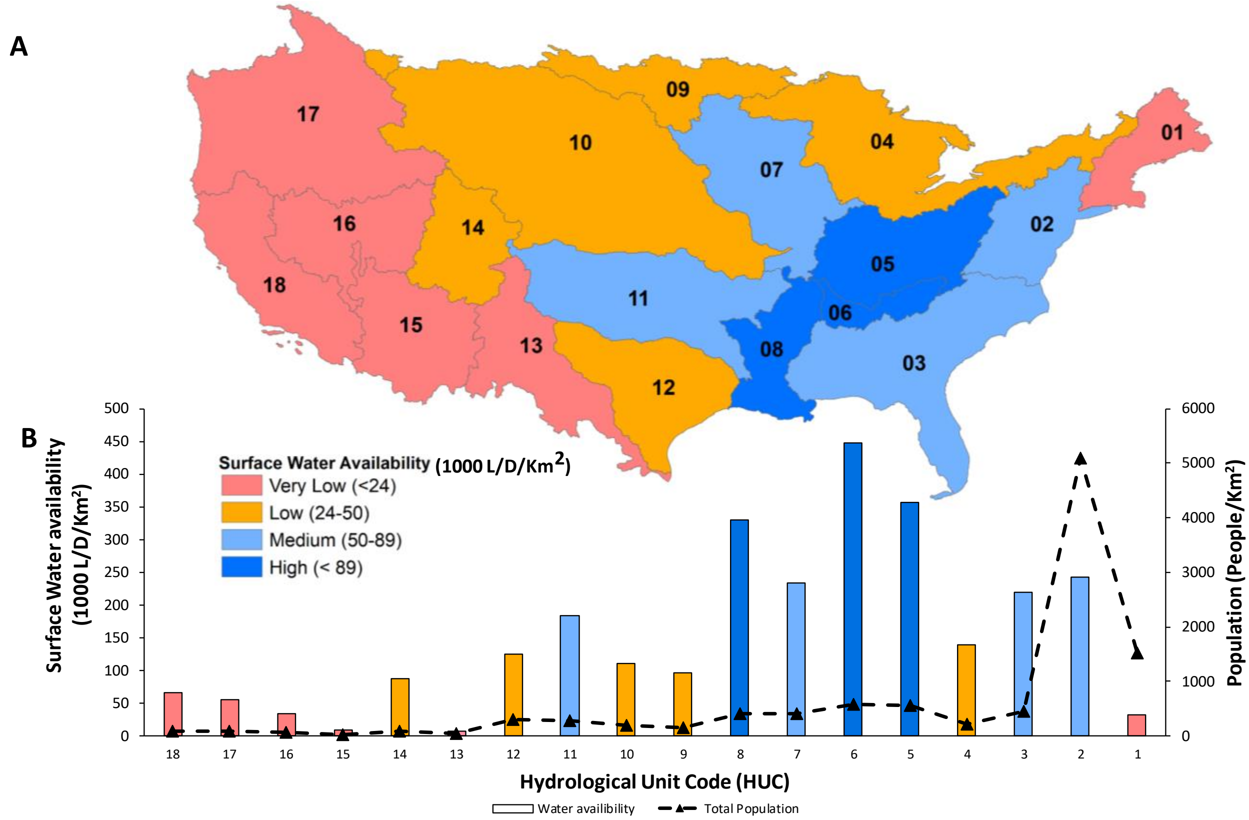


Figure (2)- 3: (A) Map representing year 2015 surface water availability in the contiguous U.S. (1000 L/D/Km^2) and (B) a comparison of the surface water availability with population density for each USGS Hydrologic Unit Code (HUC).

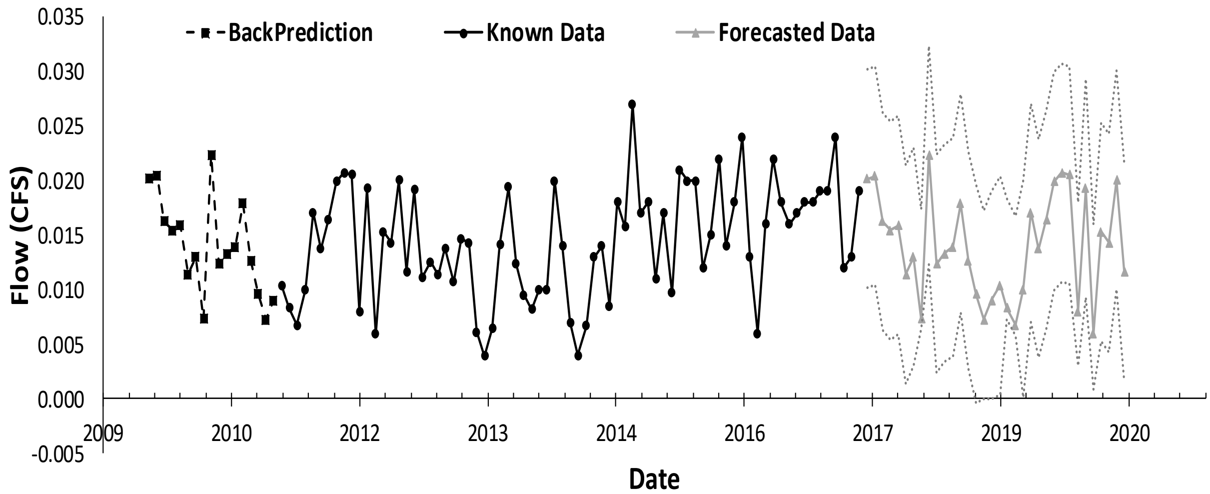


Figure (2)- 4: Wastewater treatment plant (WWTP) effluent flow (cubic feet per second, CFS) for 2011-2016 for San Antonio region with back-prediction (2009-2011) and resulting forecasted data (2017-2020) with 95% confidence interval.

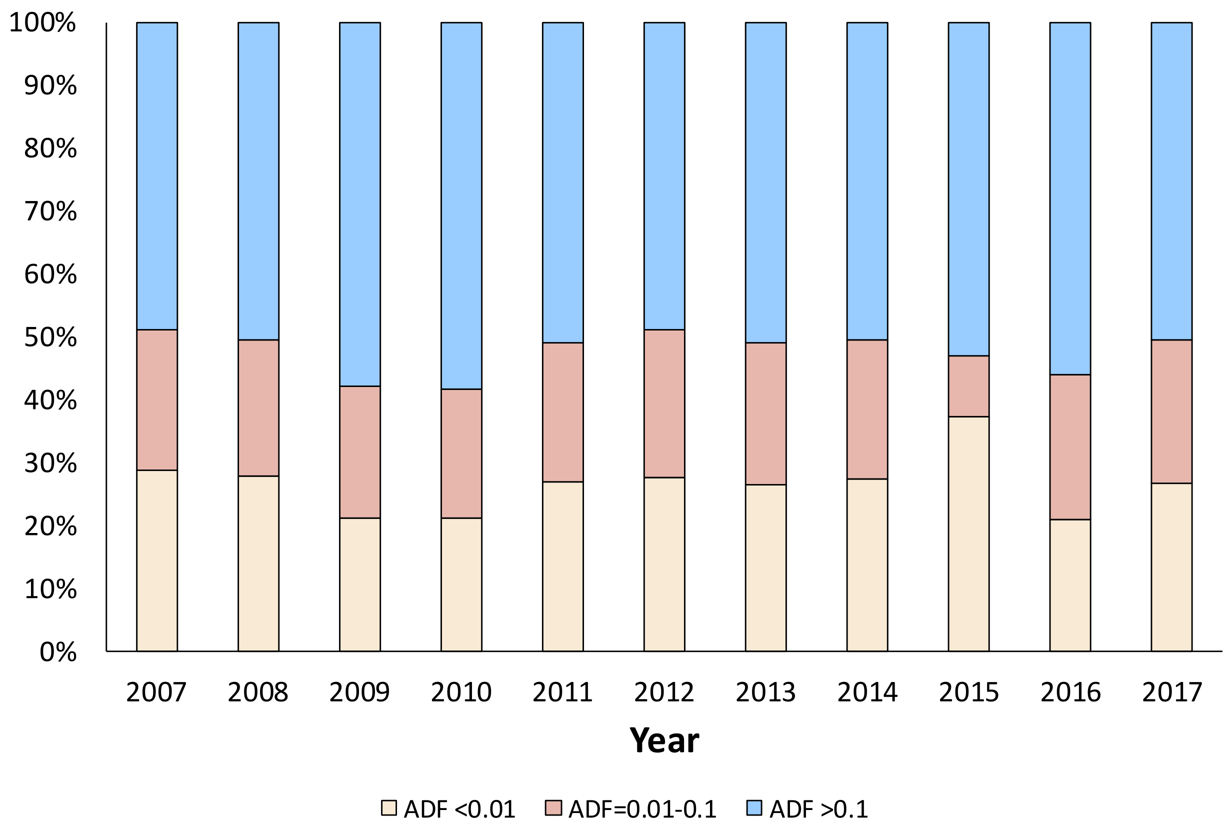


Figure (2)- 5: Percent of actual wastewater treatment plants average daily flow (ADF) (cubic feet per second) within the contiguous U.S. from 2007-2017.

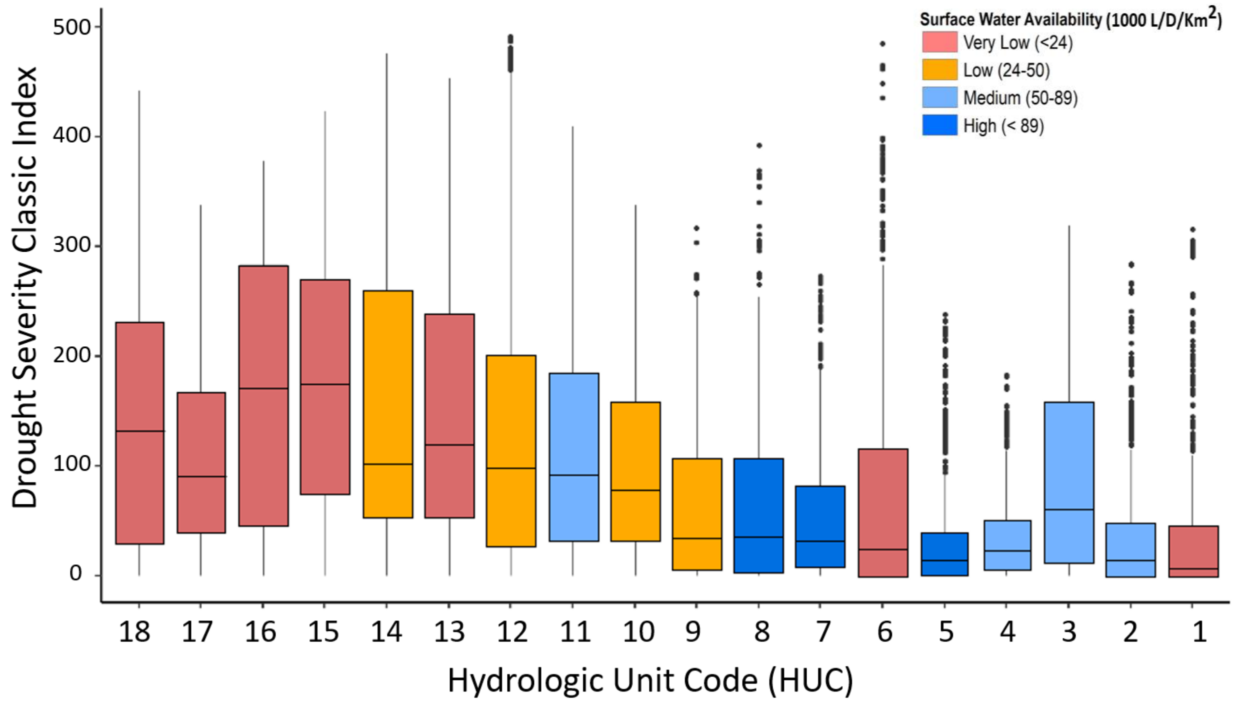


Figure (2)- 6: Weekly average Drought Severity Classification Index (DSCI) from January 2000 to September 2019 for each USGS Hydrological Unit Code (HUC). Box plot colors depict the surface water availability category of each HUC.

CHAPTER III: BIOCONCENTRATION AND DEPURATION KINETICS OF DILTIAZEM IN TILAPIA (*OREOCHROMIS MOSSAMBICUS*) AND ITS IMPLICATIONS TO AQUACULTURE

Abstract

Wastewater effluent carry various contaminants that dilute in surface waters and may result in problems throughout the receiving ecosystem and transfer possibly to humans through surface water use. Based on the traditional understanding of contaminants, soluble chemicals, like many pharmaceuticals, are not expected to bioaccumulate. However, some pharmaceuticals that ionize at an environmentally relevant pH may undergo a chemical transformation that enables bioaccumulation. This potential bioaccumulation in farmed fish is not well known. Therefore, a study was undertaken to quantify the bioaccumulation and depuration of diltiazem (DTZ) (brand names e.g., Cardizem CD, Dilacor XR), an ionizable calcium channel blocker, at environmentally relevant concentrations (1 µg L^{-1}) in tilapia (*Oreochromis mossambicus*) under controlled laboratory conditions. DTZ concentration in fish carcass, blood plasma, liver and muscle were analyzed in both exposure and depuration phase. DTZ bioconcentration was greatest in liver> plasma> carcass> muscle. Depuration rates were fastest in liver> carcass> plasma> muscle. Biological half-life ($t_{1/2}$) calculations indicated DTZ was retained longest in muscle (18.83 hrs) and shortest in liver (1.89 hrs), which is similar to stable bioconcentration factor (BCF_a) value orders. The half-life time of DTZ in muscle indicates that DTZ is processed relatively quickly in this tilapia species. Based on the 96 hr environmentally relevant concentration DTZ uptake by tilapia fingerlings, human exposure to the highest DTZ fillet concentration would be ~6 orders of magnitude below the lowest human dose, resulting in low human exposure. Transferring fish to “clean” water would further reduce concentrations. Despite the low tissue concentrations of DTZ,

concentrations of DTZ in fish plasma could be a concern for growers. Plasma concentrations were on the same order of magnitude in the fish as seen in humans after a therapeutic dose. This raises the possibility of physiological effects on tilapia at environmentally relevant diltiazem concentrations during commercial production but needs to be ascertained before any actions are taken or guidelines proposed**.**

1. Introduction

Worldwide, 631 different human and veterinary pharmaceuticals and their metabolites have been detected in wastewater effluent from 71 countries (aus der Beek et al., 2016). Typically, these compounds have low n-octanol-water partition coefficient (K_{ow}) values and therefore are excreted as the parent compound or metabolite within days of use. Consequently, after use, some portion of these chemicals make their way into the environment, mostly due to wastewater effluent, where some may be hazardous to organisms (Park, 2005; Han et al., 2006). Pharmaceuticals have a relatively short biological half-life ($t_{1/2}$) but can be pseudo-persistent in surface water under effluent dominated scenarios (Brooks et al., 2006; Daughton and Ternes, 1999). Despite the generally low log K_{ow}, some pharmaceuticals may bioaccumulate due to poor metabolism in fish (Connors et al., 2013). Pharmaceuticals are generally hydrophilic, resulting in higher solubility (e.g., diltiazem: 465 mg/L in water at 25 °C), which typically reduces their bioconcentration potential (Daughton and Brooks, 2011). To aid in their effectiveness, many pharmaceuticals are designed to be weakly ionizable, with their specific chemical structure changing with environmental pH. Their environmental fate may be predicted because K_{ow} and lipophilicity often correlate with bioavailability (Kah and Brown, 2007).

Diltiazem (DTZ) is a weak acid (pKa= 8.06), which can ionize at environmentally relevant pH, and, thus, is more bioavailable for aquatic organisms. Acids have a neutral charge at pH levels below their pKa and bases at pH levels above the pKa. The ionized fraction of the electrolyte is more polar (for dipole-dipole interaction and H-bonding capacity) and usually exhibits lower permeability into membranes and fatty tissues compared to the un-ionized fraction (Camenisch et al., 1996). Therefore, acids typically have higher toxicity and bioaccumulation at lower pH and the opposite is true for bases (Braunschweiler and Koivisot, 2000). DTZ is a calcium channel blocker drug used to treat cardiovascular disease, migraines, and arterial hypertension (i.e., high blood pressure) (Roti et al., 1988; Grossman and Messerli, 2004; Batt et al., 2008). Due to its numerous applications and incomplete degradation during wastewater treatment, DTZ is found in sewage effluent (390-425 ng/L) and surface waters (49-106 µg/L) (Khan and Ongerth, 2004; Kolpin et al., 2004; Kasprzyk-Horden et al., 2008; Meador et al., 2016; Steinbach et al., 2016b) as well as reclaimed irrigation waters (mean concentration, 111 ng/L; Wang and Gardinali, 2012). In a recent study of Gulf of Mexico fishes, DTZ plasma concentrations ranged from 1.71 ng/mL (*Ictiobus bubalus*) to 11.71 ng/mL (*Cyprinus carpio*) (Scott et al., 2016). Plasma concentrations, such as these in marine fish, may also influence aquacultured fish production, which receives 81% of the water it uses from surface waters (USDA, 2015) that are becoming increasingly dominated by wastewater effluent (Fu and Wu, 2005; Lu et al., 1995). As water resources decline, aquaculture in some parts of the U.S. will become more dependent on surface waters that have higher proportions of wastewater effluent.

