Histopathological and proteomic responses in male Chinese rare minnow (*Gobiocypris rarus*) indicate hepatotoxicity following benzotriazole exposure[☆]

Xuefang Liang^{a,*}, Jinmiao Zha^{b,c}, Christopher J. Martyniuk^e, Zijian Wang^{b,d}, Ji Zhao^a

^a School of Ecology and Environment, Inner Mongolia University, Hohhot 010021, China

^b Key Laboratory of Drinking Water Science and Technology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

^c Beijing Key Laboratory of Industrial Wastewater Treatment and Reuse, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

^d State Key Laboratory of Environmental Aquatic Chemistry, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

^e Department of Physiological Sciences and Center for Environmental and Human Toxicology, UF Genetics Institute, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611, USA

ARTICLE INFO

Article history:

Received 15 November 2016

Received in revised form

5 May 2017

Accepted 4 June 2017

Available online 30 June 2017

Keywords:

Liver proteome

Histological damage

Oxidative stress

Benzotriazole

Chinese rare minnow (*Gobiocypris rarus*)

ABSTRACT

Benzotriazole (BT) and its associated derivatives are used ubiquitously in industrial processes, and can be detected in indoor temperature coolants and in chemicals designed to inhibit corrosion. This chemical has been widely detected in aquatic environments and shows some degree of environmental persistence. Evidence has shown that BT exposure can negatively affect endocrine systems and can result in neurotoxicity in fish. However, no study has examined whether this chemical exhibits hepatotoxicity in fish, and if so, what are the underlying mechanism associated with the damage. To address this knowledge gap, we measured the liver proteome of adult male Chinese rare minnow (*Gobiocypris rarus*) exposed to either 0.05, 0.5, or 5 mg/L BT for 28 days. Overall, 17 proteins were induced and 9 were reduced in abundance following BT treatment (ratio > 1.5, $p < 0.05$). Pathway analysis revealed that cellular processes affected by BT included xenobiotic clearance, oxidative stress response, apoptosis, and translation. Moreover, transcripts related to these toxic pathways were also significantly affected by BT. In addition, rare minnows exposed to BT showed signs of hypertrophy of hepatocytes, nuclei pyknosis, and higher levels of cellular vacuolization compared to the controls, thus these early proteomic responses in the liver may be related to pathology (i.e. adverse outcome pathway). Our data demonstrate that BT dysregulates molecular responses in the liver and tissue pathology indicative of damage. This study provides new insight into BT hepatotoxicity in Chinese rare minnow.

© 2017 Elsevier Ltd. All rights reserved.

1. Introduction

There has been a significant increase in the emergence of new chemicals of concern in the aquatic environment, as improved analytical methods are now able to detect chemical contamination with higher sensitivity (Herrero et al., 2013; Richardson, 2009). Many of these chemicals enter into aquatic ecosystems and can

bioaccumulate in the aquatic food web (Liu et al., 2011; Wang et al., 2015). Benzotriazoles such as 1H-benzotriazole (BT) are industrial chemicals that are used in high volumes (~9000 tons/year worldwide) in both consumer products (e.g. additives to household dishwashing agents) and industrial compounds such as anti-corrosive and de-icing agents (Giger et al., 2006). Their wide application and resistance to biodegradation contribute to the dispersive occurrence of these compounds in the aquatic environment (Herzog et al., 2014; Liu et al., 2012). In fact, these chemicals can reach relatively high levels in some water systems. For example, BT has been detected in surface waters at concentrations ranging from 0.038 to 7.98 µg/L (Kiss and Fries, 2009; Loos et al., 2009; Voutsas et al., 2006; Wolschke et al., 2011), and higher

[☆] This paper has been recommended for acceptance by Dr. Harmon Sarah Michele.

* Corresponding author. School of Ecology and Environment, Inner Mongolia University, 235 West College Road, Hohhot, Inner Mongolia, 010021, China.

E-mail address: liangxf@imu.edu.cn (X. Liang).

levels of BT have been reported in wastewater effluent, ranging from 10 to 100 µg/L (Voutsas et al., 2006; Weiss and Reemtsma, 2005). BT has also been detected in drinking water in the UK, at a concentration of approximately 30.9 ng/L (Janna et al., 2011). In addition to water contamination, BT has also been detected in indoor dust (Wang et al., 2013) and human urine (Asimakopoulou et al., 2013a, 2013b). Thus, these compounds appear to be relatively ubiquitous chemicals in aquatic environments and may pose potential risk to human health.

Despite the persistence of BT and widespread use, data on how it affects aquatic organisms and humans are limited. The acute toxicity of BT for fathead minnows (*Pimephales promelas*) is reported to be relatively low, with a LC₅₀ value of 65 mg/L (Pillard et al., 2001). However, in contrast to acute dosing experiments, chronic exposure to BT at much lower levels (EC₁₀ values varied from 0.97 to 3.94 mg/L) has been associated with the inhibition of both growth and reproduction in aquatic invertebrates (Seeland et al., 2012), indicating that there may be uncharacterized risks to aquatic organisms. BT may also be an endocrine disruptor, and studies show that BT elicits adverse responses in marine medaka (*Oryzias melastigma*) (He et al., 2012) and rare minnow (*Gobiocypris rarus*) (Liang et al., 2014b). These studies are suggestive of an estrogenic mode of action, while antiandrogenic modes of action have been observed from studies conducted in zebrafish embryos (Fent et al., 2014). These data spur additional questions as to the mode of action for these compounds, and further studies are warranted to document the full range of any potential adverse effects.

We previously demonstrated that BT alters the mRNA levels of genes involved in the hypothalamic-pituitary-gonadal (HPG) axis and that BT can exert estrogenic disruption in rare minnow, based on alterations in the plasma E2 levels (Liang et al., 2014b). In addition, our previous proteomic study in the brain of female rare minnow has shown that BT may be related to neurotoxicity by interfering with cellular processes including cellular respiration, G-protein signal cascades, Ca²⁺-dependent signaling, cell cycle and apoptosis (Liang et al., 2016). However, hepatotoxicity and the mechanism underlying BT toxicity at the protein level have not been investigated, despite the fact that the liver is the major detoxification organ for chemicals.

The objectives of this study were to examine the proteomic response in the liver of rare minnows exposed to BT in dose-response experiments to learn more about the mechanisms of action of BT in fish. The rare minnow (*Gobiocypris rarus*) inhabits the upstream waters of the Yangtze River in China. This small bodied fish is ideal for toxicity testing because it is relatively straight forward to maintain laboratory cultures, it has a short life cycle, and it can produce hundreds of eggs. Therefore, the rare minnow is considered a premier small fish model for the assessment of chemicals in the environment in China (Zha et al., 2008). Furthermore, omics-based approaches (i.e., genomics, proteomics, and metabolomics) to inform mechanism-based toxicology have been adapted in rare minnows to better characterize toxicity and molecular pathways perturbed by chemicals (Liang and Zha, 2016). In this study, the proteomic response in the liver of rare minnow, as well as hepatic histopathology were carried out to assess the hepatotoxic potential of BT. Pathway analysis was conducted to synthesize proteome data to provide additional insight into the molecular processes that may underlie hepatotoxicity.

