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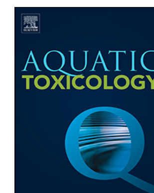

# Metabolomic analysis of short-term sulfamethazine exposure on marine medaka (*Oryzias melastigma*) by comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry

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ABSTRACT

Toxicological effects of sulfamethazine ($SM_2$) have garnered increasing concern due to its wide applications in aquaculture and persistence in the aquatic environment. Most studies have main focused on freshwater fish (i.e. zebrafish), while information regarding effects of $SM_2$ on marine species is still scarce. Here, the hepatotoxicities in marine medaka (*Oryzias melastigma*) with an increasing SM2 concentration exposures (0.01 mg/L, 0.1 mg/L and 1 mg/L) were assessed by comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry (GC × GC-TOF/MS) based metabolomics. Significant metabolites belonging to different metabolites classes were identified by multivariate statistical analysis. The increases levels of amino acids including alanine, asparagine, ornithine, proline, threonine, glutamic acid, lysine, tyrosine and phenylalanine were found in at least two exposure levels. Pathway analysis revealed that amino acids played important biological roles during $SM_2$ exposure: up-regulation of high energy-related amino acids for energy alteration; immune function disorder, oxidative stress and corresponding toxicities defenses. The down regulations of sugar and fatty acid metabolism were observed with an increasing level of SM2 exposure, suggesting that extra energy for cellular defense and detoxification was demanded in terms of different stress request. This study provided an innovative perspective to explore possible SM2 induced hepatic damages at three exposure levels on a nontarget aquatic specie.

## 1. Introduction

Abuse of antibiotics and the following drug-resistant bacteria problems have aroused considerable alarm regarding their semi-persistence and biological activity in the environment (Wollenberger et al., 2000). As one of the commonly used veterinary antibiotics to control bacterial infections in China (Zhang et al., 2015), sulfamethazine ($SM_2$) is a fairly polar and water-soluble compound with a low biodegradation rate in water and marine sediment (Bialk-Bielinska et al., 2011). Meanwhile, its poor absorption by animals and inefficient elimination in sewage treatment plants could result in broadly residues in the aquatic environment, and its potential ecotoxicity has received ongoing attention (Park and Choi, 2008). Extensive investigation of risk assessments on various trophic aquatic model species have been reported, showing that fish appear to be the least sensitive organisms, with some of their acute median effective concentrations ($E/LC_{50}$ mortality) higher than 100 mg/L (Ji et al., 2012). However, the dose-effect relationship reflects that change in phenotypes could be a cumulative result from complex toxic responses (Holford and Sheiner, 1981). Toxicological tests of $SM_2$ on zebrafish embryos have shown the abnormal embryonic development and behavioral disorders under a low concentration exposure (0.001 mg/L) (Lin et al., 2013). While for marine species, the toxicity of $SM_2$ has not been fully comprehended. The evaluations of $SM_2$ bioconcentration on *Oryzias melastigma*, a marine model fish for toxicological assessments in our previous study, have shown a great bioconcentration potency under lower exposure levels, and liver presents to be one of the main target organs in male medaka. The biomarker responses of antioxidant enzymes in liver implies that $SM_2$ can affected redox homeostasis during its short-term exposure (Zhao et al., 2016b). However, the association between ROS stimulation and $SM_2$ metabolism in liver remains unclear and present evidences are insufficient to illustrate toxic responses induced by targeted compound.

Environmental metabolomics is a power tool to assess the

*Abbreviations:* $SM_2$, sulfamethazine; GC*GC-TOF/MS, comprehensive two-dimensional gas chromatography-time-of-flight mass spectrometry, spectroscopy; QC, quality control; PCA, principle component analysis; PLS-DA, partial least squares discriminant analysis

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interactions of organisms with their environment, and have been applied widely in ecotoxicological studies (Zhao et al., 2016a). This methodology can monitor the disturbed metabolites involved in physiological and biochemical functioning caused by different factors such as genetic modifications and environmental stresses. Zebrafish has been selected for hazards investigation by metabolomics concerning sub-lethal effects (0.1 mg/L) of antibiotics. The study authors emphasize that traditional methodologies may neglect important but small-scale changes in molecular levels which could lead to the underestimation of the toxicity at low concentrations (Sotto et al., 2017). For $SM_2$, several metabolic pathways have been identified to affect energy supply, cell-membrane signaling, transport of lipid and methyl group metabolism, which could further influence their swimming behaviors such as swimming velocity, acceleration and active time. Based on the previous studies and to better understand how nontarget organisms respond to varying degrees of $SM_2$ stress, *Oryzias melastigma* is chose as a marine fish model and a short-term $SM_2$ exposure at concentrations from 0.01 mg/L to 1 mg/L was carried out with nontarget metabolomics to profile metabolic variations in different exposure circumstances. Compared with targeted metabolomics, non-targeted metabolomics can serve as a high throughput metabolites platform to gain a deeper insight into the toxicity mechanism that underlain the observed physiological and biochemical changes (Zhao et al., 2016a).