DTZ has been reported bioconcentrating through the food web to higher trophic levels, e.g. Osprey (*Pandion haliaetus*) 0.54–8.63 ng DTZ/mL plasma (Bean et al., 2018). This raises concern for potential pharmaceutical exposure to humans consuming cultured fish, which may have accumulated pharmaceutical residue from their culture water. Bioconcentration studies have examined unionizable compounds (e.g., diphenhydramine hydrochloride, Nichols et al., 2015) and found organismal accumulation that was directly correlated with chemical lipophilicity. Unionized compounds are more likely to bioconcentrate compared to ionized compounds because they are typically hydrophobic and lipophilic, resulting in partitioning to organic matter and fatty substances (Jorgensen, 2010). High lipophilicity also increases the propensity for a contaminant to cross cellular membranes (Hansch and Leo, 1979; Henderson, 1908; Nejsum et al., 2001). Therefore, bioconcentration potential can be described by log K_{ow}. However, many contaminants of emerging concern (CEC) are ionizable, which complicates traditional bioconcentration models. Ionizable compounds may exist in their ionized or unionized state depending on environmental pH (Kah and Brown, 2007), which could affect their bioconcentration potential. Therefore, it is important to study individual compounds to understand bioconcentration potential in the environment.

Once a chemical accumulates in tissue, it is important to understand its depuration mechanism to maintain product safety before reaching consumers. Depuration can be estimated through mathematical modelling or lab exposure studies. The rate constants can indicate the speed of depuration from which complete depuration time can be estimated. Biological half time ($t_{1/2}$, i.e., time taken for an organism to eliminate half of the chemical from its tissues) can be used to understand the resident time of any chemical in the organism. This indicates the persistence of that chemical in an organism's body or tissues (Wright, 2002). For fish farmers, understanding potential accumulation of CEC and depuration methods may assist with producing fish economically that are "safer" for consumers. Therefore, the objective of the study was to calculate uptake and depuration of DTZ in various tissues of a popular farmed fish (*Oreochromis* *mossambicus*) using bioconcentration factor (BCF), uptake and depuration rate constants, and $t_{1/2}$. The experimental results were used to calculate the bioconcentration factor using two different approaches in various body tissues. A single compartment box model was developed to understand the statistical uptake and depuration kinetics predictions.

2. Material and Methods

2.1 Chemicals

Diltiazem hydrochloride (CAS No. 33286-22-5) and internal standard diltiazem-d_3 hydrochloride (DTZ-d_3, CAS no. 1217623-80-7) were obtained from Toronto Research Chemicals, Canada. The LC–MS grade methanol, acetic acid, and formic acid were ordered from Fisher Scientific. For fish blood collection, Kimble® Chase 42E603 150mm Heparinized Glass Natelson Capillary Tubes (250µL) were purchased from Capitol Scientific. Oasis HLB 6cc (200 mg) extraction cartridge (30 µm particle size) was purchased from Waters (Waters Technologies Corporation, MA).

2.2 Experimental design

Tilapia (*Oreochromis mossambicus*) fingerlings (mean (n=5) body weight: 5.0 ± 0.5 g, mean, total body length: 4.5 ± 0.75 cm) were purchased from Hermann's Fish Farm, Robstown, TX. Each batch was delivered separately before each experimental exposure and acclimatized in a ~890 L (total volume) circular tank for 7 days before beginning the experiment. A semi-static flow through tank system was used that consisted of eleven independent exposure systems running simultaneously; each system was composed of a 114 L reservoir tank connected to three, 38 L tanks (Appendix C, Fig. S1). Temperature was maintained at 27 ± 1 ^{o}C and photoperiod was 24:0 (light:dark). Water quality parameters were measured using appropriate Hach spectrophotometric methods and Yellow Spring Instruments (Appendix C, Table S1). Nitrogen compound concentrations were maintained through water exchanges every 8 hours for treatments longer than 12 hours. During the acclimation and experimental periods, fish were fed a commercially prepared fish feed (Rangen) at 1% of body weight per day.

Experiments were performed following internationally accepted experimental design and standardized procedures (OECD protocol TG 305, 2012; USEPA, 2002a, 2002b, 2002c) and approved by the TAMU-CC IACUC (protocol #02-16). Tissue uptake and elimination of diltiazem on steady state BCF (BCFSS; N = 3 at each time point) was determined. Each experimental tank contained 1 µg L^{-1} DTZ with a fish to water ratio of no more than 0.3-0.5 g fish/L, which adheres to EPA guidelines (EPA, 2002 a,b,c). A control group (n=2 tanks) with clean water (no DTZ added) was maintained throughout the experiment. For the bioaccumulation part of the experiment, fish (n=5 / tank) in the treatment tanks were exposed for 8 different time intervals (1, 3, 6, 12, 24, 48, 78 and 96 hrs). After 96 hrs of exposure, depuration was examined at 4 different time intervals (1, 12, 48 and 96 hrs). For each sampling time, fish were anesthetized with buffered tricaine methane sulfonate (MS 222) at ~50 mg/L (Leary, 2013) before their caudal artery was severed and blood collected in capillary tubes. Blood was then transferred to 1.5 ml centrifuge tubes that were centrifuged at 8000 rpm for 5 minutes and plasma collected in 1.5 mL centrifuge tubes for later DTZ analysis. Liver and muscle (i.e., fillet) tissues were harvested and stored along with the remaining carcass and plasma at -40 ^{o}C until further processing. Carcass was all remaining body parts after taking blood, liver, and muscle samples.

2.3 Analytical sample preparation

Methods used were modified from Ramirez et al. (2007). Briefly, each collected tissue (carcass, liver, and muscle) sample was homogenized separately and a 1.0 g subsample transferred to a 20 mL borosilicate glass centrifuge tube, where an isotopically labelled standard, DTZ-d_3 (50 µg/L) was added. Next the tissue was extracted using 8 mL of 50:50::acetic acid: methanol (v/v), followed by shaking mechanically for 25 minutes. The mixture was then centrifuged at 16000 rpm for 40 min. The supernatant was decanted into 15 mL disposable borosilicate glass culture tube (VWR Scientific) and solvent evaporated to dryness under a stream of nitrogen at 45 ^{o}C using N-EVAP^{TM}111 Turbovap (organomation-model 5085). Samples were reconstituted in 1 mL of 0.1% formic acid. Prior to analysis samples were sonicated for 1 min and filtered using centrifuged filter tube (Micro, PTFE 0.2 µm) at 8000 rpm and transferred to an HPLC vial for final analysis. For plasma, Nichols et al. (2015) was followed with slight modifications. Briefly, a 100 µL aliquot of plasma was subsampled and DTZ-d_3 added. This solution was then diluted to 5 mL using 0.1% aqueous formic acid and mixed thoroughly using sonication. Next, the diluted sample was loaded onto a preconditioned solid-phase extraction (SPE) cartridge (Oasis HLB, 6 cc, 200 mg; Waters). The target analyte DTZ was eluted off the SPE cartridges with 5 mL of methanol. Cartridge was dried with stream of nitrogen gas followed by eluted mixture evaporated to dryness as above and reconstituted with 1 mL of 0.1% formic acid and transferred to an HPLC vial.

2.4 LC-MS analysis

The instrumental analysis protocol and method was modified from Ramirez et al. (2007) and Du et al. (2012) to meet our instrument sensitivity. An Ultimate 3000 HPLC with ISQEC mass spectrometer (Thermo Scientific) was used for DTZ analysis. Analytes were separated on a 30mm X 2.1 mm (3 µm, 175 Å) Extend-C18 selectivity column (Thermo Fisher Scientific) connected with an Uniguard^{TM} direct-connection guard cartridge (2-3 mm internal diameter) with accucore RP-MS defender guard 10 X 2.1 mm (2.6 µm) (Thermo Fisher Scientific). A binary gradient consisting of 0.1% (v/v) formic acid in water and 100% methanol was employed to achieve chromatographic separation with 10 µL of each sample for 5 min. Relevant LC-MS parameters are provided in Appendix C, Table S4

To validate the results, extraction recoveries and different detection parameters were used as described in Appendix C, Table S2-S6. For extraction recoveries, the method of Ramirez et al. (2007) was used with slight modification. Briefly, two groups of control tissue samples were used. Group 1 samples were spiked with an internal standard and DTZ and group 2 samples were spiked with the internal standard only. Both groups were processed with the same extraction proce (1 In group 2 samples, after filtration DTZ was added. Recoveries were calculated with following equation:

$$Recovery = (A_{D1}/A_{IS1})/(A_{D2}/A_{IS2}) \times 100\%$$

Where, A_{D1}, A_{IS1}, A_{D2}, A_{IS2} represents the peak areas for the DTZ (D) and internal standard (IS) in group 1 and 2, respectively.

The limit of detection (LOD) was calculated as 3 times the standards deviation in the background signal observed for replicate analysis of a tissue blank. The limit of quantification (LOQ) was calculated as 10 times the standard deviation in the background signal observed for replicate analysis of blank tissue. To validate the standard, a lack of fit test was performed and residual analysis less than 20% was used to validate the standard curve.

2.5 BCF calculation, half-life, uptake & depuration model

The bioconcentration factor (BCF) of DTZ in carcass, liver, muscle, and blood plasma was calculated in accordance with OECD Guideline No. 305 (OECD protocol TG 305, 2012) as well as by calculating the ratio of the uptake and depuration rate constants. At each sampling time, the mean concentration of DTZ in each tissue type from each experimental group was divided by the mean concentration in water from which the fish was taken (BCF_a). First-order kinetics was assumed to determine the depuration rate of DTZ in carcass, liver, muscle, and blood plasma. As described by Spacie and Hamelink (1982), an alternate method to calculate BCF_b is by dividing uptake rate constant (k_u) by the depuration rate constant (k_d). Half-life (50% depuration, $t_{1/2}$) was calculated using linear regression of the natural logarithm (ln) of the detected concentrations in liver and muscle and the value of the slope (k) of the graph: ($t_{1/2}$) =0.693/K_d (OECD protocol TG 305, 2012).

For the uptake and depuration models, Stella 10.0.6 (ISEE system Inc.) was used to prepare a single box model separately. In the uptake model, DTZ exposure time interval and dose was used to prepare drug intake concentration, which was followed by first order kinetic model constants to prepare linear regression equation for predicting the next 5 days (Appendix C, Fig. S2A). The depuration model was prepared by first order kinetic linear regression equation and used to predict 4 days (Appendix C, Fig. S2B).

The one compartment model assumes fish as a single compartment containing a mixture of lipid and water. The exchange of chemical between the ambient water and the fish is through the gills and it is assumed that within the fish the chemical accumulates preferentially in the lipid phase compared with the water phase according to its lipid/water partition coefficient, all of the lipid being equally available to the chemical. During uptake the chemical simultaneously diffuses into and out of the fish with a net flux into the fish while the chemical potential, fugacity, of the chemical is greater in the ambient water than in the fish. Equilibrium is reached with a net zero flux when the fugacities in the water and in the fish are equal. The differential equation describing uptake is:

(2

$$\frac{dC_f}{dt} = k_u\, C_W - k_d\, C_F$$

where C_F and C_W are the concentrations of chemical in the fish and water, respectively, and k_U and k_d are the uptake and elimination rate constants, respectively (Gobas and MacKay, 1987; Mackay and Hughes, 1984). Integration of this equation, with C_w remaining constant, gives the equation:

$$C_{F(t)} = C_W \frac{k_u}{k_d} (1 - \exp(-k_d t)) \quad (3$$

where t is time and $C_{F(t)}$ is concentration at time t. The ratio k_u/k_d is equal to the BCF_a and Eq. (4) can be rewritten as:

$$C_{F(t)} = C_W (BCF)(1 - \exp(-k_d t)) \quad (4$$

When fish are transferred to clean water there is a chemical concentration difference between water and fish, whereby the fish depurates the chemicals resulting in their loss from fish body. By changing tank water at a fixed interval, it is assumed that the chemical is not building up in the water, which makes $C_W = 0$ and results in the following equation:

$$C_{F(t)} = C_{F(t=0)} \exp(-k_d t)) \quad (5$$

These expressions were used to model chemical uptake and depuration with known rate constants.

3. Results and Discussion

3.1 Method performance

3.1.1 LC-MS method performance

The extraction recoveries were > 90% in water, plasma and other tissue types (Appendix C, Table S2). LCMS instrumental and statistical outcomes are provided in Appendix C, Tables S3 and S4. The LOD was > 0.02 ppb (or ng/g in fish matrices) making this method suitable given our 1 ppb exposure concentration (Appendix C, Table S3). To calculate the standard curve in addition to R^2 a standards student's lack of fitness test and residual analysis test was performed. Any data describing more than 20% in residual analysis was not used for results.

3.1.2 Uptake-depuration model performance

The model performed well for uptake and depuration of DTZ in most tissues (Appendix C, Table S5), with the exception of muscle depuration where the R^2 value was 0.36. These values are similar to those from previously published studies for whole fish and plasma in gulf killifish, *Fundulus grandis* and gold fish, *Carassius auratus* (Appendix C, Table S6) (Scott et al., 2019; Sun et al., 2006). For validation of the model, unpublished and published (Scott et al., 2019; Sun et al., 2006) research data was used and their RMSE and R^2 values were compared; both fell within a good fit of RMSE<0.1 and R^2 >0.98 for the uptake model and RMSE < 0.5 and R^2 > 0.71 for the depuration model.

3.2 DTZ concentration in tissues

Uptake. DTZ is an ionizable and moderately hydrophobic compound (log K_{ow} 2.7) with bioconcentration in fish tissues from surrounding water expected (Owen et al., 2007; Steinbach et al., 2016). Total fish DTZ concentration (i.e., sum of all tissue analysis data) was 77.5 ± 3.3 ppb after a 96 hr exposure to 1 ppb DTZ. This results in a bioconcentration factor (BCF_a) of 66.1, which is 4x higher than mosquito fish (*Gambusia holbrooki*) exposed to ~0.14 ppb DTZ for 168 hrs (Wang and Gardinali, 2013). Whole fish uptake of DTZ in this study was higher than Gulf killifish (*Fundulus grandis*; ~40 ppb uptake at 48 hrs, 8.3 pH and 1 ppb exposure) (Scott et al., 2019) and male fathead minnow (*Pimephales promelas*; 4.6 ppb uptake at 96 hrs and 1 ppb exposure) (Saari et al., 2020). However, whole fish values are limited in their ability to explain uptake and depuration dynamics.

To better understand DTZ partitioning within tilapia, the liver, muscle, plasma and remaining carcass were analyzed separately. Muscle, plasma and carcass exhibited their greatest uptake rates in the first 12-24 hrs, with uptake continuing but at a slower rate until 96 hrs (Fig. (3)- 1). However, liver concentrations increased throughout the uptake phase, resulting in a concentration ~5x higher (53.98 ppb) than the carcass (11.44 ppb) > plasma (10.83 ppb) > muscle (7.83 ppb) ($p<0.001$). Therefore, the uptake rate constants (K_u) and BCF_a were also highest for liver followed by carcass, plasma and muscle (Fig. (3)- 2A and B). The liver also had higher concentrations of DTZ (0.3 – 0.7 ppb) than muscle (0.13 – 0.15 ppb) in a survey of various unspecified wild caught species in U.S. surface waters (Ramirez et al., 2009). In that study, no detections were >0.9 ppb (in whole fish tissue), which is much lower than this study. Unfortunately, no water concentrations were reported to enable bioconcentration factor calculations. Liver concentrations were higher in rainbow trout exposed to 3 ppb DTZ for 42 days, where they were 40x higher than the muscle fillet and none was detected in plasma (Steinbach et al., 2016). Additionally, rainbow trout kidney concentrations were 3.7x higher than the liver, which is contrary to our results (Fig. (3)- 1). In the present study, kidney was included with the whole carcass during analysis, which exhibited only 1/5th DTZ accumulation as the liver. Because liver and muscle are rich in proteins and phospholipids, greater binding affinity or hydrophobicity may lead to additional sorption (Luebker et al., 2002; Vanden Heuvel et al., 1992) consequently leading to a higher distribution in these tissues when exposed to such a low concentration.

Liver concentration of other calcium channel blockers (i.e., verapamil and clozapine) was reported to be higher than in muscle tissue for fathead minnow and channel catfish (Nallani et al., 2016). Muscle concentration in this study averaged 2.75 ppb over the exposure period with maximum concentration reaching 7.8 ppb at 96 hrs, equating to 7.8 times the water concentration. For rainbow trout exposed longer (21 days vs 4 days in the present study) and to a higher concentration (3 ppb vs 1 ppb in the present study), accumulation in muscle tissue was lower (4.8 ppb, Steinbach et al., 2016).

Depuration. Diltiazem elimination was fastest during the first 1-12 hours after exposure ended. After 96 hrs of depuration, 12.5 ± 2.2 ppb remained in whole fish (i.e., calculated from all tissue analyses), equating to an approximate 84% reduction. Within the fish, depuration rates were liver >carcass > plasma > muscles (Fig. (3)- 2B, $p < 0.001$). This order is similar to the findings of Steinbach et al. (2016), where liver depurated faster than the muscle (fillet). Similar results were reported for fathead minnow exposed to 500 ppb verapamil (calcium channel blocker drug), where muscle depuration took longest among all body parts and liver depurated relatively quicker (Nallani et al., 2016). In the present study, $t_{1/2}$ indicated that DTZ spent most time in muscle (18.83 hrs) and least time in liver (1.89 hrs), which matches the BCF_a value order (Table (3)- 1) and results from other studies (e.g., Wang and Gardinali, 2013). Steinbach et al. (2016) reported comparable $t_{1/2}$ values for DTZ from 1.5 (liver) to 49 hrs (kidney) in juvenile rainbow trout These data indicate that after uptake, DTZ persists differentially in the fish before being eliminated. The data indicate that DTZ bioconcentrates and depurates at a faster rate in liver than other tissues, because of its detoxifying nature and enzyme binding properties (Kuntz, 2008).