2. Materials and methods

2.1. Experimental design and sampling

Exposure experiments followed that as previously outlined in Liang et al. (2014b, 2016). Adult male Chinese rare minnows (~10

months old) used in this experiment were maintained at the State Key Laboratory of Environmental Aquatic Chemistry, which is housed at the Research Center for Eco-Environmental Sciences and Chinese Academy of Sciences. Fish were cultured in 10 L glass aquaria with flow-through dechlorinated tap water. Water parameters were as follows: pH = 7.2–7.6, water hardness = 44.0–61.0 mg CaCO₃/L, and temperature = 25 ± 1 °C. Fish were allowed to acclimate to laboratory conditions for 2 weeks prior to the experiment. Healthy males based on visual inspection were randomly divided into 12 groups of 20 fish and were exposed to one of three concentrations of BT (0.05, 0.5, 5 mg/L) or to a water-only control as BT is readily soluble in water.

Benzotriazole (BT, CAS no. 95-14-7, purity >99%) was purchased from J&K Chemical Ltd. (USA). Stock solutions were first prepared by dissolving BT in distilled water. The stock solutions were kept in 4-L brown glass container and were diluted with aquarium water to obtain the final concentration for exposure. The stock solutions were slowly added to glass mixing vessels by means of a peristaltic pump at a rate of 60 mL/h. These solutions were then mixed with dechlorinated tap water and dispersed to the exposure tanks, at a continuous flow rate of 5 L/h. Stock solutions were renewed every second day and dosing rates were checked twice daily.

Each experimental condition included three replicates for a total of 12 tanks. During the exposure, the fish were subjected to a photoperiod of 16:8 h light: dark and were fed newly hatched brine shrimp (*Artemia nauplii*) twice daily. After 28-days of experimentation, all fish were anesthetized on ice. The fish were dissected, and livers were removed and separated into two equal parts. One half of the liver was used for protein extraction and 2D gel electrophoresis, and the second half of the liver was used for RNA isolation and real-time PCR analysis. All samples at the time of dissection were immediately frozen in liquid nitrogen and stored at –80 °C until analysis.

A second experiment, longer than the first experiment, was conducted for 42 days. This second experiment was used to determine if liver damage occurred following a longer-term exposure to BT. No significant pathology was observed in low doses (0.05 and 0.5 mg/L) after 28 d of BT exposure in a preliminary experiment (data not shown), thus 42 d was used to assess pathology and to better describe an adverse outcome pathway for BT.

2.2. Proteomic analysis

2.2.1. Protein extraction and CyDye labeling

Protein extraction and CyDye labeling were performed according to Liang et al. (2014a). Briefly, ~50 mg of frozen hepatic tissue from control and BT-treated fish were homogenized and sonicated intermittently over 5 min in 0.6 mL of ice-cold lysis buffer I (7 M urea, 2 M thiourea, 4% w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% w/v dithiothreitol (DTT), 30 mM Tris and a cocktail of protease inhibitors (Roche)). To remove remaining tissue fragments and insoluble material, the homogenates were centrifuged for 10 min at 18,000 × g, 4 °C. The supernatant was then collected and 100 µL of each sample protein extract was purified using a 2-D clean-up kit (GE Healthcare). Protein pellets were then resuspended in 50 µL lysis buffer II (lysis buffer I, 0.5% pH 4–7 IPG buffer). Protein concentration was determined using a 2-D Quant kit (GE Healthcare) according to the manufacturer's protocol. Purified protein samples were randomized and minimally labeled with Cy3 or Cy5 fluorescent dyes (50 µg protein/400 pmol dye) as per the manufacturer's instructions (GE Healthcare). Cy2 was used to label a common protein pool that contained equal amounts of proteins from the samples and this acted as an internal standard for 2D gel electrophoresis. Labeling was performed on ice for 30 min in the dark and quenched with

10 mM lysine for 10 min, on ice and in the dark.

2.2.2. Two-dimensional fluorescence difference gel electrophoresis and image analysis

The methods for the 2D-DIGE analysis proceeded as that outlined in Liang et al. (2014a). Briefly, Cy2-, Cy3-, Cy5-labeled samples were mixed together and dissolved in rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% w/v DTT, 0.5% pH 4–7 IPG buffer, and a trace of bromophenol blue). Samples ($n = 3$ biological replicates per group) were then loaded onto 18 cm immobilized pH gradient (IPG) strips (pH 4–7, GE Healthcare), and subjected to first dimension iso-electric focusing (IEF) overnight using Ettan IPGphor (GE Healthcare, USA). After IEF, each strip was equilibrated twice for 15 min with constant mixing (60 rpm). This was achieved by first using 1% DTT followed by 2.5% iodoacetamide (IAA) in order to reduce and alkylate the proteins. The SDS-PAGE was performed using an electrophoresis system (Ettan Dalt six, GE Healthcare) and 10% polyacrylamide gel. Following electrophoresis, the gels were scanned with Typhoon Trio variable mode imager (GE Healthcare, NJ) in each of the three channels that corresponded to the correct dye (488 nm for Cy2, 532 nm for Cy3, and 633 nm for Cy5). The intensity was adjusted to ensure that the maximum volume of each image was within 60–90 thousand. Image Master 2D Platinum 7.0 (GE Healthcare) software was used to analyze the images. A one-way ANOVA was used to determine if there were differences in abundance among spots. Protein spots were considered differentially expressed at a ratio ≥ 1.5 ($p < 0.05$) and were pursued for identification.

2.2.3. Protein identification

Protein identification was conducted in accordance with the method previously published (Liang et al., 2016). Following the image acquisition, gels were stained with silver nitrate and protein spots were manually excised, decolorized, dehydrated and whitened according to Liang et al. (2016). The digested peptides were resuspended with 0.8 μ l of matrix solution (50% acetonitrile, 0.1% trifluoroacetic acid, and semi-saturated α -cyano-4-hydroxycinnamic acid). All mass spectra were acquired using a 4800 Plus MALDI-TOF/TOFTM Analyzer (Applied Bio-systems, USA) in positive reflectron mode. Signal-to-noise ratios of ≥ 50 were required for tandem MS/MS analysis. The mass signals generated from the MS mode and the MS/MS mode were combined for protein identification. Mascot was set up to search an NCBI non-redundant (nr) database (zebrafish, 76,533 sequences, released Aug. 13, 2013; ray-finned fishes, 229,984 sequences, released Dec. 12, 2012) assuming the digestion enzyme trypsin. Mono-isotopic masses were used at a peptide mass tolerance of 100 ppm. Cysteine carbamidomethylation was considered as a fixed modification, and the oxidation of methionine was specified as a variable modifications. Peptide and protein identifications were accepted if Confidence Interval % (C.I. %) values were greater than 95%. The identified proteins were then matched to specific processes or functions by searching UniProt (<http://www.uniprot.org/>) and KEGG (<http://www.genome.jp/kegg/>) database.

