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Place-based screening of mixtures of dominant emerging contaminants measured in Lake Michigan using zebrafish embryo gene expression assay

Jordan Crago, Rebecca Klaper*

School of Freshwater Sciences, University of Wisconsin, Milwaukee, Milwaukee, WI 53204, USA

HIGHLIGHTS

- Assessed utility of ZFET to exposure of mixtures.
- Induction of CYP19A2 from diltiazem, fluoxetine, gemfibrozil and metformin exposure.
- Study demonstrates potential of multiple molecular endpoints in the ZFET assay.

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ABSTRACT

Determining impacts of emerging contaminants is difficult due to the different concentrations of mixtures of these chemicals over a landscape. Assessment approaches need to account for absorption, distribution, metabolism and excretion of the chemicals in an organism, and potential crosstalk between molecular pathways. The goal of this study was to assess the utility of employing a modified zebrafish embryo toxicity (ZFET) assay that assesses morphological alterations and measurements of estrogen-associated mRNA transcripts, to exposure of a mixtures of chemicals at concentrations measured in several locations in Lake Michigan. The 5 pharmaceuticals in this study were carbamazepine, diltiazem, fluoxetine, gemfibrozil and metformin. Exposures consisted of 4 concentrations of each individual chemical, mixture concentrations measured at seven locations in Lake Michigan, or 17β-estradiol. The relative expression of Estrogen Receptor-alpha, brain aromatase (CYP19A2), and gonadotropin releasing hormone 3 mRNA were measured at the end the 6-d exposure to determine estrogenicity of the individual chemical or mixture. In this study, there was significant induction of CYP19A2 in individual exposures of diltiazem, fluoxetine, gemfibrozil and metformin at concentrations measured in Lake Michigan. Exposure to 5 of the 7 chemical mixtures altered the expression of one of the three biomarkers. Transcripts varied across mixtures, indicating that biological screening of whole water samples for potential estrogenicity may need to include alternative molecular pathways other than just steroid receptor binding. This research demonstrates that pairing chemical measurements with a modified ZFET assay, twhich incorporates molecular biomarkers and morphological endpoints, could provide location and mixture specific toxic profiling.

1. Introduction

Pharmaceuticals and personal care products (PPCP) have been found in waterways across the United States (Kolpin et al., 2002; Blair et al., 2013). Unfortunately, the current analyte-by-analyte chemical identification approach used for monitoring PPCPs is limited to measuring previously identified targeted chemicals (Diotel et al., 2010; Snyder 2014). As the market continually changes for pharmaceuticals, new compounds as well as their metabolites and degradation by-products may not be identified, nor will their bioactivity in the aquatic environment be well understood (Brack et al., 2015). In addition, different mixtures of these chemicals at a variety of concentrations exists across the aquatic landscape as transport, attenuation and breakdown occur at differing rates for each compound. Monitoring data is often limited

* Corresponding author.
E-mail address: rklaper@uwm.edu (R. Klaper).

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to a select number of chemicals at any one location, and does not identify how their presence as part of a mixture impacts aquatic organisms (Snyder 2014). This is of concern, as many of these PPCPs may be more bioactive than traditional legacy contaminants as they were developed to alter specific metabolic pathways and processes in humans (Maruya et al 2015). One alternative would be anchoring current analytical measurements of pharmaceuticals and other chemicals of emerging concern in the field with effect-based molecular biomarkers, through the use of in vitro or in vivo assays.

Bioassays that target molecular initiating events (e.g. gene transactivation) have been linked to higher order physiological adverse outcomes via toxicity pathway analyses (Piersma et al., 2013; Sonneveld et al., 2006). This has the benefit of anchoring a biological pathway(s) to chemical measurements from current screening methods. These types of in vivo and in vitro assays are currently used to quantify chemical bioactivity based on mode of action (MOA), as part of the U.S. Environmental Protection Agency (EPA) ToxCast and Endocrine Disruptor Screening Programs (Dix et al., 2007; Reif et al., 2010). Effect-based receptor in vitro assays have shown promise for water quality assessment as they can assess bioactivity of a whole water sample with unknown chemicals, based on a specific mode of action, such as transactivation of estrogen receptor alpha (van der Linden et al., 2008; Leusch et al. 2010; Jobling et al., 1995). The drawback to this approach is many of the in vitro assays do not account for absorption, distribution, metabolism and excretion (ADME) of the chemicals in an organism, nor do they account for crosstalk between molecular pathways. One alternative is to modify the zebrafish OECD fish embryo acute test (ZFET) to include molecular endpoints (OECD, 2013).