3.3 DTZ uptake and depuration model

The BCF model developed by linear regression and fit into first order kinetics demonstrated a good fit to the experimental values (Appendix C, Table- S6). Root mean square error (RMSE) and R^2 were used to fit the experimental values with predicted values. As noted in section 3.1.2., RMSE is less than 1 in all tissues examined in both the uptake and depuration model, which suggests a good fit with the experimental values. Similarly, an R^2 in all tissues examined demonstrates a good fit with experimental data. The modelled uptake exhibits a similar pattern as experimental values with earlier hour exposure treatment model values related closely compared to later hour values (Fig. (3)- 3). Modelled carcass and muscle uptake started levelling off after 8 days of exposure, whereas liver uptake levelled off after 5 days and plasma took just 4 days (data not shown). The depuration model exhibited predicted values following closely to experimental values (Fig. (3)- 4). Carcass and liver modelled depuration reached 0 DTZ concentration on day 3, whereas muscles and plasma depurated before reaching day 3 (Fig. (3)- 4). When compared with RMSE and R^2 values (Appendix C, Table S5), this model provided all RMSE value less than 1. The model results follow a similar pattern as experimental values and meet experimental values closely at initial time points, which separate at later time points. The validation result from other uptake studies by fish in whole tissue and plasma demonstrated similar pattern with good fit data (Appendix C, Table S6). The model can help predict long-term chemical concentration based on short-term values. It can also be used to limit fish exposure treatments with chemicals having longer $t_{1/2}$.

3.4 Aquaculture implications

In the U.S., ~71% of water used for aquaculture is from surface water sources (Dieter et al., 2018), often with minimal or no treatment. Surface waters are often contaminated with trace level concentrations of contaminants of emerging concern (CEC), with treated wastewater effluent being a major source of the CEC (Cahill et al., 2004; Du et al., 2012; Skees et al., 2018). As a result, aquacultured organisms grown using surface water may be exposed to these contaminants. However, the specific contaminants and their concentrations are variable and influenced by many factors (e.g., distance from WWTP, volume of surface water, precipitation). The extent of contamination and its influence on both food safety and aquaculture productivity is not known. Given the observed DTZ accumulation in muscle tissue after 96 hr exposure (7.8 ± 0.4 ppb) in the present study, a fillet serving size of ~3.5 oz (100 g) and the lowest human therapeutic dose of 120 mg, a person would have to consume >153,000 servings of tilapia to receive one therapeutic dose (Medscape, 2020; USDA, 2020a). This demonstrates that human exposure to the highest DTZ muscle concentration from this study, which was the result of exposure to an environmentally relevant concentration, would be ~6 orders of magnitude below the lowest human dose. These results are based on uptake by tilapia fingerlings over only 96 hrs, rather than the muscle of a fully-grown fish exposed to the chemical throughout its life, but it provides some insight into the scale of DTZ accumulation in edible tissues. If human exposure to DTZ in tilapia muscle were a major concern, growers could reduce DTZ muscle concentrations by harvesting fish and transferring them to "clean" water for a short period of time (i.e., days), as the $t_{1/2}$ value calculated for muscle was 18.8 hrs.

While human exposure to DTZ in fish muscle is well below a human pharmacological dose, concentrations in fish plasma could be a concern for growers. In humans, a therapeutic dose results in DTZ plasma concentrations of 30 ppb (Scott et al., 2016), which is about 20 ppb higher than the 10.8 ± 0.2 ppb observed in this study. These two values are the same order of magnitude, and as proposed by Huggett et al. (2003), if these values are within 3 orders of magnitude, further research is warranted to assess potential effects in organisms. These efforts were beyond the scope of this study, but demonstrate that effects, whether positive or negative could occur at the DTZ concentrations observed in tilapia plasma. Therefore, DTZ and potentially other CECs, despite their low plasma concentrations (ppb), could influence farmed fish and therefore deserves further investigation.

4. Conclusion

Tilapia is the fourth-most consumed fish in the United States due to its low price, easy preparation, and mild taste (USDA, 2019). Therefore, understanding its contaminant uptake and depuration kinetics can help growers plan for and mitigate exposures or effects (Austin et al., 2011). Tilapia did accumulate DTZ up to 77 ppb, but much of this was in the liver and depurated. Based on 96 hr exposure at the environmentally relevant concentration of 1 ppb, DTZ uptake by tilapia fingerlings would result in potential human exposure through consumption of muscle tissue (i.e., fillet) at ~6 orders of magnitude below the lowest human therapeutic dose. The half-life of DTZ in muscle tissue was ~18.8 hrs, indicating that the compound is processed relatively quickly in tilapia. Thus, transferring fish to “clean” water would further reduce tissue concentrations. Despite the low uptake, concentrations of DTZ in fish plasma could be a concern for growers. Plasma concentrations were on the same order of magnitude in the fish as seen in humans after a therapeutic dose. This raises the possibility of physiological effects at environmentally relevant concentrations on commercial production of tilapia but needs to be ascertained before any actions are taken or guidelines proposed.

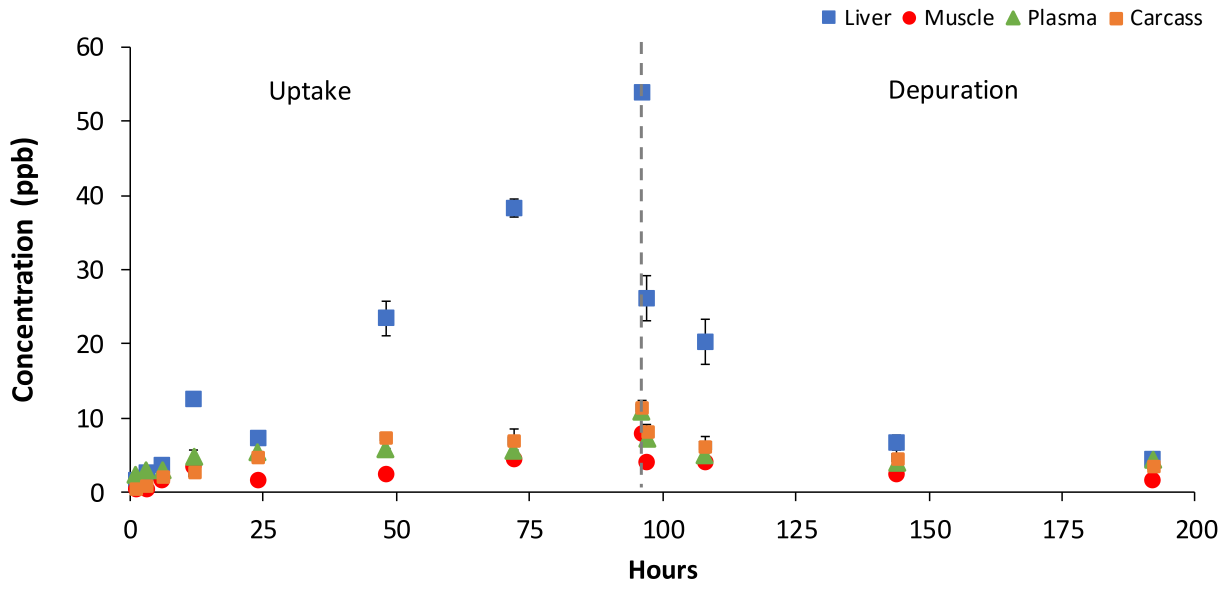


Figure (3)- 1: Measured diltiazem (DTZ) concentration (mean (± s.e., n=3)) in liver, muscle (fillet), plasma and carcass of tilapia (Oreochromis mossambicus) exposed to 1 ppb DTZ.

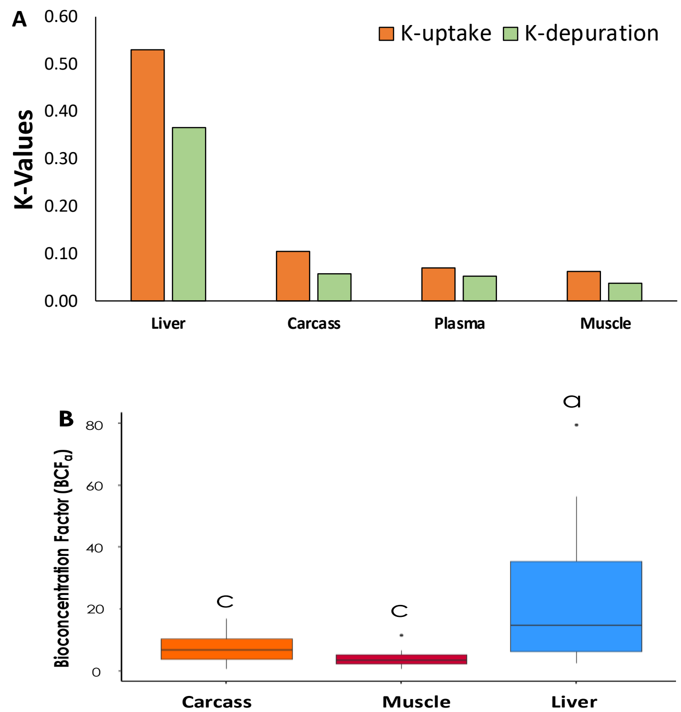


Figure (3)-2: (A) Uptake (K_u) and depuration (K_d) rate constants and (B) Bioconcentration Factor (BCF_a) in tilapia (*Oreochromis mossambicus*) liver, muscle (fillet), plasma, and carcass after exposure to 1 pbb for 96 hrs and non-exposure for 96 hrs. Bars with different lowercase letters represent statistically significant differences (HD Tukeys test, P>0.0001).

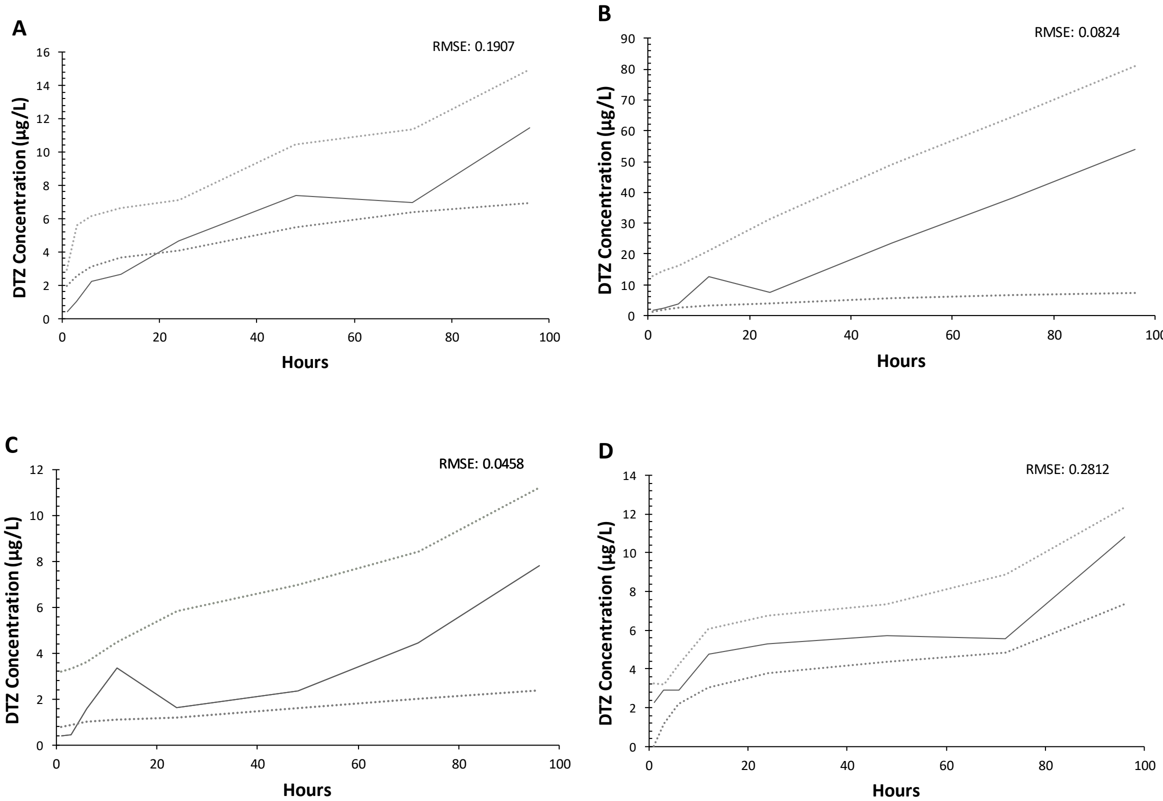


Figure (2)- 3: Diltiazem (DTZ) (uptake) values for experimental (solid) and modelled (dotted, 95% confidence interval) bioaccumulation in (A) carcass; (B) liver; (C) muscle and (D) plasma of tilapia (*Oreochromis mossambicus*) exposed for 96 hours to 1 pbb DTZ.

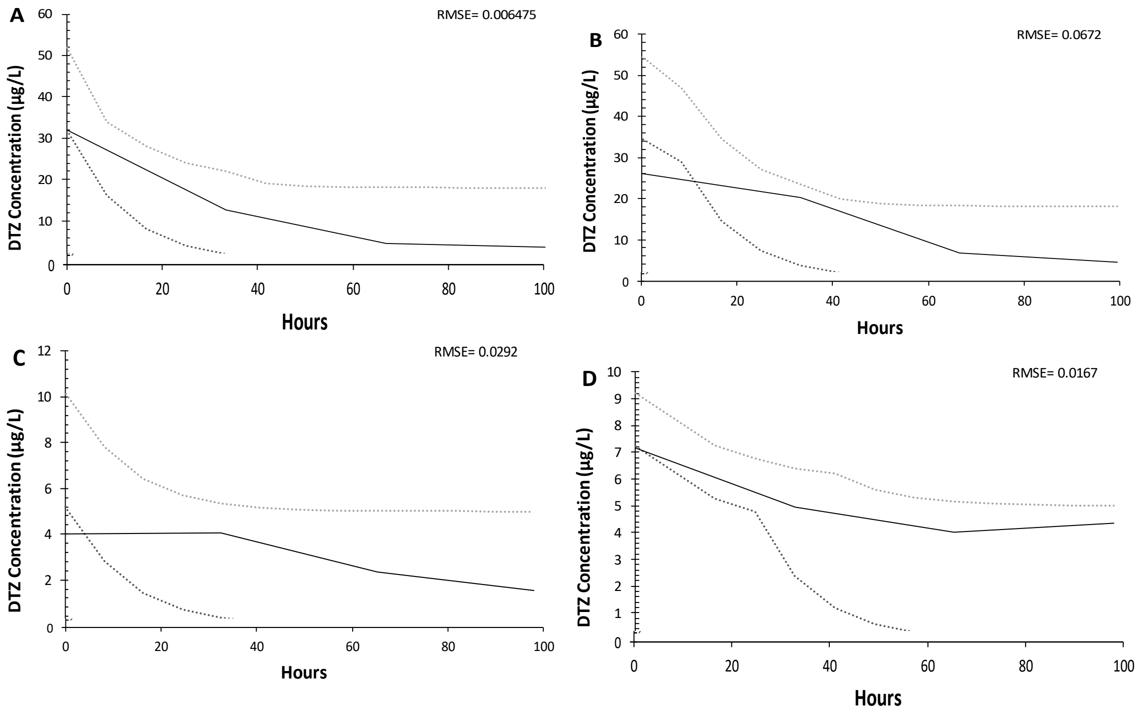


Figure (3)- 4: Diltiazem (DTZ) (elimination) values for experimental (solid) and modelled (dotted, 95% confidence interval) depuration in (A) carcass; (B) liver; (C) muscle and (D) plasma of tilapia (*Oreochromis mossambicus*) exposed for 96 hours to 1 pbb DTZ.

Table (3)-1: Bioconcentration Factor (BCF_b,), half-life ($t_{1/2}$), and change (%) in DTZ concentration at 97 hr and 192 hr in carcass, muscle, liver and blood plasma in tilapia (*Oreochromis mossambicus)* after 96 hr DTZ exposure in water at 1 pbb.

Body Parts	**BCF_b**	**$t_{1/2}$ (hr)**	**% Depuration change (from 96 hr to 97 hr)**	**% Depuration change (from 96 hr to 192 hr)**
Carcass	1.82	12.28	28.83	68.99
Muscle (Fillet)	1.69	18.83	48.69	80.49
Plasma	15.94	13.40	33.96	85.88
Liver	18.81	1.89	51.69	91.59

CHAPTER IV: COMPARISON OF BIOCONCENTRATION AND KINETICS OF GENX IN TILAPIA *OREOCHROMIS MOSSAMBICUS* IN FRESH AND BRACKISH WATER

Abstract

Contaminants of emerging concern (CEC) are causing issues from bioaccumulation in aquatic organisms to drinking water contamination. GenX (ammonium 2,3,3,3-tetrafluoro-2-heptafluoropropoxy) is one CEC occurring in the environment since it replaced other longer carbon chain perfluorinated compounds (PFC). High concentrations of GenX identified in surface waters, including estuaries, raises concern about its fate in aquatic ecosystems and potential for human exposure through consumption of wild or cultured fish. Therefore, tilapia (*Oreochromis mossambicus)* fingerlings were exposed in triplicate to 1 ppb GenX up to 96 hrs in either fresh (0 ppt) or brackish (16 ppt) water to determine uptake and bioconcentration factor values. After 96 hrs a subset of fish were exposed to non-contaminated water to determine depuration values. Bioconcentration was in decreasing order of plasma > liver > carcass > muscle, with higher distribution to liver followed by carcass and muscle. Muscle was found to have the highest half-life (1278 hrs) followed by carcass (532 hrs), plasma (106 hrs), and liver (152 hrs). The rate of uptake and depuration was positively affected by the salinity. As bioconcentration in all tissues increased with increasing salinity, this may raise concern for marine organisms and human exposure.