2.2.4. Pathway analysis

For each differentially expressed protein, the official gene symbol was manually retrieved using gene card (<http://www.genecards.org>). The official gene symbols were then used to map proteins into the Pathway Studio 9.0 (Elsevier Life Science Solutions) and ResNet 10.0 for pathway analysis. This program uses expression, binding, and regulatory data among proteins to construct interaction networks. Pathways were built using direct connections with one neighbor. Using the “Name + Alias” function, thirty proteins mapped to the program. One protein did not

successfully map as no mammalian homolog could be identified, while a second protein was excluded, as it is fish specific. In order to simplify the pathways, differentially expressed proteins across all three doses were considered as a whole for building pathways. As such, the median fold change of the protein was used to color pathways, and only proteins that were significantly altered in a BT treatment were used to calculate the median protein fold change. In the case of multiple spots (e.g. phosphorylase), the median value across all spots was used for the protein fold change relative to the control group. Sub-network enrichment analysis (SNEA) was also performed in the program to identify processes that were represented by the proteins. Criteria for an enriched cell process were that the network had to be significant (considered $P < 0.05$) and there had to be more than 10 members in the network to represent the processes most likely altered by BT exposure at the proteome level. Further details on the program can be found in Langlois and Martyniuk (2013).

2.3. Relative quantification by real-time PCR

Total RNA was extracted from minnow liver ($n = 6$ biological replicates per group) using Trizol reagent (Life Technology), and this was followed by DNase (Promega, USA) treatment. RNA integrity was measured using a 1.2% agarose gel ($0.5 \times$ TBE buffer, 150 v, 15 min) and RNA was of sufficient quality (i.e. no significant degradation based upon visual inspection of each sample). The concentration and purity of the samples were determined using a MultiskanTM GO microplate spectrophotometer (Thermo Scientific, USA). For real-time PCR experiments, samples were adjusted to a 20 μ l volume with $2 \times$ GoTaq[®] qPCR Master Mix (Promega, USA), 1 μ l of 10 μ M gene-specific primer, and ~ 100 ng first-strand cDNA synthesized from DNase-treated RNA. The relative abundance of β -actin was used to normalize the expression of target genes. All primer pairs used for real-time PCR are listed in Supplemental Table S1.

Real-time PCR reactions were assayed using the 7500 Real-Time PCR system (Applied Biosystems, USA). Each sample was analyzed in triplicate. The real-time PCR cycling parameters were as follows: initial denaturation step of 95 °C for 10 min, 40 cycles of 95 °C for 30 s, 57 °C for 40 s, and 72 °C for 30 s. To ensure amplicon specificity, a melt curve was generated at the last cycle of 95 °C for 30 s, 57 °C for 30 s, and 72 °C for 60 s. Data were analyzed according to the $2^{-\Delta\Delta C_t}$ method. Statistical analysis of variance was performed in SPSS (v18, USA). The normality of data was assessed using the Kolmogorov-Smirnov test and logarithmic transformation was performed prior to ANOVA to ensure data conformed to assumptions. The Levene's test of homogeneity of variance was used to test for adherence to ANOVA assumptions. A Dunnett's post-hoc test was used to compare each treatment with the control. All quantitative data are expressed as mean $\pm$ S.E. A probability of $p < 0.05$ was considered statistically significant.

2.4. Histopathology

Histopathology analysis followed that outlined in Liang et al. (2014b) and Chen et al. (2016). A total of 12 groups of 10 fish for each group (0, 0.05, 0.5, 5 mg/L BT) were used for histopathology analysis. Following necropsy of all male fish in the experiment, livers were excised and fixed in 4% paraformaldehyde for 24 h. Liver samples were transferred to 70% ethanol, embedded in paraffin wax, and sectioned at 3–4 μ m thick. Tissue sections were stained with hematoxylin and eosin (H and E) according to standard histological methods. Slides were visualized with an optical microscope BX53 (Olympus, Tokyo, Japan). Sections were imaged and analyzed using cellSens Standard software (Olympus, Tokyo, Japan).

To determine if the liver was compromised at the tissue level, nucleated cells and akaryotes were counted. Five fields of visions were selected at random and analyzed ($n = 6$ biological replicates per group were randomly selected from the original 10 fish). Statistical significance was considered to be $P < 0.05$ following a Student's *t*-test.

3. Results

3.1. Proteome profiles of male rare minnow liver

In order to identify sets of proteins that respond to BT exposure in male rare minnow, we compared the liver proteome of control

and treated animals using 2D-DIGE (Fig. 1). A total of 46 protein spots were found to be significantly altered in abundance (≥ 1.5 -fold) in one or more BT-treated groups compared to that of controls and 32 proteins were successfully identified (Table 1). Based upon the *p*-value and ratio criteria, there were 17 up-regulated proteins and 9 down-regulated proteins following BT treatments (Table 1). Most of these proteins were changed remarkably in the lowest concentrations of BT. Only four proteins were significantly increased (phosphoglucosyltransferase 1) or decreased (heat shock 60kD protein 1, glucose-6-phosphate dehydrogenase, and homogentisate 1,2-dioxygenase) in higher concentrations (0.5 mg/L and 5 mg/L groups), and these proteins did not show significant changes in abundance in the 0.05 mg/L group (Table 1).

Fig. 1. Representative two-dimensional gel image of the CyDye-labeled proteins from male rare minnow liver for control and BT treatments. Each gel is representative of three independent biological replicates. Numbers are allocated by the Image Master 2D Platinum 7.0 software and represent the spots with a significant variation in intensity (ratio ≥ 1.5 , $p < 0.05$). The information for the proteins corresponding to the spot numbers is presented in Table 1.

Table 1

A detailed list of protein spots identified by MALDI-TOF/TOF MS from the liver of male rare minnow following BT exposure.