The ZFET assay has been used as an alternative to adult fish in the Whole Effluent Toxicity (WET) tests (OECD, 2013). Whereas use of adult organisms in WET assays measures apical adverse outcomes, such as lethality, the ZFET assay incorporates other apical endpoints, such as malformation, heartbeat, and hatching success. A number of studies have employed zebrafish embryo screens in assessment of chemicals, such as PPCPs and PAHs using both morphological and behavioral, as well as mRNA and protein expression (Kinch et al. 2016; Reif et al. 2016). Transgenic zebrafish lines, such as tg:cyp19a1b-GFP, which have a green fluorescent protein associated with brain aromatase have also shown promise in identifying estrogenic compounds (Brion et al., 2012; Petersen et al., 2013). Allan et al. (2012) used in-stream passive samplers to determine PAH concentrations for use in zebrafish developmental toxicity exposure studies. Zebrafish developmental assays have also been shown to be a highly sensitive platform performed as part of the EPA ToxCast Phase I and II individual chemical screening assessments, and have the added ability to account for ADME and multiple pathways that are not accounted for in an in vitro assay (Sipes et al. 2011; Truong et al 2014). Inclusion of measurements of molecular initiating events with these current apical endpoints in the ZFET assay may offer an opportunity to tie measured concentrations of chemical mixtures in the environment to early biological responses in an organism. This would be particularly beneficial in environments with low-level mixtures such as stream, rivers and lakes.

The goal of this study was to assess the utility of the ZFET assay in combination with relative expression of mRNA associated with an estrogenic exposure to identify the potential estrogenic effect of location specific mixtures of several emerging contaminants measured in Lake Michigan from our previous research (Blair et al., 2013). For this study, zebrafish embryos (ZF) were exposed from 6 h post fertilization (hpf) to 144 hpf to one of five exposure regiments: a range of doses of individual chemicals, mixtures that correspond to the average concentrations found in seven locations in Lake Michigan, 17β–estradiol as a positive estrogenic control, solvent control, and control media. Exposures consisted of five pharmaceuticals, carbamazepine, diltiazem, fluoxetine, gemfibrozil and metformin. These chemicals were chosen for this study as all five were measured in the water column of Lake Michigan in our previous studies (Blair et al., 2013) (Fig. 1), and were the most prevalent prescribed non-steroidal pharmaceuticals measured in that study, both as occurance across locations and in concentration. There were 4 concentrations of each individual chemical and 7 different mixture concentrations that correspond to the average concentrations found in seven locations in Lake Michigan (Table 1). Concentrations of these chemicals measured in Lake Michigan range from the low micrograms/L, such as metformin, to the low nanograms/L, such as carbamazepine, fluoxetine, diltiazem, and gemfibrozil (Blair et al., 2013). Mortality, hatching success, and developmental deformities (pericardial edema, craniofacial, and tail) were measured.

Apart from diltiazem, the other four compounds have all been shown to alter molecular biomarkers associated with increased estrogenicity, alterations of behavior associated with reproduction or reproductive output at concentrations not normally measured in the aquatic environment (Niemuth et al., 2015; Niemuth and Klaper 2015; Galus et al., 2013; Weinberger and Klaper, 2014). Review of PubChem, a public database that houses in vitro assay results, has indicated that none of these five chemicals bind to the human estrogen receptor alpha (ERα), so other modes of action may need to be considered. The primary mode of action for these five chemicals can be divided into three categories: cell-surface ion channel/receptor modulation (carbamazepine, diltiazem, fluoxetine), energy modulation (metformin), and peroxisome proliferator activated alpha (gemfibrozil). However, it is unclear the mode of action in which these chemicals increased estrogenicity or altered reproductive output. There is some evidence that alterations in neuroendocrine ion channel flux can alter brain aromatase (CYP19A2) and gonadotropin releasing hormone expression (Chang et al. 2012, 2003, Lo and Chang, 1998; Balthazart et al., 2001, 2003). Therefore, inclusion of CYP19A2 and gonadotropin releasing hormone 3 (GnRH3) mRNA expression may be useful as a predictive biomarker in measuring estrogenicity.

To measure the potential estrogenicity of these individual chemicals and mixtures the relative expression of ERα, CYP19A2 and GnRH3 mRNA were measured at the end the exposure and compared to the response upon exposure to the well-studied positive control 17β-estradiol. ERα is a widely used molecular biomarker to measure the estrogenicity of a compound through directly binding to the estrogen receptor or through increasing estrogen biosynthesis (White et al., 1994; Rider et al., 2009). The zebrafish CYP19A2 gene has been proposed to be a suitable biomarker for exposure to xenoestrogens (Brion et al., 2012; Le Page et al. 2008), and studies have used the expression of CYP19A2 as an indicator of exposure to estrogenic chemicals (Hinfray et al., 2006; Lange et al., 2010; Petersen et al., 2013). GnRH3 is a hypothalamic releasing hormone that induces the synthesis and release of gonadotropins from the pituitary gland. GnRH3 is the initial hormone in the hypothalamus for gonadotropin release and subsequent biosynthesis of estradiol. GnRH3 neurons in the hypothalamus are responsible for incorporating a variety of environmental cues such as energy levels, photoperiod, temperature, social cues, and sex steroid hormones concentrations to regulate sex steroid production and reproduction (Vosges et al., 2010, 2012). The hypothesis of this study is that environmentally relevant mixtures of the five most common pharmaceuticals measured in Lake Michigan will cause increases in expression in CYP19A2 and GnRH3 transcripts, but not ERα. Results from this study will support the development of combining analytical measurements of water samples with effect-based measurements for estimating risk of individual chemicals