1. Introduction

Contaminants of emerging concern (CEC) cover a range of chemicals including pharmaceuticals, personal care products, plasticizers, flame retardants and pesticides (Farrington and Takada., 2014) and are, therefore, correlated with urban areas (UNEP, 1984). Chemicals that are considered to be CEC today have likely been entering surface waters since the beginning of the industrial age; advances in the last few decades in analytical chemistry have provided the resolution to detect CEC and quantify their biotic effects (Battaglin et al., 2007).

Their potential to bioconcentrate in plants and animals may have implications for food security for a global population expected to reach 9.7 billion by 2050 (USCB, 2018). An increasing human population will require more food and water use as noted by increased water use in agriculture and industry from 1960s to 1985 (150 to 355 billion ga per day) to 2010 (355,000 billion ga per day, USGS, 2018). Nutritious foods such as fish, which provide unsaturated fatty acids, essential micronutrients, vitamins, and minerals (Sahena et al., 2009; Scherz and Senser, 1994; Toppe et al., 2007) will see their demand continues to increase (Food and Agriculture Organisation (FAO), 2014). In 2012, global aquacultured fish production (90.4 million tonnes) was twice that of poultry and three times that of beef cattle (FAO, 2014). In 30 countries, fish accounted for greater than 1/3 of all animal protein consumed, out of which 22 of these countries are low income and food deficient (Kawarazuka and Béné, 2011). Farm raised fish require high-quality water resources, which are becoming increasingly dependent, or dominated directly or indirectly, by treated wastewater effluent (Alderson et al., 2015; Brooks et al., 2006; El-Gohary et al., 1995). Wastewater effluent is a source of various CEC that emanate from human activities. GenX (ammonium 2,3,3,3-tetrafluoro-2- (heptafluoropropoxy) is one such CEC used globally and recently found at high environmental concentrations (3−10 μg/L)(Heydebreck et al., 2015; Pan et al., 2017; Sun et al., 2016).

GenX is a processing aid used to prepare teflon polytetrafluoroethylene (PTFE) coated materials and wire cables (Beekman et al., 2016). It is one compound in a broader group of chemicals commonly referred to as fluorocarbons or perfluorinated compounds (PFC). They are mainly used as surfactants due to their long carbon chains containing fluorine and a terminal hydrophilic group and are more efficient at lowering the surface tension of water compared to hydrocarbon surfactants (Barbarossa et al., 2016). Two of the most commonly used synthetic fluorosurfactants are perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA). Within PFC, GenX is a fluorosurfactant and more specifically a PFOA with a pK_a = 3.82 (Beekman et al., 2016). It is predicted to have a low binding potential to sludge and soil due to Log K_{oc} values of 1.1 and 1.08, respectively, low Henry's law constant of 4.06 x 10^{-06} pa-m^3/mol, and water solubility of 207 mg/L (Beekman et al., 2016). GenX can dissociate at ambient temperature in water at neutral pH (pK_b= 8.10, OECD112 at 20 ^oC). Being a PFC, GenX shares some of the complex chemical properties including high solubility and protein-binding characteristics of ionic PFC that challenge conventional bioaccumulation assessments based on octanol-water partitioning coefficients (K_{ow}) (Wang et al., 2017).

Generally, bioconcentration factors (BCF) and bioaccumulation factors (BAF) of organic pollutants can be estimated from physicochemical properties such as Log K_{ow} (Neely et al., 1974). However, Log K_{ow} is not an appropriate parameter for PFC due to their water and oil-repelling properties (Giesy et al., 2006a). An alternate approach to assess bioaccumulation and bioconcentration of PFC is by a mechanical mass balance model (Mackay and Fraser, 2000) from laboratory studies involving animal exposure. In such models, uptake and depuration rates are quantified through a two-phase (uptake and depuration) experiment. Environmental variables, such as water salinity, alter these chemical behaviors and organismal physiology, which can also influence chemical persistence and toxicity within organisms (Dyer et al., 1989; Engel and Fowler, 1979; Hall and Anderson, 1995; Johnston and Corbett, 1985). For some organic contaminants, salinity can affect sorption behavior by changing the electrical state of the sorbent surface and by restricting water activity (Dontsova and Bigham, 2005; Higgins and Luthy, 2006; Turner and Rawling, 2001). Salt ions electrostatically attract water molecules and consequently trap them (decreasing water activity). For example, PFOS is reported to impair gill Na^+–K^+ATPase activity and reduce serum glucose level of a marine fish (Rockfish) under various salinity conditions (Jeon et al., 2010c). This kind of change has been observed in estuarine and coastal environment fishes observed with PFC bioaccumulation (Giesy and Kannan, 2001).

GenX is a short chain (C6) PFC that has replaced previously used compounds that were phased out due to concerns of their persistence and toxicity. While it has been touted as less persistent and toxic than its predecessors (Beekman et al., 2016), a recent study suggested higher toxicity of some fluorinated alternatives than their predecessors (Gomis et al., 2018). GenX is receiving significant attention after being found in the Cape Fear River in North Carolina, USA (NCDEQ (North Carolina Department Quality) and NCDHS (North Carolina Department of Health and Human Services), 2018; Pritchett et al., 2019). Instances like the Cape Fear River demonstrate the need to study its fate and impacts in aquatic systems. GenX has a safe drinking water advisory limit of 70 ppb with a provisional drinking water goal of 0.14 ppb, while higher concentrations, 91 to 631 ppb, have been detected in the aquatic environment globally (DWHA, 2016; Heydebreck et al., 2015; Sun et al., 2016). Wastewater is a major source of PFC in surface waters including estuaries, where salinity may range from 0.5 to 35 ppt (Hu et al., 2016).

Tilapia are the third most economically important fish in the USA and are able to tolerate wide salinity (0-60 ppt) and temperature ranges (20-30ºC) (USDA, 2020b; Whitfield and Blaber, 1979). This makes them an ideal species for aquaculture across diverse regions globally, but these broad tolerances also complicate our ability to understand the bioaccumulation and persistence contaminants like GenX in tilapia. A lack of knowledge on contaminant accumulation under various environmental conditions has direct human health implications.

Aquaculture is essential to addressing the food security challenges of our growing population. However, the bioaccumulation of organic contaminants, like GenX, could influence product safety. To understand the potential bioaccumulation and kinetics of GenX, tilapia was exposed for 96 hours followed by 96-hour depuration at two salinities (0 and 16 ppt). This data was used to calculate bioconcentration factors, half-life, and tissue distribution of GenX in tilapia.

2. Material and Methods

2.1 Chemicals

GenX (ammonium perfluoro (2-methyl-3-oxahexanoate, 97%; CAS No. 62037-80-3) and internal standard ammonium perfluoro(2-methyl-3-oxahexanoate) (GenX-IS; CAS No. 62037-80-3) were obtained from Apollo Scientific, UK. The LC-MS grade methanol, acetic acid, and formic acid were obtained from Fisher Scientific. Fish blood collection utilized Kimble® Chase 42E603 150mm heparinized glass Natelson capillary tubes (250µL; Capitol Scientific). Oasis HLB 6cc (200 mg) extraction cartridge (30 µm particle size) was purchased from Waters (Waters Technologies Corporation, MA). MS-222 was purchased from Fisher Scientific.

2.2 Experimental design

Tilapia (*Oreochromis mossambicus*) fingerlings (mean (n= 5) body weight: 5.0 ± 0.5 g, mean total body length: 4.5 ± 0.75 cm) were purchased from a local fish farm (Hermann's, Robstown, TX). Each group was delivered separately before each experimental exposure and acclimatized in a ~890 L circular tank with biofiltration for seven days before beginning the experiment. A semi-static flow-through tank system was prepared for the experiments. This system consisted of 11 independent exposure systems. Each system was composed of triplicate 38 L tanks (Total volume) that were connected to a single 114 L reservoir tank from which water was pumped. Water quality in each system was maintained daily through partial water exchanges every 8 hours (Appendix D, Table S1). During acclimation and the experimental period, fish were given commercial feed (Rangen) at 1% of body weight per day. Two separate treatments were performed at 0 and 16 ppt to examine the influence of salinity on uptake, depuration, and persistence of GenX in tilapia.

The experimental design followed internationally accepted and standardized procedures (OECD protocol TG 305, 2012; USEPA, 2002a, 2002c) and received institutional IACUC approval (TAMU-CC #10-19) . Each experimental tank system contained water with 1 ppb GenX and a fish to water ratio of no more than 0.3-0.5 g fish/L, which adheres to EPA recommendations (USEPA, 2002a, 2002c). Exposure was for 4 days followed by 4 days in clean water in the same experimental tanks (n=3). A control group of fish (n=2 tanks) that was not exposed to any GenX was maintained throughout the experiment. Bioaccumulation kinetics were determined by exposing different groups of fish at eight different time intervals (i.e., 1, 3, 6, 12, 24, 48, 78 and 96 hr). Depuration (i.e., elimination) kinetics were determined with different independent groups of fish that had been exposed for 96 hr and then sampled at 4 different post exposure time intervals (1, 12, 48, and 96 hrs). At each sampling time, all fish in each tank were removed and anesthetized with buffered tricaine methane sulfonate (MS 222) at ~50 mg/L (Leary, 2013) before having their caudal artery severed and blood collected for chemical analysis. Fish blood was collected using a 250µL capillary tube (Kimble® Chase 42E603 150mm heparinized glass Natelson capillary tube) and transferred to 1.5 mL centrifuge tubes. Collected blood was immediately centrifuged at 8000 rpm for 5 minutes, plasma collected into 1.5 mL centrifuge tubes and frozen at -40 ^{o}C until analysis. After blood collection, fish were decapitated quickly using a scalpel. Liver and muscle (i.e., fillet) were removed, placed in labelled plastic bags and stored on ice before storage in a freezer (-18 ^{o}C) until analysis. The remaining body parts, herein referred to as carcass, were placed in labelled plastic bags and stored on ice before storage in a freezer (-18 ^{o}C) until analysis.

2.3 Analytical sample preparation

Fish tissue samples were prepared for chemical analysis utilizing a modified method of Berger and Haukås (2005). Briefly, 1.0 g of each tissue (carcass, liver, and muscle) was homogenized separately and 50 ppb of GenX-internal standard (IS) was added. Samples were then extracted with 3 mL of a 50:50:: methanol/water (v/v) 2 mM ammonium acetate (NH_4OAc) solution in 15 mL polypropylene centrifuge tubes (VWR Scientific). Samples were homogenized followed by mixing through vortex and then extracted using sonication for 30 min at room temperature. Extracts were then separated from the tissue matrix at 16,000 rpm for 40 min. Supernatant was decanted (2 mL) into 3 mL disposable polypropylene centrifuge filter (Micro, nylon membrane 0.2 µm) tubes and centrifuged at 8,000 rpm, followed by transfer to an LC vial for storage until chemical analysis. The resulting solution was dried to <0.5 mL under a gentle stream of nitrogen at 30-40 ^{o}C and then reconstituted to 1mL using 2 mM ammonium acetate (NH_4OAc) solution.

Plasma samples were prepared by combining a 100 µL aliquot with 50 ppb of the GenX-IS and diluting it to 5 mL with 2 mM ammonium acetate before extraction using sonication. The solid-phase extraction (SPE) cartridge (Oasis HLB, 6 cc, 200 mg; Waters) were preconditioned with 5mL H_2O and 5 mL MeOH. Samples were then loaded onto a preconditioned SPE cartridge and target analyte eluted from the SPE cartridges with 5 mL of methanol. The eluent was dried as described above and reconstituted with 1 mL of 2 mM ammonium acetate in an HPLC vial.

2.4 LC-MS analysis

Sample analysis was performed using an Ultimate 3000 HPLC with ISQEC mass spectrometer (Thermo Scientific). Analytes were separated on a 30mm X 2.1 mm (3 µm, 175 Å) extend-C18 selectivity column (Thermo Fisher Scientific) with an Uniguard^{TM} direct-connection guard cartridge (2-3 mm internal diameter) and an accucore RP-MS defender guard 10X2.1 mm (2.6 µm) (Thermo Fisher Scientific). A binary gradient consisting of 20 mM ammonium acetate in water and 100% methanol was employed to achieve chromatographic separation with 5 µL of each sample for 3.5 min. Relevant LC-MS parameters are provided in Appendix D, Table S2.

Sample extraction methods, analytical parameters, and data validation from Berger and Haukås (2005) was used for this study. Briefly, two groups of control tissue samples were used. Group 1 samples were spiked with GenX-IS and GenX, whereas group 2 samples were spiked only with GenX-IS. Both groups were then processed through the same extraction procedure, however, after filtration, GenX was added to group 2 samples. Recoveries were calculated with the following equation:

$$Recovery = (A_{G1}/A_{IS1})/(A_{G2}/A_{IS2}) \times 100\% \quad (1$$

Where, A_{G1}, A_{IS1}, A_{G2}, A_{IS2} represents the peak areas for the GenX (G) and internal standard GenX-IS (IS) in group 1 and 2, respectively.

The limit of detection (LOD) was calculated as 3 times the standard deviation in the background signal observed for replicate analysis of a tissue blank. The limit of quantification (LOQ) was calculated as 10 times the standard deviation in the background signal observed for replicate analysis of blank tissue. To validate the standard, a lack of fit test was performed and residual analysis less than 20% was used to validate the standard curve. All data was statistically analyzed by ANOVA with $p < 0.05$.

2.5 BCF calculation, half-life, and tissue distribution

The bioconcentration factor (BCF) of GenX in carcass, liver, muscle, and blood plasma was calculated in accordance with OECD guideline No. 305 (OECD protocol TG 305, 2012). At each sampling time, the mean concentration of GenX in each tissue type from each experimental group was divided by the mean concentration in water from which the fish was taken (BCF_a). First-order kinetics was assumed to determine the depuration rate of GenX in carcass, liver, muscle, and blood plasma. As described by Spacie and Hamelink (1982), BCF_b may be calculated by deriving the depuration uptake and depuration rate constants, K_u and k_e. As described by Spacie and Hamelink (1982), half-life (50% depuration, $t_{1/2}$) was calculated using linear regression of the natural logarithm (ln) of the detected concentrations in liver, plasma, carcass and muscle with the value of the slope (k) of the graph: ($t_{1/2}$) =0.693/K_e (OECD protocol TG 305, 2012). To calculate tissue distribution within the body, each tissue (carcass, muscle, and liver) GenX concentration was divided by relative plasma concentration of GenX at the same time points. The tissue to plasma ratio is an indicator of the partitioning of contaminants between blood and tissues and can be used as a factor for evaluating their tissue distribution and toxicokinetics (Nichols et al., 2009). Apparent volume distribution (V_D) (L/Kg) was calculated from the ratio of whole-body GenX concentration to plasma concentration level.

3. Results and Discussion

3.1 LCMS method performance

Extraction recoveries were >85% in water and all tissue types (Appendix D, Table S3; Max recovery- 99.36%). The LOD was below 0.04 ppb (Appendix D, Table S4), which makes it suitable to provide confident results within our exposure concentration (1 ppb). A standards student's lack of fitness test and residual analysis test was performed to determine the standard curve in addition to R^2. Anything more than 20% in the residual analysis was not used for results.

3.2 Uptake, depuratation and bioconcentration

Uptake. Uptake rates were the highest in the first 12 to 24 hrs of exposure for both salinities tested with combined concentrations in tissues, not including plasma, ranging from 0.13 to 1.2 ppb (Fig. (4)- 1). Accumulation continued from 12 to 96 hrs, but at a slower rate resulting in combined tissue concentrations ranging from 1.2 to 2.5 ppb. Similar uptake patterns were observed in Pacific oyster (*Crassostrea gigas*) when exposed to PFC (perfluorooctanoic acid) at salinities ranging from 10 to 34 ppt (Jeon et al., 2010b). In fresh water (i.e., 0 ppt treatment) at 96 hrs, muscle tissue GenX concentration was significantly less than the other tissues ($p < 0.005$). At 16 ppt, GenX concentration in each tissue was significantly different in the following order: plasma > liver > carcass > muscle ($p < 0.005$). Similarly, in common carp (*Cyprinus carpio*) sampled from a river containing 5.2 - 68.5 ppb GenX, GenX bioaccumulated 3 times higher in the blood (1,510 ppb) than liver (587 ppb) (Pan et al., 2017). This same pattern was reported for another PFC (perfluoroundecanoic acid) in a lab study where plasma had 4x higher concentration than the liver in black rockfish (*Sebastes schlegeli*) exposed for 28 days to 10 ppb (Jeon et al., 2010a).

The accumulation of GenX in plasma and liver might be explained by the unique binding properties of PFC. Hydrophobic organic chemicals, such as chlorinated and brominated organic compounds and phenols, are known to accumulate preferentially in blubber, bile, liver, and intestines that have higher lipid content than blood (Ferreira-Leach and Hill, 2001; Geyer et al., 1987; Kannan et al., 2005; Moon et al., 2010). In contrast, biological monitoring studies suggest strong protein binding of PFC in biological systems, particularly in blood and liver (Giesy et al., 2006a; Han et al., 2005, 2004; Luebker et al., 2002). PFC exhibit a high binding affinity for plasma binding proteins (Bischel et al., 2010; Jones et al., 2003) and liver fatty acid-binding protein (Luebker et al., 2002). This could explain the pattern observed in our study where GenX accumulated at the highest concentrations in blood plasma and liver compared to muscle and carcass tissue reported by others (Gruber et al., 2007; Houde et al., 2006; Martin et al., 2003).