Spot ID	GI accession no.	Protein name ^a	MW/PI	Matched peptides	score	Fold change ^b (treated vs control)			Function category ^c
						0.05 mg/L	0.5 mg/L	5 mg/L	
Protein identified in multiple spots									
247	56078768	Phosphorylase	111.8/5.50	23	625	3.05	3.86	4.04	carbohydrate metabolic process/glycogen phosphorylase activity
251	56078768	Phosphorylase	111.8/5.50	19	425	1.53	1.37	1.43	
252	56078768	Phosphorylase	111.8/5.50	19	279	2.22	2.41	2.34	
254	56078768	Phosphorylase	111.8/5.50	18	535	2.89	1.92	2.62	
258	56078768	Phosphorylase	111.8/5.50	22	549	2.30	1.46	1.91	
398	56078768	Phosphorylase	111.8/5.50	18	369	−1.93	−2.09	−2.06	
405	56078768	Phosphorylase	111.8/5.50	17	445	−1.70	−2.69	−3.41	
408	56078768	Phosphorylase	111.8/5.50	15	344	−1.39	−2.12	−2.09	
320	71373043	iron regulatory protein 1	99.4/6.45	10	295	−2.33	−2.73	−3.29	metabolic process/4 iron, 4 sulfur cluster binding
361	71373043	iron regulatory protein 1	99.4/6.45	13	307	−2.08	−1.52	−2.88	
537	39645909	Glutamate dehydrogenase	60.3/8.45	17	451	1.65	1.32	1.76	cellular amino acid metabolic process/oxidoreductase activity
559	39645909	Glutamate dehydrogenase	60.3/8.45	17	572	1.78	1.6	2.09	
603	39645909	Glutamate dehydrogenase	60.3/8.45	18	721	−2.26	−3.07	−6.88	
615	39645909	Glutamate dehydrogenase	60.3/8.45	10	395	−2.33	−2.58	−3.27	
571	28278852	Methionine adenosyltransferase I, alpha	43.7/6.32	8	182	2.37	2.24	1.7	S-adenosylmethionine biosynthetic process; one-carbon metabolic process
803	28278852	Methionine adenosyltransferase I, alpha	43.7/6.32	11	227	−2.38	−3.19	−3.22	
814	82213364	Fructose-bisphosphate aldolase B	39.7/8.48	4	98	1.61	1.57	2.06	Glycolysis/fructose-bisphosphate aldolase activity
885	82213364	Fructose-bisphosphate aldolase B	39.7/8.48	6	132	−2.52	−2.79	−4.47	
923	123918122	glyceraldehyde-3-phosphate dehydrogenase	36.0/8.20	9	458	−2.31	−5.77	−7.73	Glycolysis/NAD binding; NADP binding
1374	123918122	glyceraldehyde-3-phosphate dehydrogenase	36.0/8.20	6	373	1.39	1.41	1.63	
Up-regulated proteins									
233	39645915	Heat shock protein 90, beta (Grp94), member 1	91.4/4.77	19	361	1.6	1.15	1.61	response to stress/ATP binding
261	161612168	Dmgdh protein	97.9/6.54	12	218	2.07	−0.95	0.93	
351	159155706	Heat shock protein 8	71.4/5.32	23	824	1.95	1.93	2.01	glycine catabolic process response to stress/ATP binding
435	32766553	Phosphoglucomutase 1	61.4/5.74	11	498	1.4	1.51	2.22	
448	41393103	aldehyde dehydrogenase family 9 member A1-A	56.1/6.18	9	95	1.6	−1.01	−1.01	galactose catabolic process; glycogen biosynthetic process
472	9622234	catalase	59.8/8.12	13	382	1.88	1.67	1.49	
550	20339358	Aldehyde dehydrogenase 2	57.2/6.05	8	83	2.29	2.22	2.23	aldehyde dehydrogenase (NAD) activity
618	302191716	4-aminobutyrate aminotransferase, mitochondrial	56.3/7.61	9	130	1.52	1.29	1.4	
662	28278417	S-adenosylhomocysteine hydrolase	48.5/6.33	11	563	1.54	1.48	1.56	hydrogen peroxide catabolic process/catalase activity
672	357394863	Probable imidazolonepropionase	47.3/6.05	7	231	1.94	1.6	1.05	
677	82202412	Hydroxysteroid dehydrogenase-like protein 2	44.7/7.08	9	141	1.59	1.49	2.19	cellular aldehyde metabolic process
931	45709875	Zgc:85683	32.5/5.38	4	123	2.04	1.57	1.58	
1015	528514207	Uncharacterized protein	29.3/5.2	4	130	2.36	2.18	1.24	gamma-aminobutyric acid metabolic process
1018	44890340	Phosphoglycerate mutase 1a	29.1/6.19	8	86	2.87	2.58	1.76	
1040	528500056	Hypoxanthine phosphoribosyltransferase 1	24.9/6.21	11	363	1.54	1.52	1.5	S-adenosylmethionine cycle; one-carbon metabolic process
1046	514483217	Glutathione S-transferase theta 1a [Carassius auratus]	26.4/6.09	5	186	1.52	1.47	1.28	

(continued on next page)

Table 1 (continued)

Spot ID	GI accession no.	Protein name ^a	MW/PI	Matched peptides	score	Fold change ^b (treated vs control)			Function category ^c
						0.05 mg/L	0.5 mg/L	5 mg/L	
1084	8575790	Glutathione S-transferase [<i>Pimephales promelas</i>]	22.0/7.64	4	127	2.54	2.17	1.5	transferase activity
Down-regulated proteins									
445	528496446	PREDICTED: fatty acid synthase isoform X2	276.8/6.01	17	255	−2.83	−5.7	−4.38	ND
446	528489998	Heat shock 60kD protein 1 (Chaperonin)	61.4/5.56	15	884	−1.24	−1.52	−1.03	response to stress/ATP binding
468	298201226	glucose-6-phosphate dehydrogenase, partial [<i>Gobiocypris rarus</i>]	35.6/5.87	11	250	−1.43	−1.87	−1.29	pentose-phosphate shunt/NADP binding; glucose-6-phosphate dehydrogenase activity
629	10441585	Homogentisate 1,2-dioxygenase	45.2/6.37	10	241	−1.06	−1.3	−2.14	L-phenylalanine catabolic process; tyrosine metabolic process
724	41054972	40S ribosomal protein SA	34.2/4.75	4	312	−2.05	−2.71	−5.63	cell adhesion; ribosomal small subunit assembly; translation/laminin receptor activity; structural constituent of ribosome
940	182889192	Zgc:153353	40.1/7.51	7	130	−1.62	−1.61	−1.16	hydrolase activity; metal ion binding
954	182890800	Zgc:101897 protein	27.9/8.13	6	75	−2.18	−1.49	−0.81	glutathione transferase activity
987	49900472	Sulfotransferase family 1, cytosolic sulfotransferase 6	35.8/6.19	7	125	−1.81	−1.86	−2.18	estrone sulfotransferase activity
1091	53733942	Apolipoprotein A-I	19.7/5.31	3	77	−1.94	−1.52	−1.88	cholesterol metabolic process; lipid transport; lipoprotein metabolic process

^a All proteins from *Danio rerio* database except specially noted by “[]” in the end of the protein name.

^b The average fold changes as compared to the controls. The fold changes higher than or equal to 1.5-fold are significant fold change values and are indicated by bold character. The down-regulations are noted by “−”.

^c Biological processes or molecular functions in GO terms. “ND” indicates that no function is defined for the protein.

In addition, among the protein spots identified, 6 proteins were detected in more than one spot (Table 1). For example, Protein spots 247, 251, 252, 254 and 258 were all identified as phosphorylase (PYGL), which appeared as a string of abundant spots on the gels (Supplemental Fig. S1). A shift in mass was also observed as protein spots 398, 405, and 408 were also identified as PYGL (Supplemental Fig. S1). Although each spot was significantly altered in abundance relative to control males, their expression patterns were not consistent (i.e. some modified or charged PYGL was increased in abundance while other modifications for PYGL were decreased). According to the median value across all spots and our criteria (≥ 1.5 -fold), the total abundance of PYGL was not significantly altered in the BT-treated groups compared to the control group (Table 1, Supplemental Fig. S1).