## 2. Materials and methods

### 2.1. Chemicals and reagents

Sulfamethazine (purity > 99.0%) was purchased from J&K Scientific Co., Ltd. (Beijing, China). Millipore (Millipore Corp, Bedford, MA, USA) purified water and reagent-grade methanol as well as chloroform (Sigma Aldrich, St. Louis, MO, USA) were prepared for sample extraction. Derivatization step was carried out by dissolving methoxylamine hydrochloride in anhydrous pyridine and the trimethylsilation reagent (TMS) [*N*, *O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) + trimethylchlorosilane(TMCS)] (99:1) (Sigma Aldrich, St. Louis, MO, USA).

### 2.2. Fish culture and exposure

Mature male medaka aged four months (spawn eggs daily) were obtained from the Collaborative Innovation Center for Development and Utilization of Marine Biological Resources in Xiamen University (Xiamen, China) and acclimatized to laboratory conditions in artificial seawater made by dissolving sea salt in dechlorinated municipal water treated with aeration for a day at room temperature. The salinity, dissolved oxygen, and temperature were controlled at 30‰ ± 2‰, 6.0 ± 0.2 mg/ Land 28 ± 1 °C, respectively, in a 14-h/10-h light/dark photoperiod cycle. The aquaculture quality was based on our previous study (Zhao et al., 2016b). Test fish (0.37 ± 0.06 g; 2.81 ± 0.11 cm) were randomly assigned to each group with exposure concentration at 0 mg/L, 0.01 mg/L, 0.1 mg/L, 1 mg/L (0.035 μM, 0.36 μM, 3.6 μM) for 24 h without feeding and kept in glass aquaria at a density of approximately 1.32 g fish per liter water in a 35 L glass vessel. All groups contained eight replicates. The livers were dissected immediately at the end of exposure and frozen in liquid nitrogen to cease enzymatic activity before storing at −80 °C.

### 2.3. Metabolite extraction

The metabolites extraction was based on previously described procedures (Bligh and Dyer, 1959; Lin et al., 2007; Wu et al., 2008) with slight modification. Briefly, each sample was first added with l-2-chlorophenyalanine as internal standard and homogenized in a methanol/chloroform/water mixture. Supernatants were collected from the resulting biphasic mixture and equal aliquots from each sample was combined and evenly divided into several tubes to form quality control (QC) samples. All samples were further derivatized to increase the volatility for GC × GC-TOF/MS analysis. Details of sample preparation were provided in the Supporting Information (S1).

### 2.4. Liver metabolome analysis

Each sample was randomly assigned but stratified by exposure groups (Dunn et al., 2011), and QC samples were periodically inserted into the sequence throughout the whole analytical run to monitor the reproducibility of pretreatment and instrument performance, a total of 12 QC samples were subjected to statistical analysis. In the meantime, alkanes standards (C10 to C40) were injected at the beginning and end of the sample runs during the same day to formulate retention indexes for every metabolic feature. Analytic specifications of GC × GC-TOF/MS were described in the Supporting Information (S1). The raw instrumental data from each sample were first processed by a deconvolution program developed by Chroma TOF (LECO Corp.) software to transform the two-dimensional matrices into a peak table and using the NIST/Metlab Mass Spectral Library and the LECO/Fiehn Metabolomics Library as reference libraries to identify every compound. The similarity during conversion was assigned beyond 600 to eliminate any false identification (Shi et al., 2014). Retention index match and peak alignment were analyzed by Retention Index and Statistic Compare (SC) software developed by LECO Corp. Peak sorting algorithm was accomplished by R 3.4.0 statistical software to search peak tables for peaks generated by the same compound using several search criteria. All peak areas were normalized to the internal standard before submitting to multivariate statistical analysis.

### 2.5. Multivariate statistical and metabolic pathway analysis

Statistical analysis was performed using MetaboAnalyst 3.0 online (Xia et al., 2012). Briefly, principle component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were both applied to classify variations among different groups to select potential biomarkers. Statistical significance changes were then determined with a *t*-test (two-tailed, equal variances, $P <= 0.05$) (Nagato et al., 2016) corrected by a false discovery rate (FDR) (Benjamini and Hochberg, 1995). Potential biomarkers were further identified and confirmed by the HMDB database (http://www.hmdb.ca), and KEGG (http://www.genome.jp/kegg/compound/). Pathway analysis was then used for data interpretation to find biological correlations among selected metabolites with the impact value threshold set at 0.1 (Wang et al., 2016).

## 3. Results

### 3.1. GC × GC-TOF/MS performance in metabolomics

The contour plot of total ion current (TIC) GC × GC chromatogram was shown in Fig. 1, where over 3000 peaks were detected including reagent artifacts and column bleeding, which consisted of products added by BSTFA and impurities from the matrix of the samples (Li et al., 2009). In the whole TIC zone, it was clear that the two-dimensional chromatogram had complemented further separation for peaks that co-eluted on both axes, so most of the peaks could be well separated except for some that existed in high abundance. This could result in some tailing observed in the $^2$D dimension, but these did not influence the data processing. The electron ionization detector with fast acquisition rate successfully enabled the maximum deconvolution and identification of the overlapping peaks (Almstetter et al., 2012).

The reproducibility of the method had been checked with the retention time (RT) shifts of QC samples. The relative standard deviation (RSD) of the RT in the 1D dimension and 2D dimension were within 0 to 2.133% and 0 to 0.460%, respectively, which were considered as acceptable ranges. These results showed that during sample preparation

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