Similarly, differences in tissue partitioning of GenX observed between the 0 and 16 ppt treatments may be at least partially explained through the “salting-out” effect, where ions in water reduce the solubility of organic molecules (Schwarzenbach et al., 2002). When two natural systems are compared, a higher PFC concentration in salt water (0.0094–0.0312 ppb, German Bight, Ahrens et al., 2009) was observed (< 0.00024–0.0055 ppb, Pearl River Delta, China, So et al., 2004). PFC can ionize at environmentally relevant pH, forming a strong ion pair with cations that results in increasing hydrophobicity of chemicals due to neutralized charged moieties and thus partitions to particulate matter (Giesy et al., 2006). In a salinity gradient from estuarine to coastal waters, the salting-out effect can impact the fate of PFC by changing sorption properties that may affect bioaccumulation in aquatic organisms (Giesy and Kannan, 2001; Jeon et al., 2011).

Salt water and brackish water fish take in larger water volumes compared with freshwater fish to maintain their osmotic balance (Copeland, 1950; Fritz and Garside, 1974; Marshall et al., 1999; Potts and Evans, 1967). This could lead to higher contaminant exposures that result in greater bioconcentration of compounds, like PFC, that salt out depending upon the water salinity (Marshall et al., 1999; Potts and Evans, 1967). Heydebreck et al. (2015) reported GenX concentrations in saltwater at European monitoring sites from non-detectable to 0.086 ppb (median < 0.003 ppb) while concentrations observed at freshwater sites in China ranged from non-detectable to 3.1 ppb (median < 0.1 ppb). The discharge of GenX in the Xiaoqing River, China was estimated to be 4.6 t yr^{-1} (22% of total target PFC discharge) (Pan et al., 2017).

In the present study, bioconcentration of GenX was relatively low (BCF_a value < 2, Fig. (4)- 3) in all tissues sampled, suggesting lower uptake than observed in previous studies of GenX and PFC (Jeon et al., 2010a; Pan et al., 2017). BCF_a values were greatest in liver> carcass > muscle, whereas the order for BCF_b values was carcass > plasma > muscle > liver (Fig. (4)- 3; Appendix D, Table S5). When comparing tissues across treatments, muscle exposed to 16 ppt treatment had a significantly ($p < 0.005$) higher BCF_a as compared to the 0 ppt treatment (Fig. (4)- 3A).

Depuration. GenX depuration over 96 hrs was low, ranging from 20.37 to 51.52% and 26.06 to 47.89% decrease in concentration in the 0 and 16 ppt treatments, respectively. As with uptake, depuration rates were highest during first 12 hrs, followed by little or no depuration from 12 to 96 hrs (Fig. (4)- 1 and 2). Total depuration amount (by concentration) was greatest in the plasma > liver > carcass > muscle at both the salinities (Fig. (4)- 2; $P < 0.005$). The depuration rate constant increased with increasing salinity resulting in higher depuration at 16 ppt compared to 0 ppt salinity (Appendix D, Fig. S1B). Despite the higher depuration rates, each tissue exposed to 16 ppt had a significantly higher GenX concentration after 96 hrs of depuration (Fig. (4)- 2). No other published study was found that reported GenX depuration, however, similarly low depuration rate for a mixture of PFC was observed in black rockfish (*Sebastes schlegeli*) tissues over a much longer period (60 days) (Jeon et al., 2010a).

BCF and Half-life ($t_{1/2}$). The half-life values, at both tested salinities, were greatest in muscle > carcass > liver> plasma ($p < 0.005$) (Fig. (4)- 3B). The tissue BCF_a order was inverse of half-life (liver> carcass> muscle, Fig. (4)-3A) as expected. Despite liver and plasma having the highest GenX accumulation concentrations at both 0 and 16 ppt (Fig. (4)-1), the liver and plasma have shorter half-life values than carcass and muscle (Fig (4)- 3B). The results indicate tissues and plasma have longer $t_{1/2}$ at 0 ppt compared to 16 ppt salinity, where muscle had the greatest difference (i.e., 568 hrs) and plasma had the least difference (i.e., 24 hrs) (Fig (4)- 3B) between treatment salinities. Similar results were reported in blackrock fish (*Sebastes schlegeli*) where $t_{1/2}$ was in highest in carcass (124.8 hrs), followed by plasma (108 hrs) and least in liver (93.6 hrs) (Jeon et al., 2010a).

3.3 Tissue distribution

For determining the biological fate of chemicals, toxicokinetics in plasma (concentration-time curves) is crucial (Vermeire et al., 2007). The ratios of GenX in sampled tissues to blood plasma were calculated at both salinities at each time point (Fig. (4)- 4; Table (4)- 1). The GenX distribution in the liver is significantly higher than other tissues ($p<0.005$, Tukey's test), which is similar to tissue distribution (tissue:blood ratios) for the PFOA hexafluoropropylene oxide trimer acid reported in common carp (*Cyprinus carpio*) where liver was ~30% higher compared to muscle tissue (~10 %)(Pan et al., 2017); this suggests that most PFC, including GenX, share similar mechanisms of distribution.

Because liver and muscle (or carcass) are rich in proteins and phospholipids, greater binding affinity or hydrophobicity may lead to additional sorption (Luebker et al., 2002; Vanden Heuvel et al., 1992) consequently leading to a higher distribution in tissues when exposed to such a low concentration. GenX has been reported to bind with human liver fatty acid binding protein (hl-FABP), one of the most abundant proteins in the liver and additionally low dissociation constant (K_d = 4.36 ± 1.17), suggesting a stronger binding affinity of GenX to hL-FABP (De Silva and Mabury, 2006). Vanden Heuvel et al., (1992) suggested that in rats the high hepatic accumulation of PFC was due partly to the binding of PFC to intracellular proteins, such as fatty acid-binding proteins.

In the present study, the mean total apparent volume distribution (V_D) for tilapia exposed to GenX in saltwater was greater than tilapia exposed to GenX in freshwater (Table (4)- 1).This demonstrates preferential distribution of GenX in tilapia at 16 ppt compared to 0 ppt is higher within intravascular fluid and plasma compared to whole-body tissue.

Muscle concentration of GenX at 96 hrs for tilapia at 0 and 16 ppt was ~0.14 ppb and 0.312 ppb respectively (Fig. (4)- 2). The human subchronic daily exposure limit is 0.2 ppb as recommended by USEPA (2018b). If the values in the present study were for a harvestable tilapia, a fillet serving size of ~3.5 oz (100 g) would contain either 14.0 or 31.2 µg (ppb) GenX depending on salinity. Therefore, a single serving would expose a person to 69 (0 ppt) or 155 (16 ptt) times the subchronic oral reference dose (USEPA, 2018b).

4. Conclusion

The different uptake and depuration rate constants for GenX in tilapia at different salinities can assist with determining the fate of GenX in an estuarine environment. A similar pattern was observed in oysters and other marine fishes, which provides a constructive fate of PFC under different salinity conditions. The major concern with GenX is with its depuration, as depuration is not as fast as bioaccumulation. In the present study, half of the GenX that accumulated in the fish still remained after 96 hours of depuration, with little change in tissue concentration after the first few hours. Other PFC have been noted to return to original levels with extended depuration time (> 30 days) (Jeon et al., 2010b, 2010a). In regard to aquaculture, it is probably not economic to depurate for such a long time. Another concern with GenX is it has a very long half-life in muscle. Why this occurs was not an objective of the present study but needs to be examined to assist with aquaculture management and understanding the potential impact of GenX in natural ecosystems.

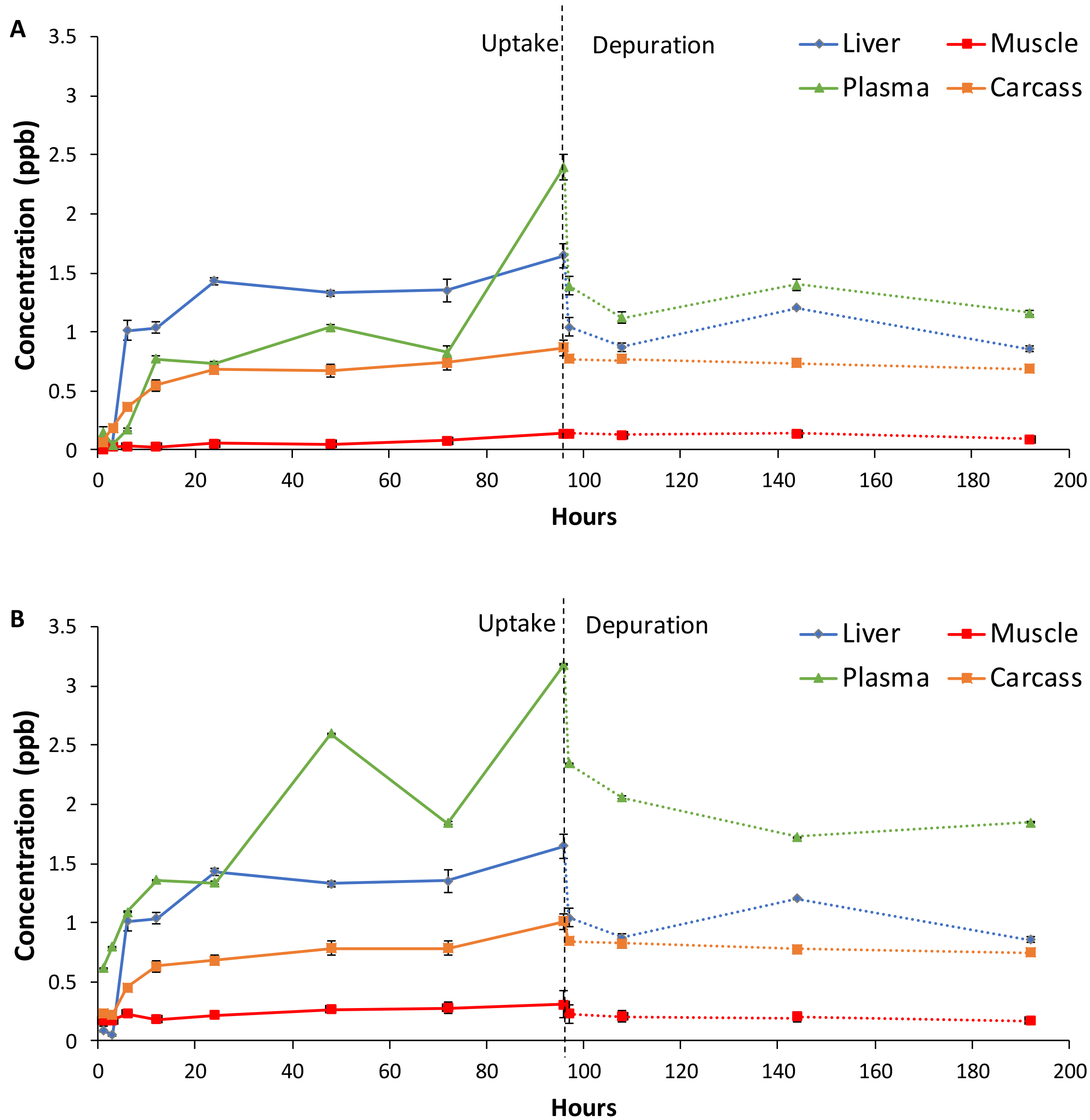
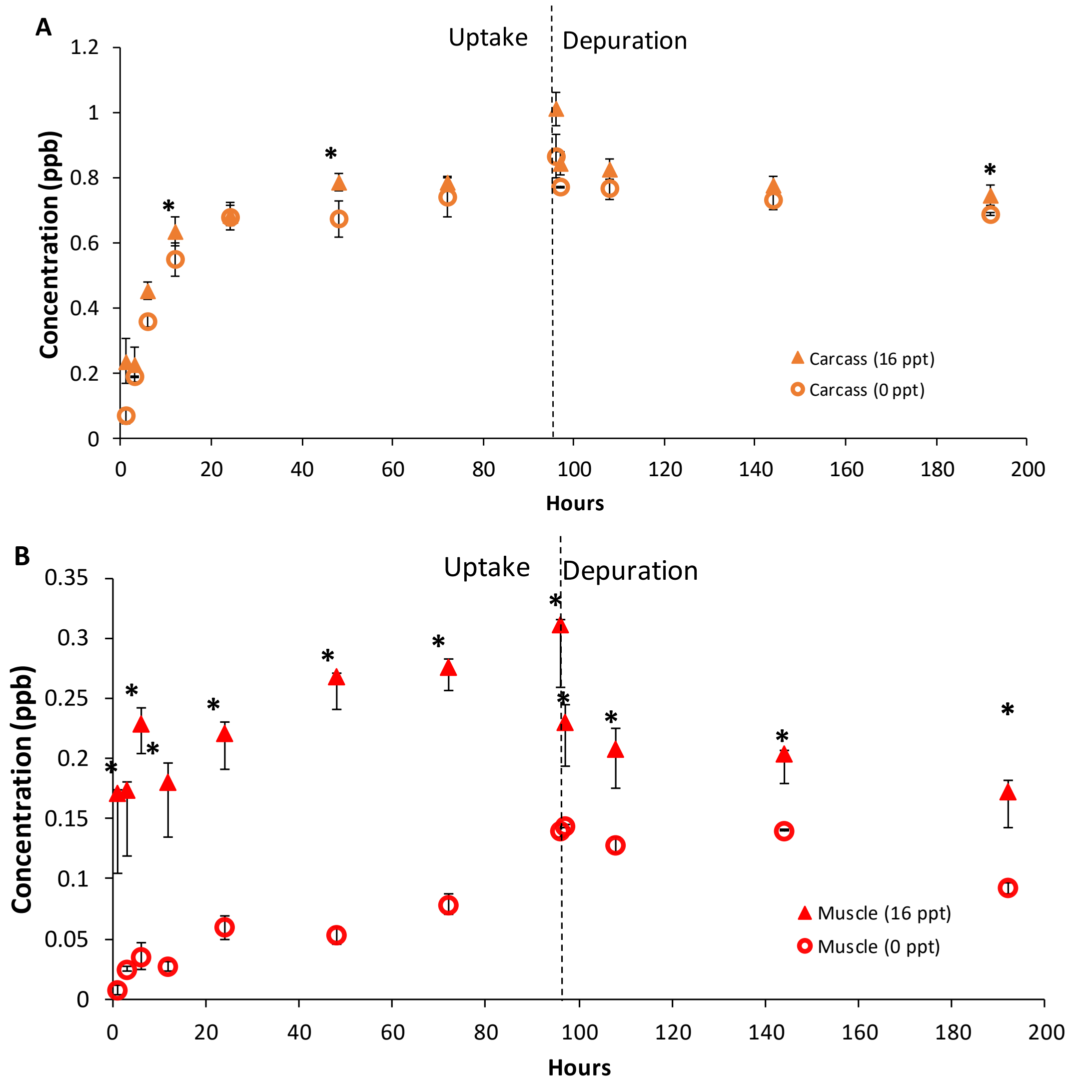


Figure (4)- 1: GenX uptake and depuration, each 96 hrs, in tilapia (*Oreochromis mossambicus*) liver, muscle (fillet), plasma and carcass exposed to 1 ppb in A) 0 and B) 16 ppt salt. Individual data points are given as the mean ± standard error (n=3, pooled samples).


A
Uptake
Depuration
Concentration (ppb)
Hours
Carcass (16 ppt)
Carcass (0 ppt)
B
Uptake
Depuration
Concentration (ppb)
Hours
Muscle (16 ppt)
Muscle (0 ppt)

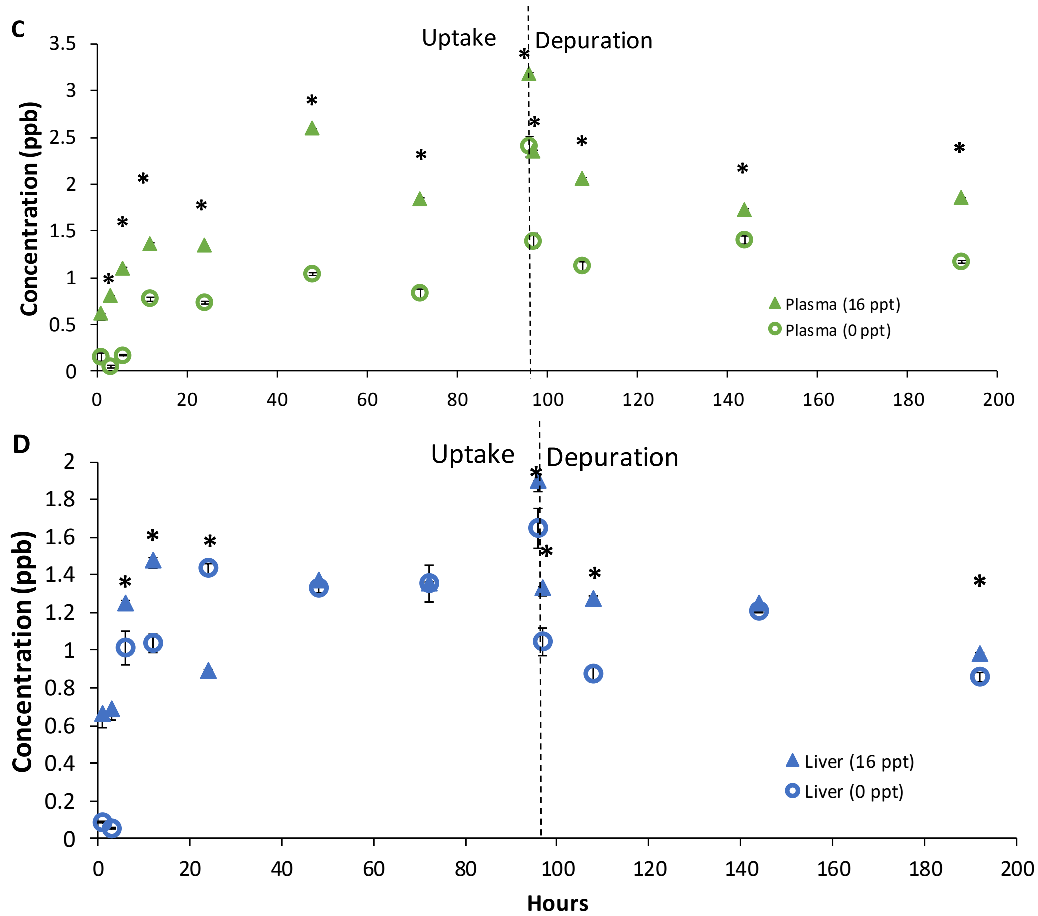


Figure (4)- 2: GenX uptake and depuration tissue comparison in tilapia (*Oreochromis mossambicus*) A) carcass; B) muscle (fillet); C) plasma and D) liver exposed to 1 ppb at 0 ppt and 16 ppt salinity. Individual data points are mean ± standard error (n=3, pooled samples). Circles represent 0 ppt salinity and triangles are 16 ppt salinity. * indicates statistically significant difference ($p<0.05$, Tukey's test) at that time point.