3.2. Differentially expressed proteins and pathway analysis

Thirteen proteins had a direct relationship among each other and these included heat shock 70 kDa protein 8 (HSPA8), heat shock protein 90 kDa beta (Grp94), member 1 (HSP90B1), and catalase (CAT) (Supplemental Fig. S2). These proteins increased in abundance following BT treatment and are involved in the oxidative stress response. Thus, these proteins comprise a sub-network that is regulated by BT. Proteins regulated by BT were also associated with xenobiotic clearance. Specific proteins related to xenobiotic clearance included glutamate dehydrogenase 1 (GLUD1), aldehyde dehydrogenase 9 family, member A1 (ALDH9A1), 4-aminobutyrate aminotransferase (ABAT), and S-adenosylhomocysteine hydrolase (AHCY) (Supplemental Table S2). ABAT, ALDH9A1 and AHCY were all up-regulated in 0.05 mg/L group, while GLUD1 was down-

regulated by 1.58 fold (according to the median value across spots 537, 559, 603, and 615) in 5 mg/L group (Table 1). Fig. 2 shows the relationship between proteins and cell processes related to apoptosis, reactive oxygen species generation, and xenobiotic clearance. Lastly, the process of translation was also significantly affected at the level of the proteome, and proteins such as GAPDH, FASN, and ACO1 were decreased in the network following BT treatments while proteins such as HPRT1, CAT and HSPA8 were increased in this network (Fig. 3). All abbreviations for the protein networks are found in the Figure caption.

3.3. Validation by qPCR

To validate the alteration of proteins identified after BT exposure, 6 proteins were selected for qPCR analysis (Fig. 4). The transcripts that were assessed included 4-aminobutyrate aminotransferase (*abat*), probable imidazolonepropionase (amidohydrolase domain containing 1, *amdhd1*), apolipoprotein A-I (*apo1a*), glutamate dehydrogenase 1 (*glud1*), phosphorylase (*pygl*), and sulfotransferase family 1, cytosolic sulfotransferase 6 (*sult1st6*). Transcripts in general showed correspondence with that of the protein abundance (Fig. 4). Here we point out that transcriptional and translation regulation is different, and discrepancies in the regulation of molecular targets by BT can be explained by these two different cell processes. Nevertheless, congruence between gene and protein responses following BT in multiple doses is stronger evidence for disruption of the target. Consistent with the proteomic data, the mRNA levels of *abat* and *amdhd1* presented a marked up-regulation in lower concentrations of BT (Fig. 4). The pattern of *apo1a* and *sult1st6* mRNA in 0.5 and 5 mg/L exposure concentrations was in

Fig. 2. Protein network for differentially expressed proteins with cell processes related to apoptosis, reactive oxygen species generation, and xenobiotic clearance. Red indicates an increase in relative protein levels while green indicates a decrease in relative protein levels. Abbreviations are as follows; ABAT, 4-aminobutyrate aminotransferase; ACO1, aconitase 1, soluble; AGMAT, agmatine ureohydrolase (agmatinase); AHCY, S-adenosylhomocysteine hydrolase; ALDH2, aldehyde dehydrogenase 2 family (mitochondrial); ALDH9A1, aldehyde dehydrogenase 9 family, member A1; ALDOB, aldolase B, fructose-bisphosphate; AMDHD1, amidohydrolase domain containing 1; APOA1, apolipoprotein A-I; CAT, catalase; DMGDH, dimethylglycine dehydrogenase; FAHD2A, fumarylacetoacetate hydrolase domain containing 2A; FASN, fatty acid synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUD1, glutamate dehydrogenase 1; GSTO2, glutathione S-transferase omega 2; GSTT1, glutathione S-transferase theta 1; G6PD, glucose-6-phosphate dehydrogenase; HGD, homogentisate 1,2-dioxygenase; HPGD, hydroxyprostaglandin dehydrogenase 15-(NAD); HPRT1, hypoxanthine phosphoribosyltransferase 1; HSDL2, hydroxysteroid dehydrogenase like 2; HSPA8, heat shock 70 kDa protein 8; HSPD1, heat shock 60 kDa protein 1 (chaperonin); HSP90B1, heat shock protein 90 kDa beta (Grp94), member 1; MAT1A, methionine adenosyltransferase I, alpha; PGAM1, phosphoglycerate mutase 1 (brain); PYGL, phosphorylase, glycogen, liver; RPSA, ribosomal protein SA; SULT1C2, sulfotransferase family, cytosolic, 1C, member 2.

Fig. 3. Protein network for differentially expressed proteins with the process of translation following BT treatments. Red indicates an increase in relative protein levels while green indicates a decrease in relative protein levels. Abbreviations follow that given in the Fig. 2 caption. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

accordance with that of the protein data (Fig. 4). However, in the 0.05 mg/L group, the *apoA1a* and *sult1st6* mRNA levels were not significantly changed while corresponding protein levels were decreased (Fig. 4). Again, this highlights the complexity underlying gene-protein regulation. In the case where proteins were detected

in multiple spots in 2D-DIGE gels, the median value across all spots was used for the protein fold change relative to the control group. Both *glud1* and *pygl* were chosen to validate the protein abundance levels for multiple spot proteins. *GLUD1* was down-regulated by 1.58-fold at 5 mg/L BT group while *PYGL* was not significantly altered following all three exposure concentrations of BT. The mRNA levels of *glud1* and *pygl* showed comparable response patterns when compared to protein data generated with DIGE (Fig. 4).

3.4. Histopathology

Normal livers in rare minnow typically show compact and tightly arranged hepatocytes with homogeneous cytoplasm, and a large spherical nucleus with one nucleolus (Fig. 5A). Notable histological changes were observed in the livers of the rare minnow following exposure to 0.05, 0.5 and 5 mg/L BT for 42 d (Fig. 5B–D) when compared to normal liver histology. Hypertrophy of the hepatocytes, nuclei pyknosis, and increases in cellular vacuolization were observed in all three treatment groups (Fig. 5B–D). Additionally, in the highest BT treatment group, the hypertrophy of the hepatocytes was even more prevalent than the other BT-treated groups (rectangle area in Fig. 5D).

4. Discussion

Benzotriazole is an emerging contaminant and is relatively widespread in aquatic environments. Evidence indicates that BT has endocrine disrupting potential (Fent et al., 2014; He et al., 2012; Liang et al., 2014b) and may be neurotoxic (Liang et al., 2016). To

Fig. 4. Protein and relative mRNA levels of selected proteins in the livers of male rare minnow following BT treatments. The magnified images of protein spots from the 2D-DIGE gels are shown in the upper part of each panel. The bar charts below show the protein levels based upon 2D-DIGE and mRNA levels using real-time PCR. The values represent the average fold changes. Significant changes of mRNA levels as compared to the controls ($p < 0.05$) are indicated by asterisks (*), and pound sign (#) refers to an average increase or decrease higher than 1.5-fold in protein levels compared to the controls.

Fig. 5. Light microscopy images of hepatic tissue of male rare minnow (H & E, 40 ×). Hepatic tissue of male fish from (A) control, (B) 0.05 mg/L BT treatment group, (C) 0.5 mg/L BT treatment group, and (D) 5 mg/L BT treated group were examined. Pathology indicating damage of the liver was observed after BT treatment, including swelling of hepatocytes (arrow), vacuolization (arrow ahead) and deformed cell nuclei (solid arrow). Scale bar is 20 μm.

characterize the liver proteome and to better understand the mechanism of hepatic toxicity of BT, 2D-DIGE coupled MALDI-TOF/TOF-MS was employed. A total of 32 differentially expressed proteins were identified in one or more of the treatment groups. Of these proteins, 6 were identified in the spot strings as the same protein, which suggests that BT may play a role in protein modification (McCarthy et al., 2003). Although no apparent shifts with MW or PI were observed in exposure gels compared to the control, the protein level of each spot was changed dramatically after BT treatment. We speculate that significant changes in protein modification for some proteins (e.g. phosphorylation) might be induced in response to BT (Lv et al., 2014; Thio et al., 2013), although further studies are needed to verify the extent of phosphorylation.

