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Zebrafish embryo toxicity of 15 chlorinated, brominated, and iodinated disinfection by-products

David Hanigan[1,2,*], Lisa Truong[3], Michael Simonich[3], Robert Tanguay[3], Paul Westerhoff[2]

1. Department of Civil and Environmental Engineering, University of Nevada, Reno, NV 89557-0258, United States
2. School of Sustainable Engineering and the Built Environment, Arizona State University, Tempe, AZ, 85287-3005, United States
3. Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR, 97333, United States

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ABSTRACT

Disinfection to protect human health occurs at drinking water and wastewater facilities through application of non-selective oxidants including chlorine. Oxidants also transform organic material and form disinfection by-products (DBPs), many of which are halogenated and cyto- and genotoxic. Only a handful of assays have been used to compare DBP toxicity, and researchers are unsure which DBP(s) drive the increased cancer risk associated with drinking chlorinated water. The most extensive data set employs an *in vitro* model cell, Chinese hamster ovary cells. Traditionally, most DBP research focuses on the threat to human health, but the effects on aquatic species exposed to DBPs in wastewater effluents remain ill defined. We present the developmental toxicity for 15 DBPs and a chlorinated wastewater to a model aquatic vertebrate, zebrafish. Mono-halogenated DBPs followed the *in vivo* toxicity rank order: acetamides > acetic acids > acetonitriles ~ nitrosamines, which agrees well with previously published mammalian *in vitro* data. Di- and tri-halogenated acetonitriles were more toxic than their mono-halogenated analogues, and bromine- and iodine-substituted DBPs tended to be more toxic than chlorinated analogues. No zebrafish development effects were observed after exposure to undiluted or non-concentrated, chlorinated wastewater. We find zebrafish development to be a viable *in vivo* alternative or confirmatory assay to mammalian *in vitro* cell assays.

Introduction

Disinfection is intended to protect human health *via* a reduction in pathogen loading and is applied to both drinking water and treated wastewater, with the latter intended to protect recreational use of the receiving waterway. Disinfection is achieved with non-selective oxidants such as free chlorine, monochloramine, chlorine dioxide, or ozone. These oxidants compromise the cell, or in the case of viruses, damage DNA, and both mechanisms disrupt proliferation (Venkobachar et al., 1977; Wigginton et al., 2012). However, oxidants also react with other organic matter in the water, producing disinfection by-products (DBPs), several of which are thought to be human carcinogens (Richardson et al., 2007).

Human health risk from DBP exposure has been assessed by combining occurrence data with bacterial and animal toxicity studies. Early research suggested that chloroform occurred in chlorinated drinking water at greater concentrations than non-chlorinated water (Bellar et al., 1974; Rook, 1974) and that chloroform caused epithelial tumors in Osborne–Mendel rats and hepatocellular carcinoma in B6C3F mice (National Cancer Institute, 1976). Recent studies have

* *Corresponding author*. E-mail: DHanigan@UNR.edu (David Hanigan).

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assessed DBP toxicity using Chinese hamster ovary (CHO) AS52 and *Salmonella typhimurium* cells (Jeong et al., 2015; Plewa et al., 2002, 2004a; Wagner et al., 2014). Using DNA electrophoretic mobility as a measurement of DNA damage after chronic exposure, genotoxicity followed the order: haloacetic acids > haloacetamides > haloacetonitriles > halonitromethanes > haloacetaldehydes > nitrosamines > trihalomethanes. Chronic exposure cytotoxicity (growth inhibition) follows the rank order: haloacetamides > haloacetaldehydes > halonitromethanes > haloacetic acids > haloacetonitriles > trihalomethanes > nitrosamines (Jeong et al., 2015; Wagner et al., 2014). Iodinated analogues tend to be more toxic than brominated, which are in turn more toxic than chlorinated (Richardson et al., 2007). While these assays serve their intended purpose (*i.e.*, to provide comparative insight into human toxicity), they provide little insight into ecological risk. Because of higher concentrations of organic matter and bromide in wastewaters, their disinfection results in orders of magnitude greater DBP concentrations than for disinfected drinking waters (S.W. Krasner et al., 2016). Despite the higher DBP levels in wastewater effluents, previous research lacks data on DBP effects in terms of ecological endpoints. Therefore, there exists a potential ecological risk from exposure to DBPs in wastewater effluents, and there is little data available to inform the magnitude of this risk.