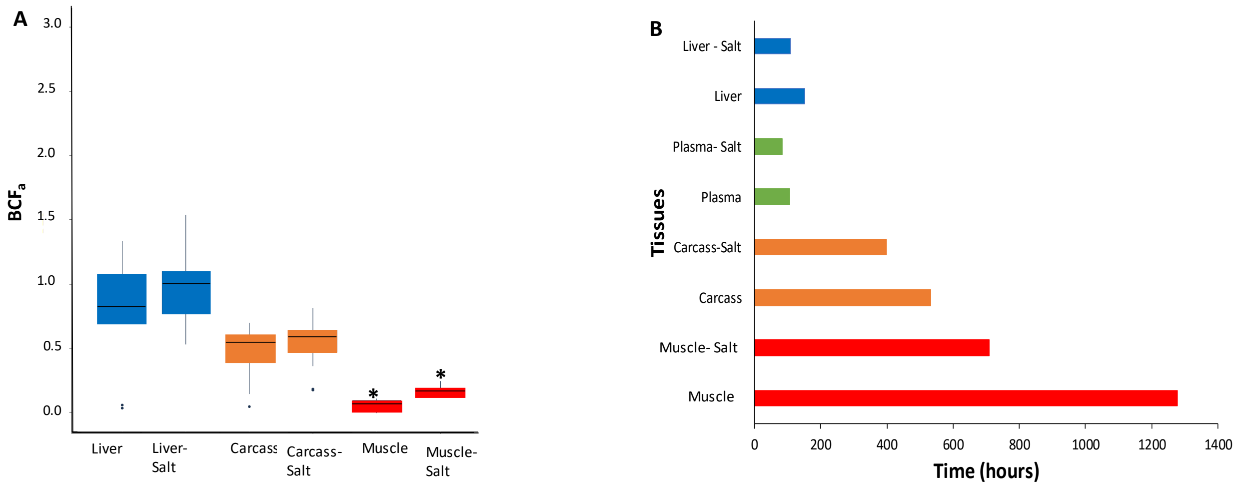


Figure (4)-3: A) Steady-state bioconcentration factor (BCF_a) in carcass, muscle (fillet) and liver and (B) half-life ($t_{1/2}$) of GenX in carcass, plasma, muscle (fillet) and liver of tilapia (*Oreochromis mossambicus)* exposed to 1 ppb GenX for 96 hrs at salinities of 0 or 16 (salt). * denotes statistically significant difference from other tissues (P<0.05)

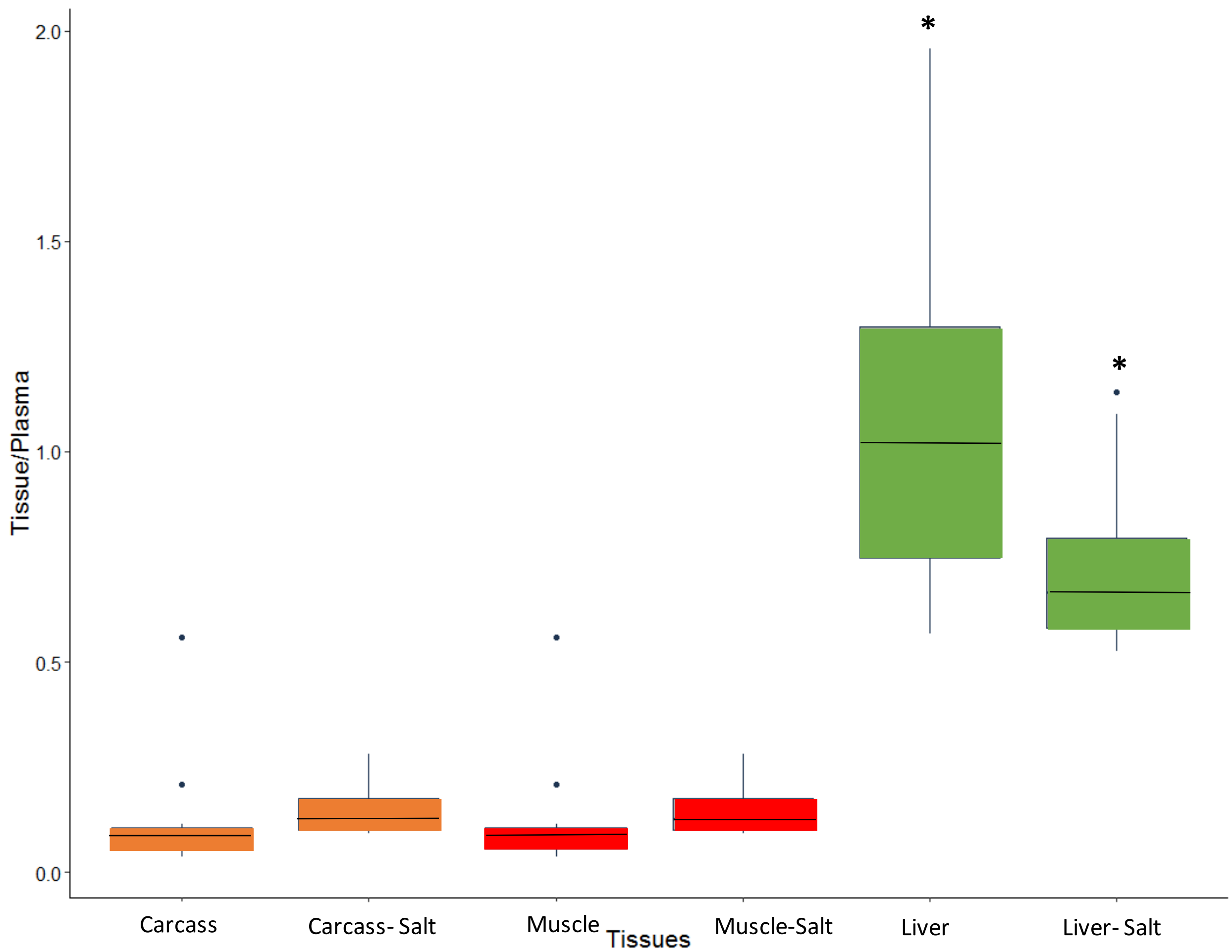


Figure (4)- 4: Tissue distribution (i.e., ratio of tissue concentration to plasma concentration) of GenX in tilapia (*Oreochromis mossambicus*) exposed to 1 ppb GenX for 96 hrs at two salinities (0 and 16). * denotes statistically significant difference from other tissues (P<0.005).

Table (4)-1: Mean (SD) (n=3) apparent volume distribution (V_D) (L Kg^{-1}) values of tilapia fingerlings (*Oreochromis mossambicus*) exposed for 96 hrs to 1 ppb GenX.

Time points (Hours)	V_D (0 ppt) (SD)	V_D (16 ppt) (SD)
1	2.39 (0.34)	1.68 (0.37)
3	7.63 (0.22)	1.46 (0.43)
6	8.47 (0.52)	1.78 (0.39)
12	6.83 (0.64)	1.71 (0.18)
24	1.92 (0.37)	1.35 (0.30)
48	3.57 (0.70)	0.98 (0.18)
72	2.42 (0.17)	1.33 (0.22)
96	2.85 (0.50)	1.10 (0.39)

CONCLUSION

Four USGS hydrological unit codes (HUCs 13, 15, 16 and 18) had the lowest average wastewater dilution factor (WWFD) over the last decade and were in the top 10 U.S. regions for population growth from 2010-2016; a trend that is projected to continue. This growth will further increase wastewater effluent discharge volumes, which will result in greater wastewater proportions in surface waters. Therefore, more CEC and higher concentrations of these compounds are likely to occur. Downstream users of this water, whether they perform treatment or not, may need to consider the presence of CEC and potential effects depending on the intended water use. These considerations will be most important during abnormally dry or drought conditions, when surface-water dilution of wastewater is limited. This may require monitoring of CEC, particularly those that pose a risk to humans and aquatic organisms, and possibly targeted treatment where dictated based on risk assessments.

Surface water availability depends upon a variety of factors. Changing climate and population effect surface water availability especially in high water demand areas. During 2015, the southwest and western U.S. (10 HUCs), where populations are growing and there is low precipitation, contained 5 of the 6 VLWA and 4 of the 5 LWA regions. Population growth may increase surface water withdrawals, which can reduce river baseflows, but also at least partially recharge those flows due to increasing volumes of lower quality WWTP effluent. This may result in the overall water quality of a system declining, which will be most evident in rivers that are permanently or periodically effluent-dominated or dependent. In the early 2000’s, 23% of regulated U.S. effluent releases into streams receive less than a 10-fold dilution; under low-flow conditions it may go up to 60% (Brooks et al., 2006). For example, 285 of 582 regulated discharges in Texas, Oklahoma, New Mexico, Arkansas, and Louisiana enter surface water bodies in which effluent accounts for >90% of the instream flow (Brooks et al., 2006). As climate change accelerates and populations grow, what is considered low flow today may become the norm in parts of the U.S., increasing the number of effluent-dominated or -dependent rivers and streams.

Tilapia is the fourth-most consumed fish in the United States due to its low price, easy preparation, and mild taste. Therefore, understanding its contaminant uptake and depuration kinetics can help growers plan for and mitigate exposures or effects (Austin et al., 2011). Tilapia fingerlings accumulated DTZ up to 77 ppb, but much of this was in the liver and depurated. Based on 96 hr environmentally relevant concentration DTZ uptake, human exposure to the highest DTZ muscle concentration would be ~6 orders of magnitude below the lowest human therapeutic dose, thus resulting virtually in no human exposure. Additionally, the half-life of DTZ in muscle was 18.83 hrs, indicating that even when accumulated, the compound is processed relatively quickly in tilapia. This suggests that transferring fish to "clean" water would further reduce concentrations. Despite the low uptake, concentrations of DTZ in fish plasma could be a concern for growers. Plasma concentrations were on the same order of magnitude in the fish as seen in humans after a therapeutic dose. This raises the possibility of effects at environmentally relevant concentrations on commercial production of tilapia.

The different uptake and depuration rate constants with different salinities can assist with understanding the fate of GenX in an estuarine environment. Salinity changes found in an estuary may change the kinetics of GenX daily and result in higher toxicity in the marine environment as compared to freshwater conditions. A similar pattern has been observed in oysters and other marine fishes, which provides a constructive fate of PFC under different salinity conditions. A major concern with GenX is with its depuration, as depuration was not as fast as bioaccumulation. GenX did not return to initial values when depurated for 96 hours after exposure. Other PFC have demonstrated a return to original levels when depuration was extensive (>30 days) (Jeon et al., 2010b, 2010a). In regard to aquaculture, it is probably not economical to depurate for such a long time. Another concern with GenX is that with a long half-life, how may this affect organisms in natural environments through trophic transfer.

Today, when wastewater is discharged into surface waters, regulations (e.g., National Pollutant Discharge Elimination System) require that it is diluted by a specified factor within a specified distance from the discharge point in order to minimize negative environmental effects. Currently, treated wastewater is not accepted for direct reuse in U.S. aquaculture, but *de facto* reuse is occurring. Regions with the greatest temperature increases, precipitation declines, and population growth will experience increasing wastewater effluent loading, resulting in CEC concentrations that may require assessment of exposures and possible impacts to farm-raised fish and to humans. The present study examined data to assess trends in wastewater dilution as well as short-term projections that can be used by policy makers as well as stakeholders for planning. It is imperative to make informed projections of future water quality and quantity and determine the potential for CEC to bioaccumulate in farmed aquatic organisms in order to ensure their continued health benefits for human consumers.

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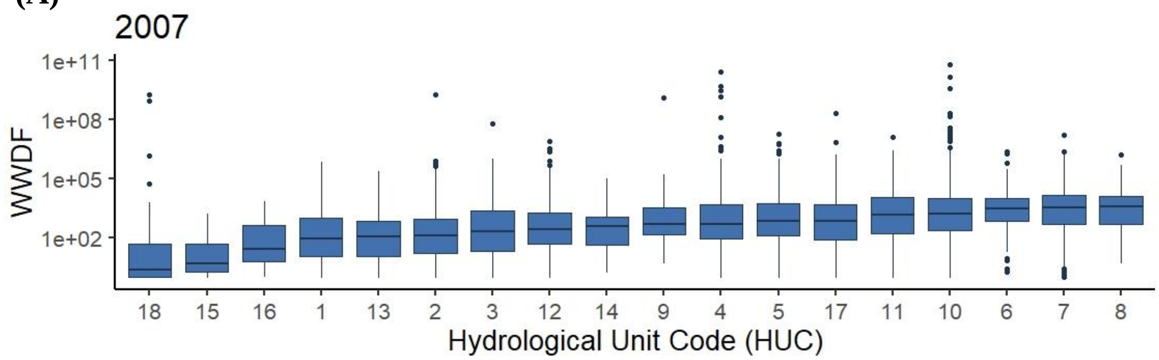
LIST OF APPENDICES

Appendix A. Chapter I Supplementary information

(A)



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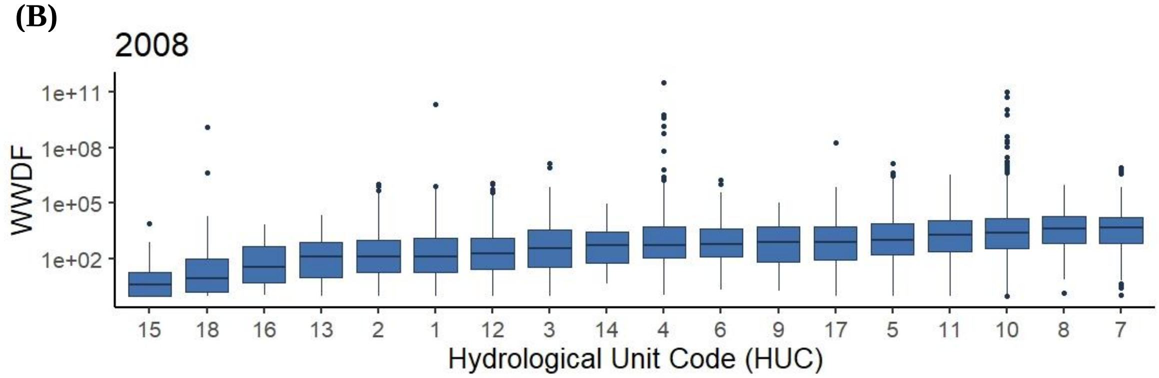

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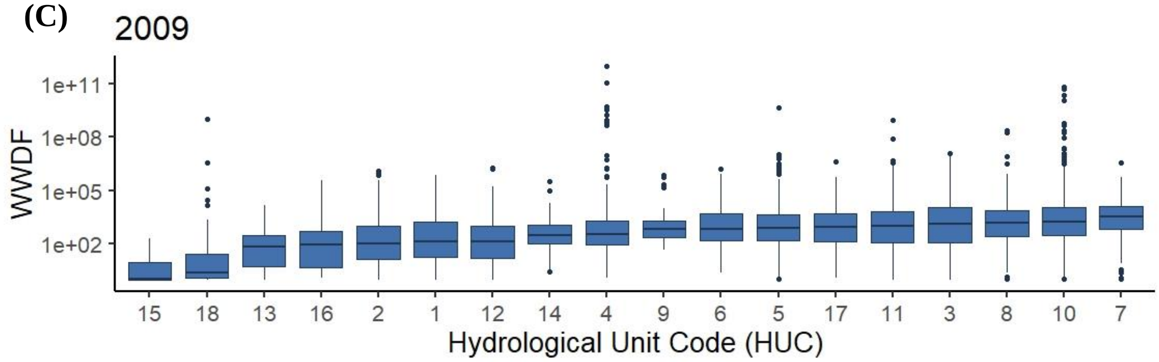