Pathway analysis revealed that BT-responsive proteins were mainly involved in the process of xenobiotic clearance, oxidative stress response, apoptosis, and translation. In the present study, three primary proteins related to xenobiotic clearance were identified, including SULT1ST6, ABAT, and GLUD1. Our data revealed that SULT1ST6 protein and mRNA were significantly decreased in abundance after BT exposure. SULT1ST6 is a member of sulfotransferases and exhibits sulfating activity toward endogenous compounds of both endocrine and nervous systems in fish (Gao et al., 2014; Liu et al., 2010). Thus, SULT1ST6 is active during detoxification and is also important in metabolizing environmental estrogens (Liu et al., 2008). Previous studies have also shown a significant decrease in *sult1st6* mRNA levels in male rare minnow exposed to bisphenol A (Gao et al., 2014) and in zebrafish embryos exposed to chlorpyrifos (Jeon et al., 2016). These results suggest

that the hepatic detoxification might be repressed by BT, and further support our previous study reporting on the estrogenic potential of this compound (Liang et al., 2014b).

Additionally, both the transcription and protein levels of ABAT and GLUD1 were significantly increased and decreased in response to 0.05 and 5 mg/L BT, respectively. As a signaling molecule and neurotransmitter, γ -Aminobutyric acid (GABA) is produced from glutamate by glutamate decarboxylase and is further metabolized into succinic semialdehyde by ABAT. During this process, GLUD1 converts glutamate to α -ketoglutarate, followed by further oxidation in the tricarboxylic acid (TCA) cycle to generate ATP (Bouché et al., 2003; McKenna et al., 1996). According to this metabolic process, we hypothesize that in the current study, the upregulation of ABAT increases GABA, resulting in the downregulation of GLUD1. Aberrant glutamatergic transmission has been associated with neuronal pathology and neurodegenerative diseases (Kim et al., 2011). Environmental chemicals have also been shown to disrupt the normal balance of these enzymes. For example, in the brain of methylmercury-exposed zebrafish, the transcript levels of *glud1a* and *abat* were decreased and increased, respectively (Cambier et al., 2012). In addition to the brain, GABA signaling also exists in vertebrate liver, and activation of this GABAergic system can protect the liver against toxic injury (Wang et al., 2017). Li et al. (2016) reported that a repression of this amino acid metabolism pathway is associated with liver impairment in zebrafish exposed to arsenic. In another study, GLUD1 was reported to be down-regulated in male rare minnow (*Gobiocypris rarus*) liver after exposure to pentachlorophenol (Fang et al., 2010). Thus, altered

protein and transcript levels of ABAT and GLUD1 indicate that BT may disrupt hepatic GABA signaling and induce oxidative stress.

Conversion of glutamate in TCA cycle increases the production of anti-oxidant molecule such as NADPH, thus GABA shunt pathway plays an important role in antioxidant defense (Ramond et al., 2014). Decreased GLUD1 coupled a general induction of heat shock proteins (HSP90B1 and HSPA8) and CAT indicates that there is a general and oxidative stress response in the liver following BT exposure. HSPs are well characterized molecular chaperones that assist in protein folding and are bio-indicators for their sensitivity to environmental stress (Gupta et al., 2010). Catalase is ubiquitous in tissues and acts to reduce hydrogen peroxide and to diminish the effects of reactive oxygen species (ROS) (Nishikawa et al., 2009). An increase in HSPs and catalase activity has been documented in the liver of fathead minnows (*Pimephales promelas*) responding to 17 β -trenbolone (an androgen receptor agonist) and flutamide (an androgen receptor antagonist) (Martyniuk et al., 2009). Furthermore, a wide variety of chemicals affect proteins related to ROS production in fish species, including pesticides (Atamaniuk et al., 2013; Martyniuk et al., 2010), and heavy metals (Carlson et al., 2013; Lushchak, 2016). These studies support the hypothesis that BT can induce oxidative stress and adversely affect rare minnow liver at the molecular level.

As a consequence of oxidative stress, cell death and apoptosis may occur and this can result in the hepatotoxicity of BT. Cell death and apoptosis were two of the top pathways identified with sub-network enrichment analysis and therefore, appear to have a prominent role in BT toxicity. Notably, APOA1, the primary protein constituent of high-density lipoprotein (HDL) and a protein that mediates anti-apoptotic responses in mammals (Rütti et al., 2009), was decreased in each of the exposure concentrations of BT in this study. Inhibition of *apoa1* transcripts has also been observed previously in the liver of male goldfish exposed to fluoxetine (FLX) and 17 α -ethinylestradiol (EE2) (Silva de Assis et al., 2013), as well as in male zebrafish livers following exposure to waterborne EE2 (Martyniuk et al., 2007). In addition, proteomic analysis in the male rare minnow liver identified proteins that are also associated with the process of translation. These proteins are also involved in apoptosis, suggesting impairment of both protein synthesis and cell death. For example, methionine adenosyltransferase I, α (MAT1A) is an essential enzyme that is responsible for the biosynthesis of S-adenosylmethionine. Reduced MAT1A expression in hepatocellular carcinoma contributes to increased apoptosis and enhanced tumorigenesis (Yang et al., 2013). Moreover, the histological damage in the liver of male rare minnow was observed at each concentration in this study and these protein responses may be associated with pathology of the liver in extended exposures. In a previous study, histological damage in the liver of rare minnow exposed to 5 mg/L BT for 28 days was observed while no significant impacts were examined after 0.05 and 0.5 mg/L BT treatments (Liang et al., 2014a). These results suggest that the hepatic lesions are caused by BT via disturbing the apoptosis pathway; this damage may be caused at relative low concentrations of BT after longer exposure period.

In conclusion, 2D gel electrophoresis identified proteins affected by BT that are involved in xenobiotic clearance, oxidative stress response, apoptosis, and translation. Furthermore, histological damage in the liver of male rare minnow was observed across all exposure groups following BT exposure. The results presented here provide novel insight into the mechanisms of hepatotoxicity of BT in male rare minnow. We hypothesize that BT induced cellular stress impairs protein synthesis and induces the apoptosis pathway, resulting in ROS production and liver lesions. However, additional experimental validation is required and the biological impacts of long-term BT exposure remain to be examined.

Acknowledgements

This work was supported by the National Natural Science Foundation of China [grant numbers 21507064, 21677165]; and the Natural Science Foundation of Inner Mongolia Autonomous Region of China [grant number 2015MS0202].

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.envpol.2017.06.013>.