Zebrafish (*Danio rerio*) are genetically and physiologically similar to higher order vertebrates (Howe et al., 2013; Kimmel et al., 1995), and their short embryogenesis period (<5 days) presents a rapid *in vivo* developmental toxicity platform with environmental relevance that is not captured using other invertebrate or *in vitro* assays. Zebrafish can be robotically staged in 96-well plates, further increasing the throughput of this model organism. This has led to *in vivo* toxicity assessments (mortality, mobility, and developmental) of thousands of anthropogenic chemicals (Reif et al., 2016). Despite the rapid proliferation of hazard assessment using this model organism, published literature observing zebrafish exposed to DBPs is sparse. In these limited studies, three publications focus on two DBPs, dichloroacetonitrile and 2,2-dichloroacetamide, and found that they are toxic to zebrafish (lowest observable effect level [LOEL] = 0.9 μM and 50% population lethality [LC_{50}] = 2.7 mM, respectively) and may bioaccumulate in the organism (Lin et al., 2016a, 2016b; Yu et al., 2015). Another study exposed zebrafish to ten DBPs and found that trihalomethanes were more developmentally toxic and caused greater mortality than acetic acids and bromate (half maximal effective concentration [EC_{50}] and LC_{50} between 0.2 mM and >42.8 mM for the 10 compounds) (Teixidó et al., 2015). Both studies did not observe toxic effects until the animals were exposed to concentrations several orders of magnitude greater than nM concentrations expected in environmental waters.

The goal of this research was to explore a mature assay, the embryonic zebrafish, as a new platform to assess the toxicity of DBPs to whole organisms. Some ecological conclusions may be drawn from the results but the intent of this study was not to directly provide an ecological risk assessment of released DBPs. We present the morphological outcomes of the animals statically exposed to 15 DBPs (two regulated in drinking water) for 6 hr to 5 days post fertilization and compare the toxicity rank order of the DBPs to published research. We showed that several DBPs cause mortality, photomotor inhibition, and developmental malformations at relatively low concentrations (<100 μM), but chlorinated wastewater itself did not cause significant mortality or developmental malformations.

1. Experimental

1.1. DBP reagents and samples

Fourteen separate DBPs were purchased from Sigma Aldrich (St. Louis, MO, USA) at ≥98% purity. One additional DBP, dibromoacetonitrile, was only available at a maximum purity of 94% from Alfa Aesar (Tewksbury, MA, USA). We intentionally selected DBPs with relatively low vapor pressure (Table 1) because the zebrafish embryo exposures are conducted in well plates sealed only with film. The as-purchased DBPs, in either solid or liquid form, were diluted directly into Milli-Q water (>18.2 MΩ-cm). This was conducted using a balance to measure the mass of the solids or of the viscous liquids, followed by addition of water. Because this is a direct measurement of the mass of the compounds added to a known volume of solution, no additional verification of the concentration was conducted.

Wastewater was collected in July 2015 from the overflow weir of a secondary clarifier at a treatment facility in Phoenix, AZ and transported to Arizona State University where it was stored in a refrigerator for less than two weeks before testing. The sample was collected before exposure to oxidants but after biological treatment and settling. Seven days before zebrafish exposures, 2.0 mgCl_2/L of NaOCl was applied to one part of a split wastewater sample in sealed headspace free amber vials. We chose this low dose to intentionally form DBPs without leaving residual free chlorine (*i.e.*, all the chlorine reacted with organic matter in the sample in a short period before the animals were exposed to the sample). The other part of the split sample was not reacted with chlorine and exposed to zebrafish directly. The goal of the experiment with treated wastewater was to determine if chlorinated or non-chlorinated wastewater itself is toxic to zebrafish embryos (*not* to determine at which concentration factors it becomes toxic), and thus we chose not to use established mutagenic organic matter pre-concentration methods (Grabow et al., 1981; Vartiainen et al., 1987). Additionally, we intended to determine if there were additional drivers of toxicity in chlorinated or non-chlorinated wastewater that were not captured by the 15 DBPs selected, which were expected to form at low concentrations in the chlorinated sample, although they were not measured.

1.2. Zebrafish platform

Tropical 5D wild-type adult zebrafish embryos were housed at Oregon State University Sinnhuber Aquatic Research Laboratory. The fish were kept at standard laboratory conditions and spawned, collected, and staged according to Kimmel et al. (1995). For 12 of the 15 DBPs and the wastewater samples, the embryo chorions were removed at 4 hr post fertilization (hpf)

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