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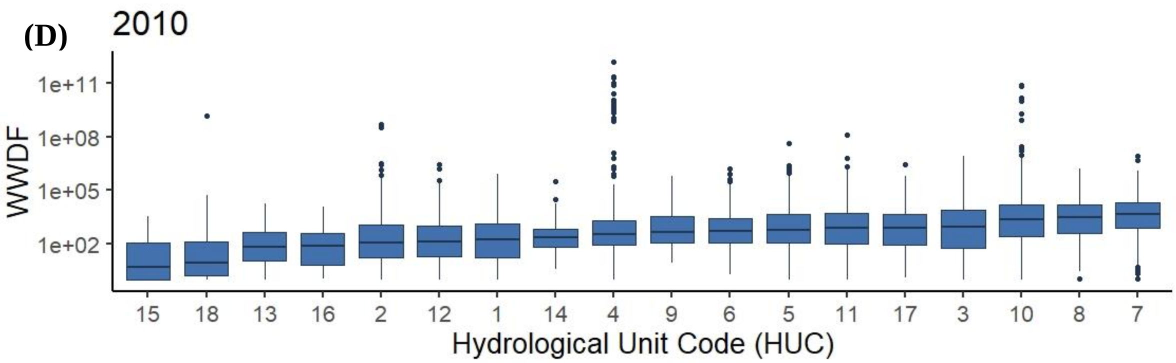
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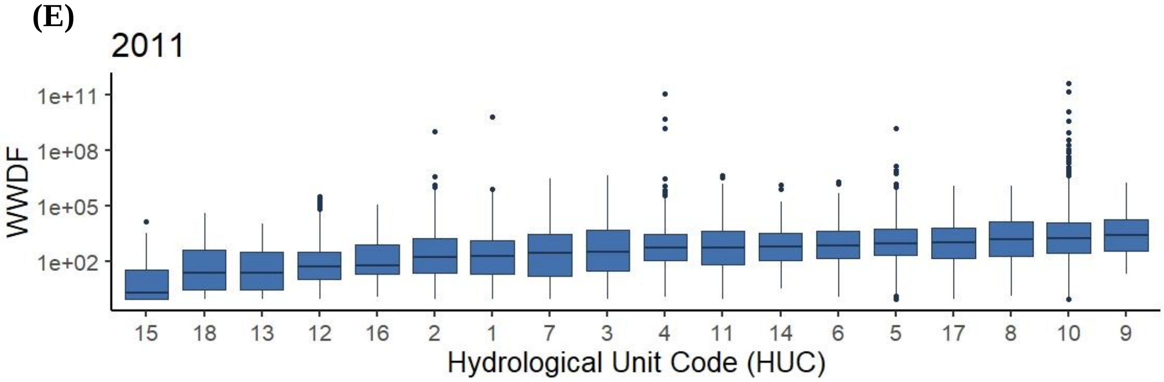
(E)


2011
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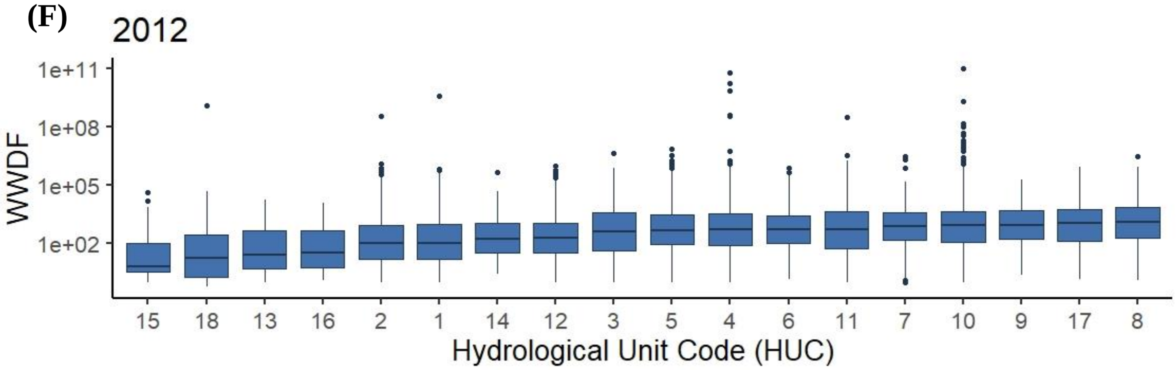
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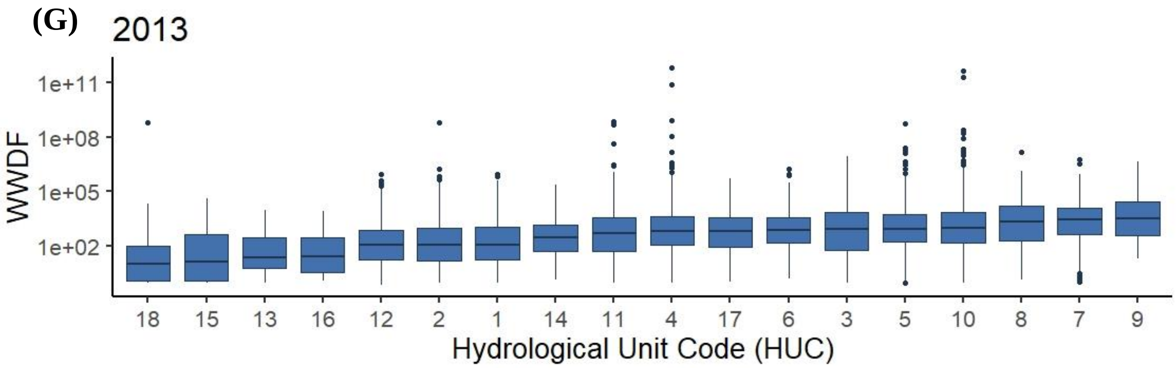
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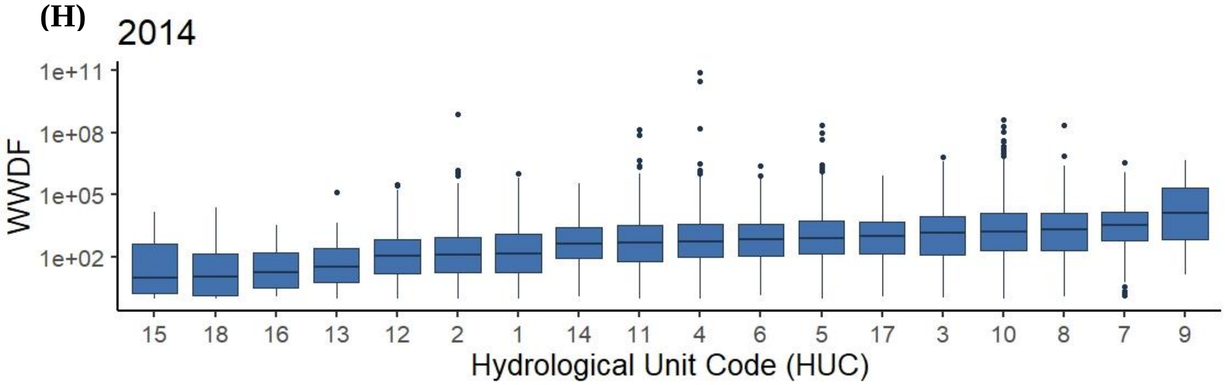
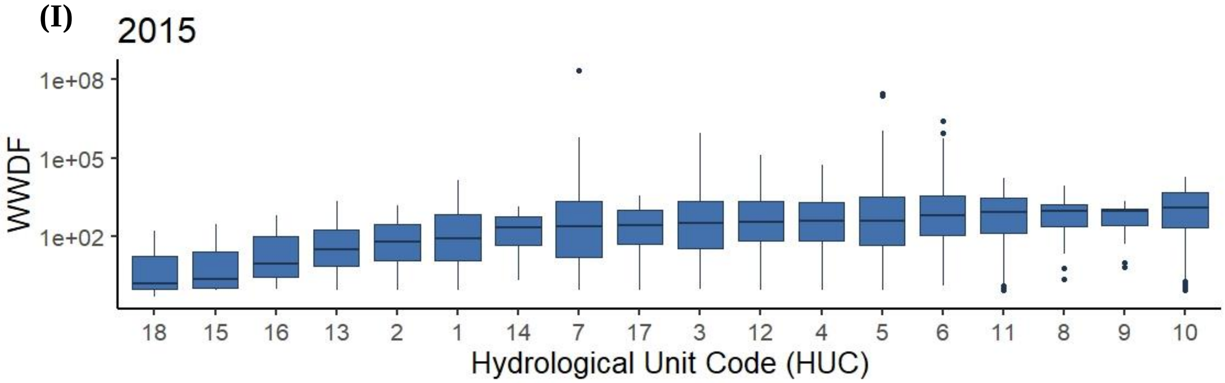


(I)



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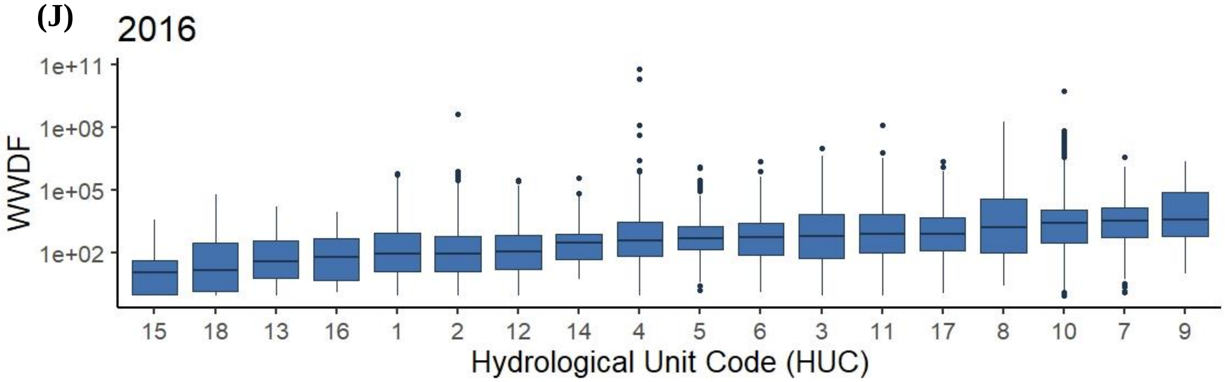

Fig. S1 Average WWDF from 2007 to 2016 (A-J) for each U.S. hydrologic region. The top, middle and bottom lines of box represent the 75th, 50th and 25th percentile with points representing outliers beyond the 10th and 90th percentile. Top and bottom vertical lines represent largest and smallest value within 1.5 times interquartile range above 75th Percentile and below 25th percentile respectively

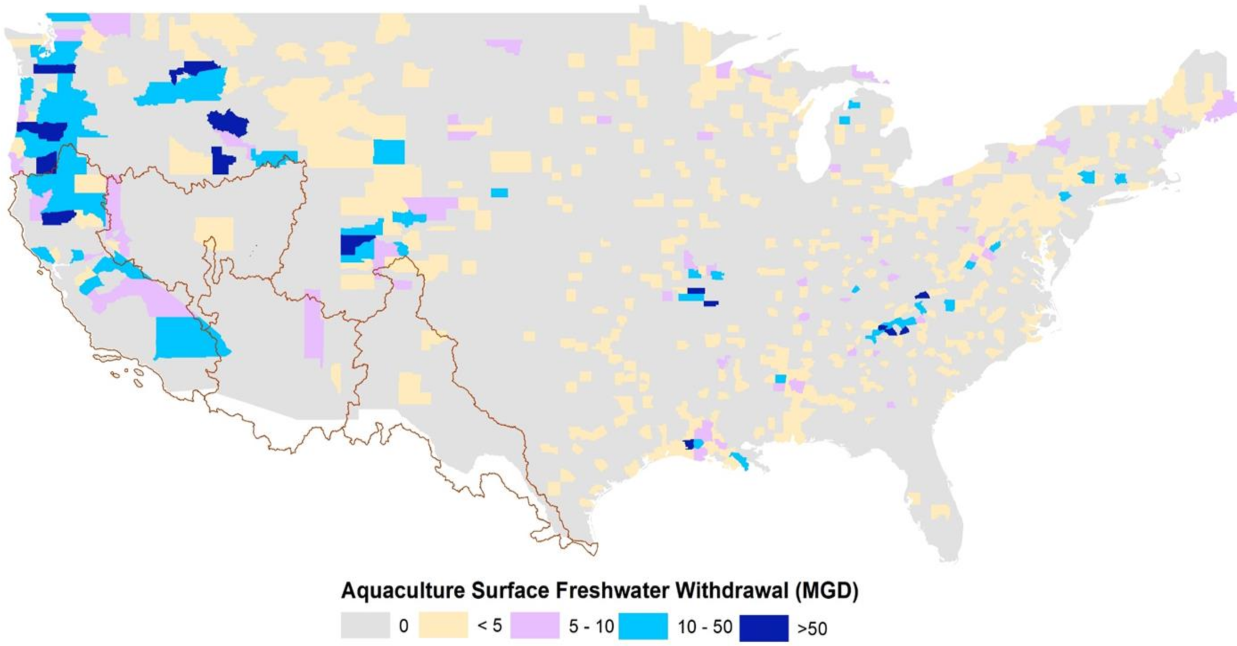

Fig. S2 Total aquaculture surface freshwater withdrawal in million gallon per day (MGD) for the contiguous U.S. by county in 2015 with HUC 13, 15, 16, and 18 outlined (USDA, Census of Aquaculture, 2018).

Appendix B. Chapter II Supplementary information.

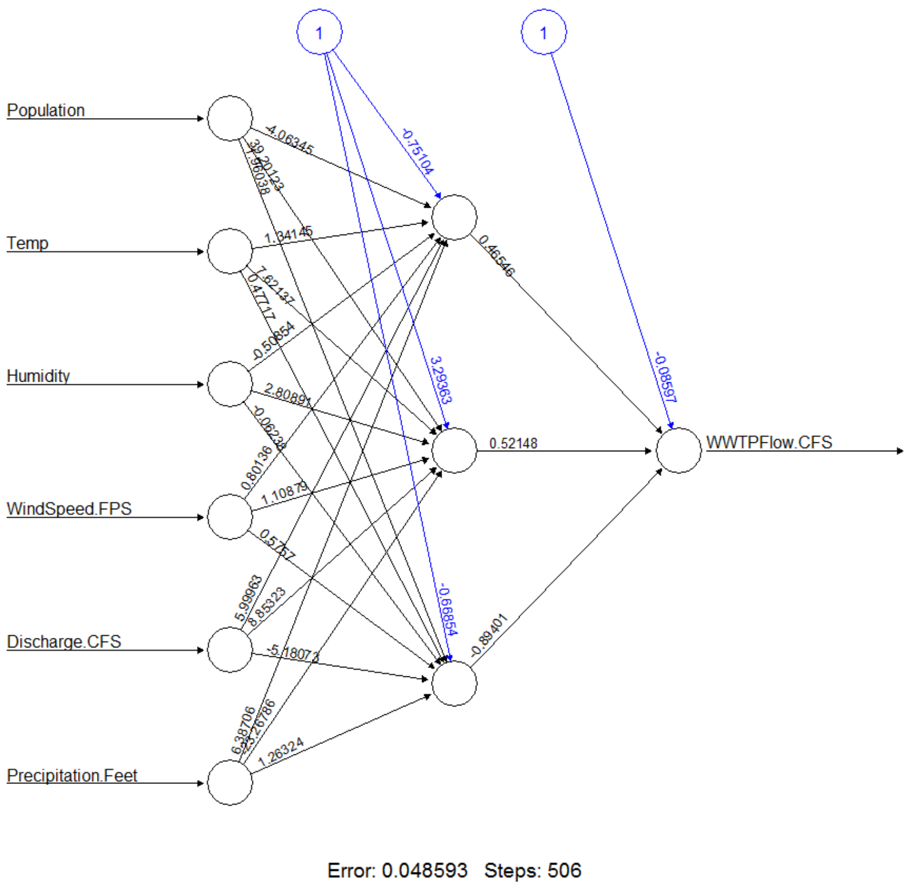

Fig. S1 Outcome of artificial neural net (ANN) model run 100 times in R.

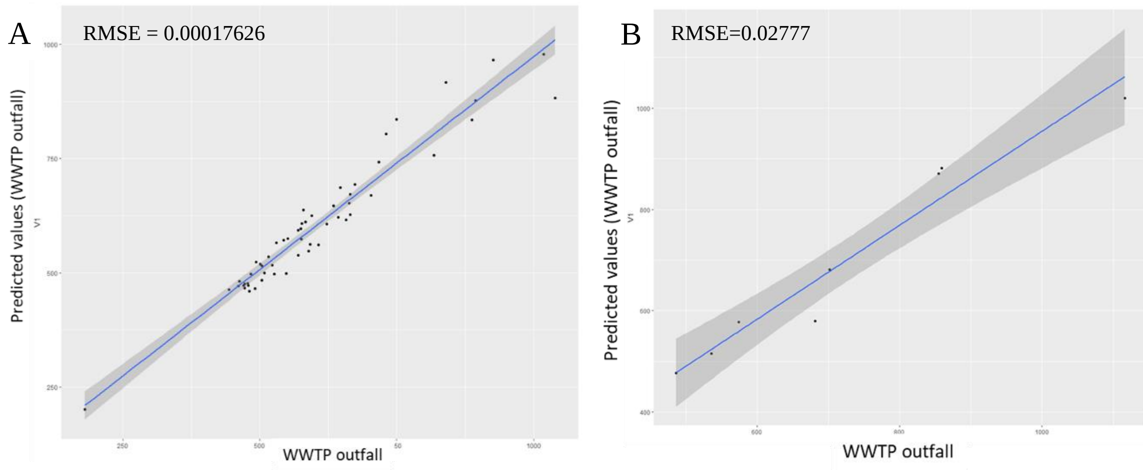

Fig. S2 Root mean square error (RMSE) value calculated for A) training data and B) validation data.

