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Assessment of endocrine disruptors effects on zebrafish (*Danio rerio*) embryos by untargeted LC-HRMS metabolomic analysis

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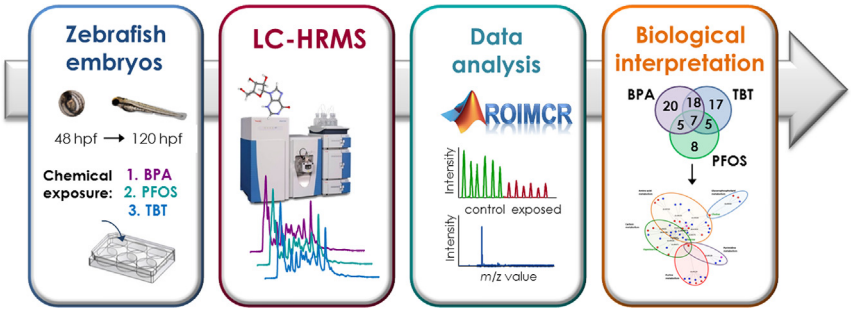
HIGHLIGHTS

- Metabolic disruption in zebrafish embryos by environmentally relevant EDCs.
- BPA, PFOS and TBT effects were revealed by LC-HRMS metabolomics.
- Chemometric analysis allowed the assessment of EDCs effects.
- EDC treatments showed a considerable overlap of the most altered metabolic pathways.
- Specific metabolic disruption of these EDCs was also identified.

GRAPHICAL ABSTRACT



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ABSTRACT

Bisphenol A (BPA), perfluorooctane sulfonate (PFOS), and tributyltin (TBT) are emerging endocrine disruptors (EDCs) with still poorly defined mechanisms of toxicity and metabolic effects in aquatic organisms. We used an untargeted liquid chromatography-high resolution mass spectrometry (LC-HRMS) metabolomic approach to study the effects of sub-lethal doses of these three EDCs on the metabolic profiles of zebrafish embryos exposed from 48 to 120 hpf (hours post fertilization). Advanced chemometric data analysis methods were used to reveal effects on the subjacent regulatory pathways. EDC treatments induced changes in concentrations of about 50 metabolites for TBT and BPA, and of 25 metabolites for PFOS. The analysis of the corresponding metabolic changes suggested the presence of similar underlying zebrafish responses to BPA, TBT and PFOS affecting the metabolism of glycerophospholipids, amino acids, purines and 2-oxocarboxylic acids. We related the changes in glycerophospholipid metabolism to alterations in absorption of the yolk sack, the main source of nutrients (including lipids) for the developing embryo, linking the molecular markers with adverse phenotypic effects. We propose a general mode of action for all three chemical compounds, probably related to their already described interaction with the PPAR/RXR complex, combined with specific effects on different signaling pathways resulting in particular alterations in the zebrafish embryos metabolism.

Abbreviations: AIF, All-ion fragmentation; BPA, Bisphenol A; CE-MS, Capillary electrophoresis-mass spectrometry; EDC, Endocrine disrupting chemical; GC-MS, Gas chromatography-mass spectrometry; HCA, Hierarchical cluster analysis; FTMS, Hybrid Fourier transform mass spectrometry; IS, Internal standard; LC-MS, Liquid chromatography-mass spectrometry; LC-HRMS, Liquid chromatography-high resolution mass spectrometry; LOEC, Lowest observed effect concentration value; MCR-ALS, Multivariate curve resolution by alternating least squares; PFOS, Perfluorooctane sulfonate; Q-TOF, Quadrupole time-of-flight; rMANOVA, Regularized multivariate analysis of variance; ROI, regions of interest; TBT, Tributyltin; WWTP, Wastewater treatment plant; YSA, Yolk sack area.

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1. Introduction

The concern about endocrine disrupting chemicals (EDCs) effects on human and wildlife health is currently increasing (Diamanti-Kandarakis et al., 2009). EDCs are exogenous compounds that initiate abnormal endocrine system processes by activating or inactivating endocrine target receptors or disturbing hormonal balances and metabolism, eventually disrupting homeostatic mechanisms, and finally affecting reproduction and development (Mallozzi et al., 2017). There is a broad range of potential EDCs, including organochlorines, dioxins, organotins (*e.g.,* tributyltin, TBT), polyfluoroalkyl compounds (*e.g.,* perfluorooctane sulfonate, PFOS), brominated flame retardants, alkylphenols, bisphenols (*e.g.,* Bisphenol A, BPA) and phthalates (Casals-Casas and Desvergne, 2011; Oliveira et al., 2016), in addition to natural or synthetic hormones (*e.g.,* estradiol, estrone or ethinyl estradiol), which are being released into the environment. These compounds could reach organisms through oral ingestion, respiratory inhalation or dermal absorption, depending on their physicochemical properties (Woodruff, 2011). Aquatic organisms can uptake them directly from water by gills or skin, *via* uptake of suspended particles or through the consumption of contaminated food (van der Oost et al., 2003). EDCs are considered an important threat to the human health, wildlife and environment at environmentally relevant concentrations (Xu et al., 2013). For instance, PFOS is generally found in the environment at low concentrations. However, concentrations up to 600 $ng \cdot L^{-1}$ have been reported in the Tennessee River downstream (Huang et al., 2010; Hansen et al., 2002).