References

- Asimakopoulos, A.G., Bletsou, A.A., Wu, Q., Thomaidis, N.S., Kannan, K., 2013a. Determination of benzotriazoles and benzothiazoles in human urine by liquid chromatography-tandem mass spectrometry. *Anal. Chem.* 85, 441–448.
- Asimakopoulos, A.G., Wang, L., Thomaidis, N.S., Kannan, K., 2013b. Benzotriazoles and benzothiazoles in human urine from several countries: a perspective on occurrence, biotransformation, and human exposure. *Environ. Int.* 59, 274–281.
- Atamaniuk, T.M., Kubrak, O.I., Storey, K.B., Lushchak, V.I., 2013. Oxidative stress as a mechanism for toxicity of 2,4-dichlorophenoxyacetic acid (2,4-D): studies with goldfish gills. *Ecotoxicology* 22, 1498–1508.
- Bouché, N., Lacombe, B., Fromm, H., 2003. GABA signaling: a conserved and ubiquitous mechanism. *Trends Cell Biol.* 13, 607–610.
- Cambier, S., Gonzalez, P., Mesmer-Dudons, N., Brêthes, D., Fujimura, M., Bourdineaud, J.P., 2012. Effects of dietary methylmercury on the zebrafish brain: histological, mitochondrial, and gene transcription analyses. *Biometals* 25, 165–180.
- Carlson, P., Smalley, D.M., Van Beneden, R.J., 2013. Proteomic analysis of arsenic-exposed zebrafish (*Danio rerio*) identifies altered expression in proteins involved in fibrosis and lipid uptake in a gender-specific manner. *Toxicol. Sci.* 134, 83–91.
- Chen, R., Liu, C., Yuan, L., Zha, J., Wang, Z., 2016. 2, 4-Dichloro-6-nitrophenol, a photolysis product of 2, 4-dichlorophenol, caused anti-androgenic potency in Chinese rare minnows (*Gobiocypris rarus*). *Environ. Pollut.* 216, 591–598.
- Fang, Y., Gao, X., Zha, J., Ning, B., Li, X., Gao, Z., Chao, F., 2010. Identification of differential hepatic proteins in rare minnow (*Gobiocypris rarus*) exposed to pentachlorophenol (PCP) by proteomic analysis. *Toxicol. Lett.* 199, 69–79.
- Fent, K., Chew, G., Li, J., Gomez, E., 2014. Benzotriazole UV-stabilizers and benzothiazole: antiandrogenic activity in vitro and activation of aryl hydrocarbon receptor pathway in zebrafish eleuthero-embryos. *Sci. Total Environ.* 482, 125–136.
- Gao, J., Zhang, Y., Yang, Y., Yuan, C., Qin, F., Liu, S., Zheng, Y., Wang, Z., 2014. Molecular characterization of PXR and two sulfotransferases and hepatic transcripts of PXR, two sulfotransferases and CYP3A responsive to bisphenol A in rare minnow *Gobiocypris rarus*. *Mol. Biol. Rep.* 41, 7153–7165.
- Giger, W., Schaffner, C., Kohler, H.P.E., 2006. Benzotriazole and tolyltriazole as aquatic contaminants. 1. Input and occurrence in rivers and lakes. *Environ. Sci. Technol.* 40, 7186–7192.
- Gupta, S.C., Sharma, A., Mishra, M., Mishra, R.K., Chowdhuri, D.K., 2010. Heat shock proteins in toxicology: how close and how far? *Life Sci.* 86, 377–384.
- He, T., Liang, B., Liu, W., Shin, P.K.S., Wu, R.S.S., 2012. Estrogenic potential of benzotriazole on marine medaka (*Oryzias latipes*). *Ecotoxicol. Environ. Saf.* 80, 327–332.
- Herrero, P., Borrull, F., Pocurull, E., Marcé, R.M., 2013. Efficient tandem solid-phase extraction and liquid chromatography-triple quadrupole mass spectrometry method to determine polar benzotriazole, benzothiazole and benzenesulfonamide contaminants in environmental water samples. *J. Chromatogr. A* 1309, 22–32.
- Herzog, B., Lemmer, H., Helmreich, B., Horn, H., Müller, E., 2014. Monitoring benzotriazoles: a 1 year study on concentrations and removal efficiencies in three different wastewater treatment plants. *Water Sci. Technol.* 69, 710–717.
- Janna, H., Scrimshaw, M.D., Williams, R.J., Churchley, J., Sumpter, J.P., 2011. From dishwasher to tap? xenobiotic substances benzotriazole and tolyltriazole in the environment. *Environ. Sci. Technol.* 45, 3858–3864.
- Jeon, H.J., Lee, Y.H., Kim, M.J., Choi, S.D., Park, B.J., Lee, S.E., 2016. Integrated biomarkers induced by chlorpyrifos in two different life stages of zebrafish (*Danio rerio*) for environmental risk assessment. *Environ. Toxicol. Pharmacol.* 43, 166–174.
- Kim, K., Lee, S.G., Kegelman, T.P., Su, Z.Z., Das, S.K., Dash, R., Dasgupta, S., Barral, P.M., Hedvat, M., Diaz, P., 2011. Role of excitatory amino acid transporter-2 (EAAT2) and glutamate in neurodegeneration: opportunities for developing novel therapeutics. *J. Cell. Physiol.* 226, 2484–2493.
- Kiss, A., Fries, E., 2009. Occurrence of benzotriazoles in the rivers Main, Hengstbach, and Hegbach (Germany). *Environ. Sci. Pollut. Res.* 16, 702–710.
- Langlois, V.S., Martyniuk, C.J., 2013. Genome wide analysis of *Silurana* (*Xenopus*) tropical development reveals dynamic expression using network enrichment analysis. *Mech. Dev.* 130, 304–322.
- Li, C., Li, P., Tan, Y.M., Lam, S.H., Chan, E.C.Y., Gong, Z., 2016. Metabolomic

