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# Transcriptional and physiological response of fathead minnows (*Pimephales promelas*) exposed to urban waters entering into wildlife protected areas

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ABSTRACT

The mission of protected areas is to conserve biodiversity and improve human welfare. To assess the effect of urban waters entering into protected areas, we performed 48-h whole-effluent exposures with fathead minnows, analyzing changes in steady state levels of mRNAs in the livers of exposed fish. Raw wastewater, treated city wastewater, and treated wastewater from a university were collected for exposures. All exposed fish showed altered mRNA levels of DNA damage-repair genes. Fish exposed to raw and treated wastewaters showed down-regulation of transcripts for key intermediates of cholesterol biosynthesis and elevated plasma cholesterol. The type of wastewater treatment influenced the response of gene transcription. Because of the relevance of some of the altered cellular pathways, we suggest that these effluents may cause deleterious effects on fish inside protected areas that receive these waters. Inclusion of research and mitigation efforts for this type of threat in protected areas management is advised.



## 1. Introduction

Aquatic systems are the final destination of pollutants which have largely unknown long-term effects on aquatic and terrestrial organisms (Schwarzenbach et al., 2006; Zanden and Rasmussen, 1996). About 7.4% of the wastewater is reused in the U.S. leaving about 93% of the wastewater for discharge into the nation's streams/rivers (Miller, 2006). Typical wastewaters contain many compounds associated with human use, including domestic-use chemicals, veterinary and human pharmaceuticals, personal care products, and mixtures of contaminants as well as unknown contaminants (Fent et al., 2006; Kolpin et al., 2002). The use of advanced treatment (e.g., reverse osmosis, ozone treatment) can improve removal of emerging contaminants in wastewater, but this is not widely practiced due to cost, and even when used these methods do not remove all contaminants from wastewater (Lee et al., 2004). For some contaminants (e.g. short chain perfluoro alkyl acids) there are no procedures available for their removal (Eschauzier et al., 2012).

There is evidence linking water pollution caused by urban development to the loss of biodiversity in water ecosystems through toxicity and eutrophication, among other processes (SCBD, 2010). In addition, man-made changes to hydrological features in waterbodies have contributed to the decline of these areas and resulted in higher pollutant loading (Brown et al., 2009; Paul and Meyer, 2008; Walsh et al., 2005). Freshwater fish have the highest extinction rate worldwide among all vertebrate classes (Burkhead, 2012). Among the causes of fish extinction, about 17% are attributable to water pollution, the third leading cause after habitat loss (32%) and introduction of non-native fish (29%) (Burkhead, 2012). Since different types of green areas, like parks and urban forests, are the major sources of biodiversity in the urban landscape, it is fundamental to provide a network of green spaces capable to preserve and enhance urban biodiversity (Niemela, 1999).

To estimate the impact of water pollution on wildlife species or on ecosystems is a gigantic task. The complex mixtures of chemicals found in urban waters makes it difficult to elucidate the impact of individual chemicals in biota (Denslow et al., 2007). Nonetheless,

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the fact that toxicity is preceded by alterations in transcriptional responses allows the use of approaches like microarrays for early detection of toxic effects (Nuwaysir et al., 1999). DNA microarray analysis can measure the expression of thousands of genes at a time, potentially accelerating the discovery of toxicant pathways (Waters et al., 2002). Also, transcriptional response profiling provides more specificity than the use of traditional biomarkers, helping to establish links among the mechanisms of action exerted by certain chemicals (Poynton et al., 2008). Thus, a toxico-genomics approach is useful to determine transcriptional responses to assess effects produced by complex environmental mixtures of chemicals (Snape et al., 2004).

Among the typically found compounds in surface waters with wastewater influence are pesticides, insect repellents (e.g., DEET), and pharmaceuticals (e.g., carbamazepine) (Fent et al., 2006; Kolpin et al., 2002). Pharmaceuticals, for instance, are designed to exert effects at low doses, so their effects on aquatic biota may be more potent than historical environmental contaminants (Arnold et al., 2014). While it is known that chemicals such as carbamazepine and DEET can be lethal to fish at very high levels (60 mg/L and 110 mg/L, respectively) (Brooke et al., 1984; Malarvizhi et al., 2012), there is less knowledge about the sub lethal effects that might be observed at the concentrations found in the environment.

One group of chemicals commonly found in water with wastewater influence is the set of perfluoroalkyl substances (PFASs). Persistent and amphiphilic PFASs are widely used in several applications such as textiles, food packaging, firefighting foams, and cookware, among others. PFASs are detected in surface waters (typically ranging up to 400 ng/L) due to their wide use and their environmental persistence (for review see Ahrens, 2011). Several studies in fish have reported that liver is a main target tissue of PFASs (Lau et al., 2007; Martin et al., 2003a, b) with the disruption of fatty acid metabolism, lipid and cholesterol transport with adverse effects at the mg/L level (Wei et al., 2008). Cholesterol is the most important simple lipid in fish (Tocher, 2003). Potential impacts of PFASs on the metabolism of cholesterol is particularly relevant to reproduction because of the connection between cholesterol and the biosynthesis of sex hormones, an area that requires further research (Ankley et al., 2005). Cholesterol also plays a fundamental role in fish cell membrane architecture (Tocher, 2003).