EDCs are intensely investigated in ecotoxicological and environmental fields in an attempt to discover their possible adverse effects on aquatic organisms, and also to evaluate the consequences of the environmental exposures to these pollutants for the human population. These different families of chemicals are extensively used for industrial applications (including food and clothing industry), ending up in wastewater treatment plants (WWTPs). As most of WWTPs are unable to eliminate these compounds totally, EDCs finally arrive to the environment causing diverse physiological effects in many aquatic species (Chang et al., 2016; Mihaich et al., 2009). This harmful impact on marine and freshwater ecosystems leads to different restrictions and banning legislations through the world (Martínez et al., 2017; Grün et al., 2006). Despite these limitations, remnant levels in aquatic systems could still promote some endocrine disruption effects, such as adipogenic activity upon TBT exposure (Grün et al., 2006; Inadera and Shimomura, 2005).

Owing to the extensive list of described metabolic disorders, including endocrine, reproductive, hormone balance, and immune systems and the toxicity effects caused by EDCs in wildlife (Gore et al., 2015; Giulivo et al., 2016), a more comprehensive view of their complex toxic effects using several methodologies is needed in biological and environmental research. Omic approaches play a crucial role in the understanding of the toxic effects and mechanisms of action of EDCs. Accordingly, they are becoming a key field in the assessment of the occurring processes to characterize specific metabolic disruption (Messerlian et al., 2017). Among omic sciences, the untargeted analysis of changes on the concentration of metabolites (metabolomics) has emerged as a powerful tool to study signaling pathways (Bundy et al., 2008; Baxter et al., 2007), leading to complex metabolic responses in model organisms, such as the zebrafish (*Danio rerio*) and its embryos.

Metabolites are considered as the downstream product of gene expression revealing relative changes at transcriptomic and proteomic levels (Urbanczyk-Wochniak et al., 2003). Therefore, metabolomics reflects the underlying biochemical activity and it gives insight into the understanding of the physiology and molecular phenotype of the investigated organisms (Viant, 2007). However, metabolomic research is a complex field due to the broad range of low molecular weight compounds (metabolites, mass range of 50–1500 dalton (Da)) with large structural diversity due to the different involved cell biological processes. Hence, development and application of different high-throughput analytical platforms are essential for metabolomic studies. Among these analytical platforms, hyphenated mass spectrometry-based techniques present advantageous properties for metabolomics, since they provide higher sensitivity and selectivity than other used platforms. Liquid chromatography-mass spectrometry (LC-MS), gas chromatography–mass spectrometry (GC-MS) and capillary electrophoresis-mass spectrometry (CE-MS) offer easy and reliable metabolic profiling of biological systems (Zhang et al., 2012). Moreover, recent advances in MS-based technologies have allowed covering a larger variety of chemical compounds, which is especially useful in untargeted omics. In fact, in untargeted metabolomics, high-resolution mass spectrometry techniques such as, time-of-flight (TOF) (Wilson et al., 2005), quadrupole time-of-flight (Q-TOF) (Weaver et al., 2007) or hybrid Fourier transform mass spectrometry (FTMS) have gained importance in relation to the conventional low-resolution mass spectrometry platforms. For instance, Orbitrap instruments provide the very advantageous all-ion fragmentation (AIF) option for metabolite identification without the need of selecting the precursor ion and of reanalyzing samples (Geiger et al., 2010).

However, data analysis in untargeted omic studies is still a challenging issue. Untargeted metabolomics generates massive amounts of full scan MS data, requiring the use of advanced data analysis methods. Thereby, several data analysis tools have emerged to handle these large datasets. The "regions of interest" (ROI) type of approaches have been proposed with the goal of reducing the size of metabolomics datasets without any loss of mass accuracy (Tautenhahn et al., 2008). In this way, the ROI compression pretreatment in combination with the multivariate curve resolution alternating least squares (MCR-ALS) procedure (in the so-called ROIMCR procedure) has demonstrated to be a powerful strategy to get very complete metabolic profiling of the investigated systems (Gorrochategui et al., 2016; Gorrochategui et al., 2015a).

The main aim of this work is to assess metabolomic responses of zebrafish (*D. rerio*) embryos exposed to sub-lethal doses of EDCs. Untargeted LC-HRMS ROIMCR metabolomic approach allows identifying potential biomarkers related to toxicity mechanisms of investigated pollutants in these aquatic organisms.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used in the preparation of buffers and solutions were analytical reagent grade. Acetic acid (glacial), methanol (HPLC grade) and acetonitrile (HPLC and MS grade) were purchased from Merck (Darmstadt, Germany). Chloroform was supplied by Carlo Erba (Peypin, France). Ammonium acetate (MS grade), dimethyl sulfoxide (DMSO), water (HPLC and MS grade), calcium sulfate dihydrate ($CaSO_4 \cdot 2H_2O$), bisphenol A (BPA), perfluorooctane sulfonate (PFOS), tributyltin (TBT) and methionine sulfone and piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES), used as the internal standards (IS), were provided by Sigma-Aldrich (St. Louis, MO, USA).

2.2. Animal maintenance and rearing conditions

Adult zebrafish were maintained under standard conditions in fish water, composed of reverse-osmosis purified water containing 90 $\mu g \cdot mL^{-1}$ of Instant Ocean (Aquarium Systems, Sarrebourg, France) and 0.58 mM $CaSO_4 \cdot 2H_2O$ at a temperature of 28 (±1°C). Fish were fed twice a day with dry flakes (TetraMin, Tetra, Germany). Embryos from wild-type zebrafish were obtained by natural mating placing six males and three females on 4-L breeding tanks with a mesh bottom. At 2 hpf (hours post fertilization), eggs were collected and rinsed. Fertilized viable eggs were then randomly distributed in 6-well multiplates (10 embryos/well). Embryos were raised at 28.5 °C with a 12 Light:12 Dark photoperiods in fish water (3 mL/well). All experiments were