- characterizations of liver injury caused by acute arsenic toxicity in zebrafish. *PLoS One* 11, e0151225.
- Liang, X., Martyniuk, C.J., Cheng, G., Zha, J., Wang, Z., 2014a. Pyruvate carboxylase as a sensitive protein biomarker for exogenous steroid chemicals. *Environ. Pollut.* 189, 184–193.
- Liang, X., Martyniuk, C.J., Zha, J., Wang, Z., 2016. Brain quantitative proteomic responses reveal new insight of benzotriazole neurotoxicity in female Chinese rare minnow (*Gobiocypris rarus*). *Aquat. Toxicol.* 181, 67–75.
- Liang, X., Wang, M., Chen, X., Zha, J., Chen, H., Zhu, L., Wang, Z., 2014b. Endocrine disrupting effects of benzotriazole in rare minnow (*Gobiocypris rarus*) in a sex-dependent manner. *Chemosphere* 112, 154–162.
- Liang, X., Zha, J., 2016. Toxicogenomic applications of Chinese rare minnow (*Gobiocypris rarus*) in aquatic toxicology. *Comp. Biochem. Physiol. D. Genomics Proteomics* 19, 174–180.
- Liu, T.A., Bhuiyan, S., Liu, M.Y., Sugahara, T., Sakakibara, Y., Suiko, M., Yasuda, S., Kakuta, Y., Kimura, M., Williams, F.E., Liu, M.C., 2010. Zebrafish as a model for the study of the phase II cytosolic sulfotransferases. *Curr. Drug Metab.* 11, 538–546.
- Liu, T.A., Bhuiyan, S., Snow, R., Yasuda, S., Yasuda, T., Yang, Y.S., Williams, F.E., Liu, M.-Y., Suiko, M., Carter, G., Liu, M.-C., 2008. Identification and characterization of two novel cytosolic sulfotransferases, SULT1 S7 and SULT1 S8, from zebrafish. *Aquat. Toxicol.* 89, 94–102.
- Liu, Y.S., Ying, G.G., Shareef, A., Kookana, R.S., 2011. Simultaneous determination of benzotriazoles and ultraviolet filters in ground water, effluent and biosolid samples using gas chromatography–tandem mass spectrometry. *J. Chromatogr. A* 1218, 5328–5335.
- Liu, Y.S., Ying, G.G., Shareef, A., Kookana, R.S., 2012. Occurrence and removal of benzotriazoles and ultraviolet filters in a municipal wastewater treatment plant. *Environ. Pollut.* 165, 225–232.
- Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S., Bidoglio, G., 2009. EU-wide survey of polar organic persistent pollutants in European river waters. *Environ. Pollut.* 157, 561–568.
- Lushchak, V.I., 2016. Contaminant-induced oxidative stress in fish: a mechanistic approach. *Fish. Physiol. Biochem.* 42, 711–747.
- Lv, D.W., Subburaj, S., Cao, M., Yan, X., Li, X., Appels, R., Sun, D.F., Ma, W., Yan, Y.M., 2014. Proteome and phosphoproteome characterization reveals new response and defense mechanisms of brachypodium distachyon Leaves under salt stress. *Mol. Cell. Proteomics* 13, 632–652.
- Martyniuk, C.J., Alvarez, S., McClung, S., Villeneuve, D.L., Ankley, G.T., Denslow, N.D., 2009. Quantitative proteomic profiles of androgen receptor signaling in the liver of fathead minnows (*Pimephales promelas*). *J. Proteome Res.* 8, 2186–2200.
- Martyniuk, C.J., Gerrie, E.R., Popescu, J.T., Ekker, M., Trudeau, V.L., 2007. Microarray analysis in the zebrafish (*Danio rerio*) liver and telencephalon after exposure to low concentration of 17 α -ethinylestradiol. *Aquat. Toxicol.* 84, 38–49.
- Martyniuk, C.J., Kroll, K.J., Doperalski, N.J., Barber, D.S., Denslow, N.D., 2010. Genomic and proteomic responses to environmentally relevant exposures to dieldrin: indicators of neurodegeneration? *Toxicol. Sci.* 117, 190–199.
- McCarthy, J., Hopwood, F., Oxley, D., Laver, M., Castagna, A., Righetti, P.G., Williams, K., Herbert, B., 2003. Carbamylation of proteins in 2-D electrophoresis—myth or reality? *J. Proteome Res.* 2, 239–242.
- McKenna, M.C., Sonnenwald, U., Huang, X., Stevenson, J., Zielke, H.R., 1996. Exogenous glutamate concentration regulates the metabolic fate of glutamate in astrocytes. *J. Neurochem.* 66, 386–393.
- Nishikawa, M., Hashida, M., Takakura, Y., 2009. Catalase delivery for inhibiting ROS-mediated tissue injury and tumor metastasis. *Adv. Drug Deliv. Rev.* 61, 319–326.
- Pillard, D.A., Cornell, J.S., DuFresne, D.L., Hernandez, M.T., 2001. Toxicity of benzotriazole and benzotriazole derivatives to three aquatic species. *Water Res.* 35, 557–560.
- Ramond, E., Gesbert, G., Rigard, M., Dairou, J., Dupuis, M., Dubail, I., Meibom, K., Henry, T., Barel, M., Charbit, A., 2014. Glutamate utilization couples oxidative stress defense and the tricarboxylic acid cycle in *Francisella* phagosomal escape. *PLoS Pathog.* 10, e1003893.
- Rütti, S., Ehses, J.A., Sibler, R.A., Prazak, R., Rohrer, L., Georgopoulos, S., Meier, D.T., Niclauss, N., Berney, T., Donath, M.Y., von Eckardstein, A., 2009. Low- and high-density lipoproteins modulate function, apoptosis, and proliferation of primary human and murine pancreatic β -cells. *Endocrinology* 150, 4521–4530.
- Richardson, S.D., 2009. Water analysis: emerging contaminants and current issues. *Anal. Chem.* 81, 4645–4677.
- Seeland, A., Oetken, M., Kiss, A., Fries, E., Oehlmann, J., 2012. Acute and chronic toxicity of benzotriazoles to aquatic organisms. *Environ. Sci. Pollut. Res.* 19, 1781–1790.
- Silva de Assis, H.C., Simmons, D.B.D., Zamora, J.M., Lado, W.E., Al-Ansari, A.M., Sherry, J.P., Blais, J.M., Metcalfe, C.D., Trudeau, V.L., 2013. Estrogen-like effects in male goldfish co-exposed to fluoxetine and 17 α -ethinylestradiol. *Environ. Sci. Technol.* 47, 5372–5382.
- Thio, C.L.P., Yusof, R., Abdul-Rahman, P.S.A., Karsani, S.A., 2013. Differential proteome analysis of chikungunya virus infection on host cells. *PLoS One* 8, e61444.
- Voutsas, D., Hartmann, P., Schaffner, C., Giger, W., 2006. Benzotriazoles, alkylphenols and bisphenol A in municipal wastewaters and in the Glatt River, Switzerland. *Environ. Sci. Pollut. Res.* 13, 333–341.
- Wang, L., Asimakopoulos, A.G., Kannan, K., 2015. Accumulation of 19 environmental phenolic and xenobiotic heterocyclic aromatic compounds in human adipose tissue. *Environ. Int.* 78, 45–50.
- Wang, L., Asimakopoulos, A.G., Moon, H.B., Nakata, H., Kannan, K., 2013. Benzotriazole, benzothiazole, and benzophenone compounds in indoor dust from the United States and East Asian countries. *Environ. Sci. Technol.* 47, 4752–4759.
- Wang, S., Xiang, Y.Y., Zhu, J., Yi, F., Li, J., Liu, C., Lu, W.Y., 2017. Protective roles of hepatic GABA signaling in acute liver injury of rats. *Am. J. Physiol. Gastrointest. Liver Physiol.* 312, G208–G218.
- Weiss, S., Reemtsma, T., 2005. Determination of benzotriazole corrosion inhibitors from aqueous environmental samples by liquid chromatography–electrospray ionization–tandem mass spectrometry. *Anal. Chem.* 77, 7415–7420.
- Wolschke, H., Xie, Z., Möller, A., Sturm, R., Ebinghaus, R., 2011. Occurrence, distribution and fluxes of benzotriazoles along the German large river basins into the North Sea. *Water Res.* 45, 6259–6266.
- Yang, H., Cho, M.E., Li, T.W.H., Peng, H., Ko, K.S., Mato, J.M., Lu, S.C., 2013. MicroRNAs regulate methionine adenosyltransferase 1A expression in hepatocellular carcinoma. *J. Clin. Invest.* 123, 285–298.
- Zha, J., Sun, L., Zhou, Y., Spear, P.A., Ma, M., Wang, Z., 2008. Assessment of 17 α -ethinylestradiol effects and underlying mechanisms in a continuous, multi-generation exposure of the Chinese rare minnow (*Gobiocypris rarus*). *Toxicol. Appl. Pharmacol.* 226, 298–308.