Previous (ACEPD, 2010) and current analyses of water samples from the urban waters investigated here (Treated City Waste Water and On Campus Treated Waste Water) showed the consistent presence of several PFASs as well as sporadic occurrence of other contaminants typically found in WWTPs, such as DEET and carbamazepine. These waters enter into wildlife and nature preservation areas. The objectives of this research were to use a toxico-genomic approach to 1) determine if the treated water that enters into the wildlife preserves can alter relevant genes and cellular pathways in the liver of fathead minnows, and 2) explore the linkages between chemicals that are present in three different types of wastewater and the observed transcriptional and physiological responses. The results are discussed in the context of water pollution impact on protected areas.

## 2. Materials and methods

### 2.1. Study site and water collection

Three sites were selected for the collection of wastewaters within the Orange Creek Basin, Gainesville, Florida, USA. The sites for this study were chosen based on their differences in water treatment systems (from advanced treatment to no treatment) and because they discharge their effluents into protected areas.

#### 2.1.1. Raw wastewater (RW)

This water comes from a 180-acre lake, located in the Tumblin Creek watershed, which covers of urban Gainesville, with 60% of the basin corresponding to impervious areas and nearly 80% corresponding to residential and commercial areas (Scholl, 1985). The lake itself was designated as a State of Florida wildlife sanctuary (Bill No. 1356 Chap. 65 1005). Water quality at this site is impaired due to the illegal wastewater dumping; stormwater runoff containing fertilizers from residential, commercial, and agricultural activities; leakage from a landfill, sanitary sewer lines and septic tank systems. The outflow of the lake then flows to a State Preserve where it enters the Florida Aquifer via the LaChua Sink.

#### 2.1.2. Treated city wastewater (TCW)

Surface water was collected in Sweetwater Branch creek. The Sweetwater Branch watershed encompasses 5.3 square kilometers with the following major land uses: 60% low density residential, 20% commercial, 14% mixed forests and wetlands. The City of Gainesville wastewater reclamation facility is a tertiary-level domestic wastewater treatment plant, which treats domestic wastewaters from southeast and northeast areas of Gainesville. This plant uses a conventional activated sludge with secondary clarifiers and tertiary filtration (Tennant et al., 2010). After treatment, water is discharged to Sweetwater Branch creek where it flows across Payne's Prairie State Preserve. The wastewater treatment plant provides about 67% of the flow of Sweetwater Branch creek. The maximum amount of discharge permitted is 7.5 million gallons per day (mgd) (28.4 million liters per day) (ACEPD, 2010).

#### 2.1.3. On-campus treated wastewater (OTW)

This site is a 1.08 million gallons per day (4.1 million liters per day) wastewater treatment facility on a university campus with a population of approximately 49,000 students and 5100 faculty members (UF Mater Plan, 2006). About 90% of the irrigation water used on campus is reclaimed water from the wastewater treatment plant, which is treated using a Krurger BioDenipho process to Class I water quality standards (potable water). The BioDenipho process improves both nitrogen and phosphorus removal by periodically changing the flow path through two parallel aeration tanks (UF Physical Plant, 2005). The irrigation runoff plus other storm waters are directed to Lake Alice, an on campus nature conservation area (Wells, 2005), which also receives about 5% of the treated wastewater for wetland enhancement (UF Mater Plan, 2006).

Water from each site was collected two days prior to the fish exposure experiment using Chemfluor® tubing and 120-L steel barrels coated with polyester resin (gel coat) to avoid chemical losses. Basic parameters such as temperature, pH, dissolved oxygen, and electrical conductivity were measured on site using field meters (Table S1, Supplementary information). Water from the barrels was transported to the laboratory and pumped into four fiberglass cylinders in the aquatic toxicology facility. Water from each cylinder was then pumped into four replicate aquariums per treatment (Fig. 1) and kept for 1 day without fish (pre-treatment); high pH (>9) was adjusted to pH 6.5 for one of the effluent waters.

### 2.2. Water analyses

Water samples (1 L) were collected from the collection barrels for each effluent in amber glass bottles and stored at 4 °C until analysis for nutrients and metals. EPA standard methods 300.1 and 3120 (EPA, 1993) were used to analyze non-acidified samples for chlorine, conductivity and pH; sulfuric acid was added to preserve nutrients and nitric acid was added to preserve trace metals. Water samples (500 ml) were collected using EPA Method 537 in polypropylene bottles for analysis of 10 types of perfluorinated