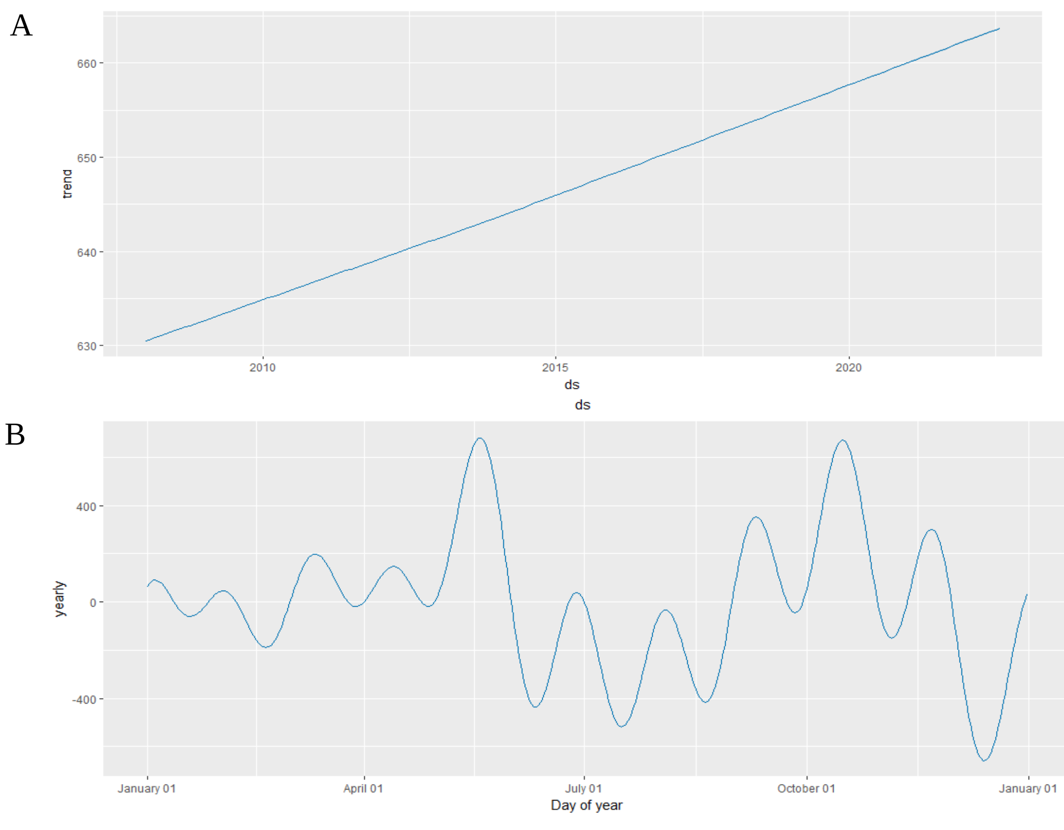

Fig. S3 Wastewater treatment plant (WWTP) effluent flow component graphs for the forecasting model representing A) trends and B) seasonal patterns for the entire forecasting period (2007-2020) from San Antonio, TX region.

Appendix C. Chapter III Supplementary information.

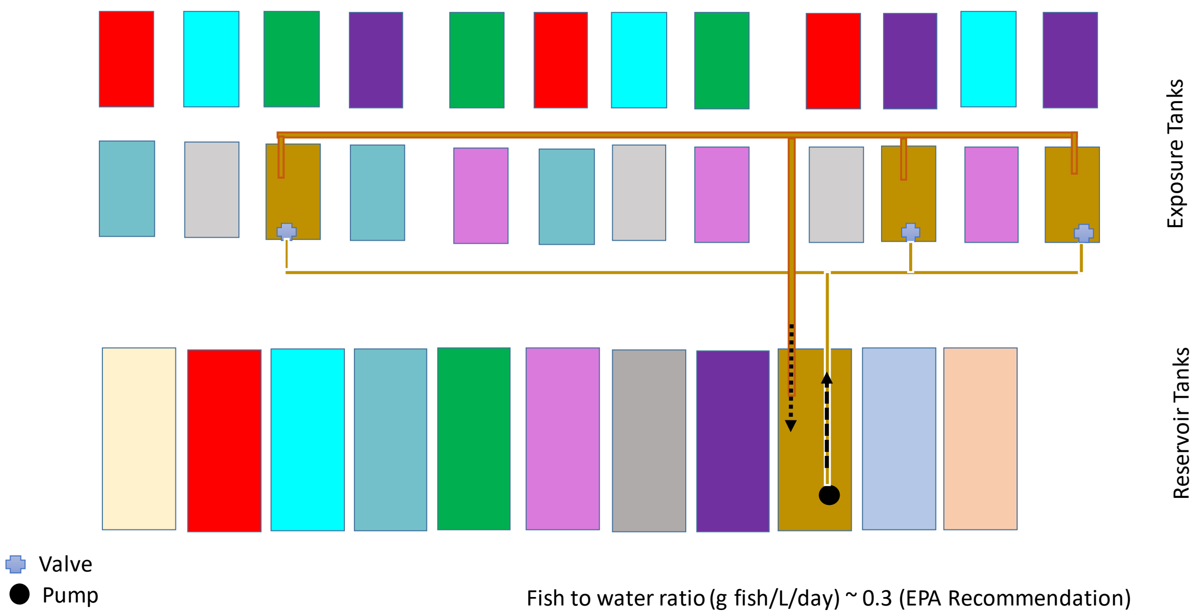

Fig. S1 Semi-static flow-through system. Each color reservoir tank represents its exposure tank replica and random experimental setup (e.g., dark amber is one complete system).

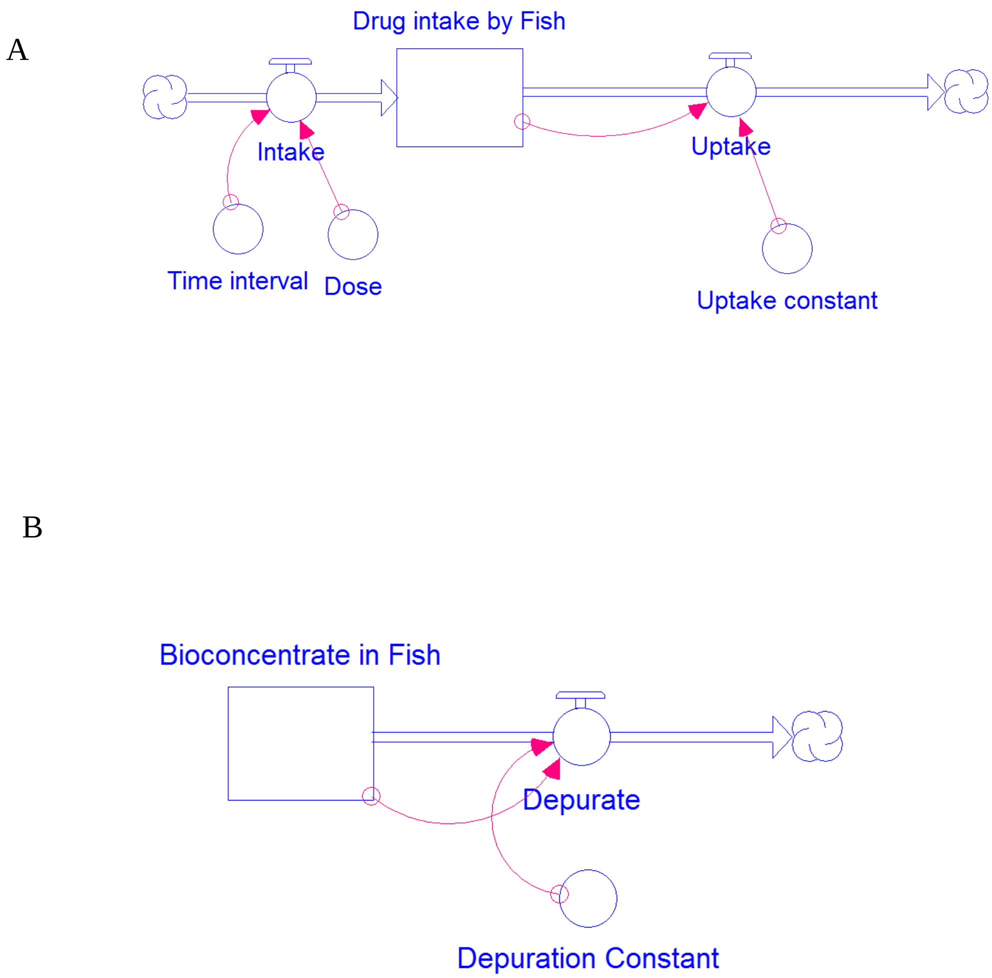

Fig. S2 Stella model for (A) Uptake and (B) Depuration of diltiazem exposure to fish.

Table S1 Mean ± SD (n=11) values for water quality parameters for experimental tanks of tilapia (*Oreochromis mossambicus*) exposed to 1ppb diltiazem for up to 96 hrs.

Dissolved oxygen (ppm)	pH	Temperatur e (°C)	Nitrite (ppm)	Nitrate (ppm)	Unionized Ammonia (ppm)
8.5 ± 0.5	7.7 ± 0.5	26 ± 1	0.50 ± 0.02	2.00 ± 0.05	0.200 ± 0.002

Table S2. Extraction recoveries (%) of diltiazem from water and tissues of tilapia (*Oreochromis mossambicus*) exposed to three diltiazem concentration (n=5, mean ± SD).

	Water	Carcass	Liver	Fillet	Plasma
Extraction recoveries	92.97 ± 5.1	95.88 ± 6.2	86.96 ± 8.2	111.54 ± 10.1	116.78 ± 11.4

Table S3. Linear range water and tissue concentration of diltiazem with limit of quantification (LOQ) and limit of detection (LOD), lack of fit test (F-calculated) parameters.

	Linear range	LOQ	LOD	F-calculated
Water (µg L^{-1})	0.1-12	0.112	0.026	0.005
Tissue (ng g^{-1})	0.1-12	0.136	0.041	0.0025

Table S4. LC-MS analytical parameters for diltiazem (DTZ) and its internal standard (DTZ-d_3).

	m/z	Ion Polarity	Vaporization Temperature (^oC)	Ion transfer tube temperature (^oC)	Source voltage positive ion (V)	Sheath gas pressure (psig)	Aux gas pressure (psig)	Total pump flow (mL/min)
DTZ	415.2	Positive	300	300	3500	20	15	0.2
DTZ-d_3	418.2	Positive						

Table S5. Calculated uptake and depuration model error (RMSE) and their R^2 values.

Tissues	Uptake		Depuration	
	RMSE	R^2	RMSE	R^2
Carcass	0.197	0.77	0.492	0.94
Liver	0.082	0.87	0.067	0.67
Muscle	0.046	0.83	0.292	0.36
Plasma	0.281	0.68	0.017	0.95

Table S6. Validation results (RMSE and R^2) for model graphs for DTZ uptake and depuration in fish from different studies (gulf killifish, *Fundulus grandis* and goldfish, *Carassius auratus*)

Tissues	Uptake		Depuration	
	RMSE	R^2	RMSE	R^2
Whole fish	0.072[a]	0.94[a]	0.019[a] 0.147[b]	0.72[a] 0.77[b]
Plasma	0.093[c]	0.99[c]	0.008[c]	0.88[c]

[a] unpublished data, B. Brooks, Baylor University; [b]Sun et al., 2006; [c]Scott et al., 2019

Appendix D. Chapter IV Supplementary information.

Table S1. Mean ± SD (n=11) values for water quality parameters for experimental tanks of tilapia (*Oreochromis mossambicus*) exposed to 1ppb GenX for up to 96 hrs.

Dissolved oxygen (ppm)	pH	Temperature (°C)	Nitrite (ppm)	Nitrate (ppm)	Unionized Ammonia (ppm)
8.3 ± 0.4	7.7 ± 0.3	27 ± 1	0.50 ± 0.02	1.99 ± 0.02	0.200 ± 0.002

Table S2. LC-MS quantitative information for GenX and its internal standard (GenX-IS).

	m/z	Ion Polarity	Vaporization Temperature (°C)	Ion transfer tube temperature (°C)	Source voltage Positive ion (V)	Sheath gas pressure (psig)	Aux gas pressure (psig)	Total pump flow (ml/min)
GenX	415.2	negative	124	150	2000	20	15	0.2
GenX –IS	418.2	negative						

Table S3. Extraction recoveries (%) from water and tissues of tilapia (*Oreochromis mossambicus*) exposed to three GenX concentration (n=5, mean ± SD).

	Water	**Carcass**	**Liver**	**Fillet**	**Plasma**
Extraction recoveries	90.97 ± 4.06	93.88 ± 3.05	88.96 ± 6.00	99.36 ± 9.01	98.96 ± 16.00

Table S4. LC-MS parameter mean water and tissue concentration with limit of quantification (LOQ), limit of detection (LOD), and lack of fit test (F-calculated) parameters.

	Linear range(ng/g)	LOQ (ng/g)	LOD (ng/g)	F-calculated
Water	0.2-10.01	0.151	0.045	0.004
Tissue	0.2-9.01	0.125	0.039	0.003

Table S5. Bioconcentration factor (BCF_b) values in carcass and different tissues of tilapia (*Oreochromis mossambicus*) after 96 hr exposure to GenX and 96 hr depuration.

Tissues	0 ppt	16 ppt
Carcass	5.58	4.31
Plasma	2.97	2.94
Muscle	2.20	1.56
Liver	0.26	1.54

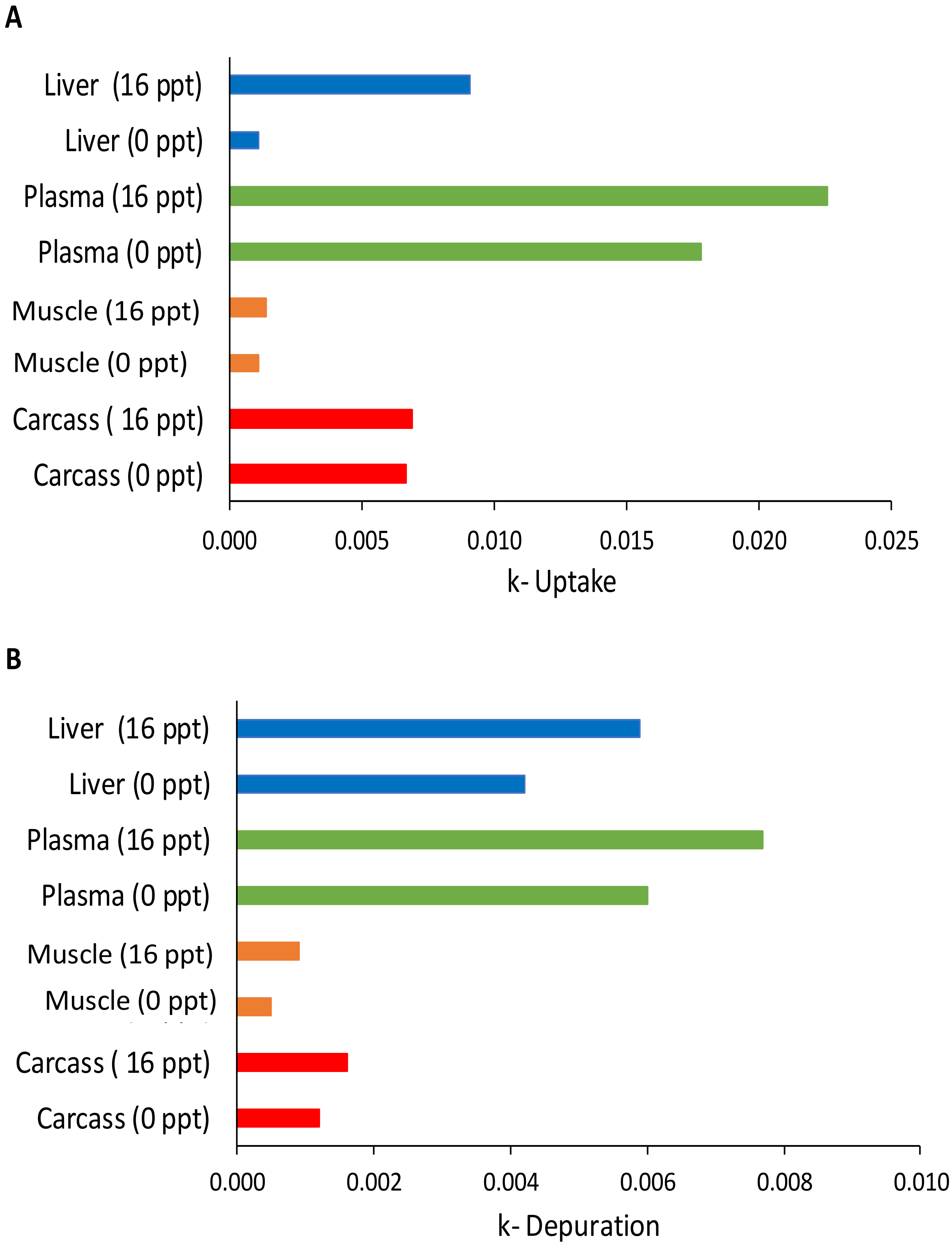

Fig. S1. (A) Uptake (K_u) and (B) depuration (K_e) rate constants for GenX in tilapia (*Oreochromis mossambicus*) blood plasma, liver, muscle, and carcass at 0 and 16 ppt.