Science of the Total Environment 563–564 (2016) 273–281

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Multi-biomarker responses as indication of contaminant effects in *Gambusia affinis* from impacted rivers by municipal effluents

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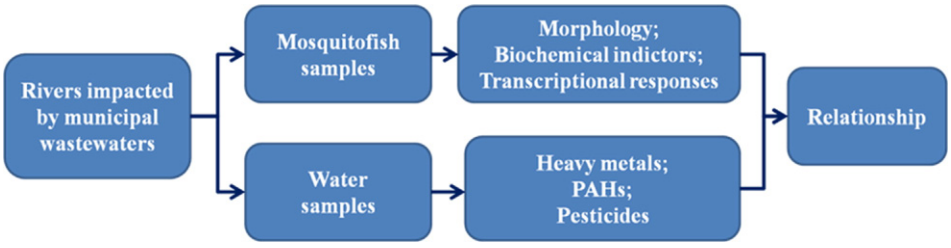
HIGHLIGHTS

- Wild mosquitofish was surveyed through a multi-biomarker approach.
- Metallothionein in mosquitofish were consistent with heavy metal exposure.
- Heavy metals and pesticides might affect the hormonal effects in mosquitofish.
- Multiple biological endpoints should be considered in environmental risk assessment.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 4 March 2016
Received in revised form 7 April 2016
Accepted 17 April 2016
Available online 30 April 2016

Editor: D. Barcelo

Keywords:
Mosquitofish
Biomarker
Chemicals
Metals
Pesticides
Toxic effects

ABSTRACT

This study investigated toxic effects in mosquitofish from two urban rivers of South China impacted by municipal effluents by using multiple biomarkers including fish morphology, biochemical indicators and transcriptional responses, and explored potential cause-effect relationship with a list of chemicals (metals, polycyclic aromatic hydrocarbons (PAHs) and pesticides). The results showed significant alterations in metallothionein (MT) protein and mRNA expression in mosquitofish collected from the two rivers and a strong association between MT protein and mRNA expression levels and heavy metals in the river water. Both ethoxyresorufin-*O*-deethylase (EROD) activity and cytochromes P450 1A (CYP1A) mRNA expression were significantly enhanced in mosquitofish at most sampling sites. There existed a strong correlation between EROD activity and CYP1A mRNA expression levels, but no clear correlations between these responses and PAHs in the river water possibly because of the presence of many other agonists of the aryl hydrocarbon receptor in the two rivers. Significant acetylcholinesterase (AChE) inhibition was observed in mosquitofish brain samples. The pesticides in the two rivers showed an influence on the AChE activity, which was also found to be significantly negatively correlated to fipronil concentrations. Moreover, the result also indicates that metals and pesticides present in the two rivers might cause the observed estrogenic and androgenic effects in mosquitofish. The findings from this study clearly showed morphological, biochemical and transcriptional responses in mosquitofish due to chemical contamination of the two urban rivers. This multi-biomarker approach using mosquitofish can be applied to evaluate contamination of riverine environments.

1. Introduction

Municipal wastewater effluents represent a major source of loading for chemical contaminants in the aquatic environment (Holeton et al., 2011). These effluents are derived from a range of complex discharges such as domestic and industrial wastewaters, as well as runoffs

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http://dx.doi.org/10.1016/j.scitotenv.2016.04.127
0048-9697/

(Lavado et al., 2006), which are known to contain pharmaceuticals, personal care products, hormones, metals and persistent organic chemicals (Holeton et al., 2011; Houde et al., 2013). Contaminants from municipal wastewater discharges may be taken up by aquatic animals, leading to the impairment of organism health conditions (Gagnon et al., 2006).

There is growing awareness of the need to assess the effects of multiple contaminants in organisms in aquatic environments (Fernandes et al., 2007). Among the available techniques, the use of biomarkers based on variations at the morphological, cellular and molecular levels in indicator species appears as a valuable tool to detect environmental stress of multiple contaminants in aquatic environments (Fernandes et al., 2007; Frenzilli et al., 2008; Lavado et al., 2006). Assessment of biomarkers in fishes can act as an early warning system predicting the pollution effects (Mierzejewski et al., 2014). More importantly, some biomarkers can be specific to particular chemical classes such as heavy metals, planar aromatic compounds, pesticides, and environmental estrogens (Roberts et al., 2005). For example, in certain animals, including fish, metallothionein (MT) is low molecular-mass cysteine-rich metal-binding protein that is capable of being induced by heavy metals (Hansen et al., 2006; Linde-Arias et al., 2008). Cytochrome P4501A (CYP1A) plays an important role in catalyzing oxidative metabolism of organic pollutants and has been used as a specific biomarker for exposure to aryl hydrocarbon receptor (AhR) agonists in fish such as PAHs and polychlorobiphenyls (PCBs) (Olivares et al., 2010; Maes et al., 2013). Both ethoxyresorufin *O*-deethylase activity (EROD) and CYP1A mRNA expression have been successfully used as common biomarkers for exposure to PAHs and PCBs in fish (Corsi et al., 2003; Quiró et al., 2007). Additionally, the inhibition of AChE activity is a specific biomarker for exposure to carbamate and organophosphorus pesticides (Corsi et al., 2003). However, although some biomarkers can be specific to particular chemical classes, they may also be affected by other chemical classes. Therefore, the integrated use of a wide battery of selected biomarkers is a sound procedure to minimize misinterpretation in complex environmental pollution situations (Linde-Arias et al., 2008).

Many urban rivers in the Pearl River Delta region of South China have been heavily polluted by municipal wastewaters and runoffs (Jiang et al., 2015; Pan et al., 2014). However, the effects of water quality in aquatic biota have been little studied in this region. In the present study, this field investigation focuses on the two urban rivers (Shima River and Danshui River), which are the tributaries of the Dongjiang River (part of the Pearl River system). They were selected because they are good examples of overexploited rivers, receiving extensive municipal wastewater discharges from the fast growing cities like Shenzhen and Dongguan (Chen et al., 2014; Ying et al., 2012). In a previous study, (xeno)estrogens and androgens were present at same sampling sites in the Shima River and Danshui River and some of those chemicals could contribute to severe feminization and masculinization of western mosquitofish (Huang et al., 2016). However, information related to the presence of heavy metals or persistent organic pollutants and their toxicological effects in inhabiting fish in these rivers is quite scarce. Therefore, toxicant-induced changes in biological systems in these rivers require further investigation.

Mosquitofish were selected as aquatic sentinel species because they are widely distributed throughout South China. Although mosquitofish have been reported as an ideal species for the study of endocrine disrupting effects in aquatic environments (Huang et al., 2016), multi-biomarker studies for detection of possible effects in mosquitofish due to other chemical pollutants are still limited. This study aimed to examine if potential biomarker responses could be observed in the two urban rivers Shima River and Danshui River of South China by measuring a suite of selected biomarkers (condition factor (CF), hepatosomatic index (HSI), MT protein content, MT mRNA expression, EROD activity, CYP1A mRNA expression, AChE activity and glutathione *S*-transferase (GST) activity), and to evaluate if potential biomarker responses could be connected to relevant chemical contaminants. Moreover, some main data related to hormonal activities and effects from a previous study (Huang et al., 2016) have also been integrated in this study, with the aim of investigating if the estrogenic and androgenic effects in mosquitofish were responsive to changes in heavy metals, PAHs and pesticides and if the chosen biomarkers in this study are affected by (xeno)estrogens and/or androgens.

2. 2. Materials and methods

2.1. Sample collection

Western mosquitofish (*Gambusia affinis*) and surface water samples were collected from one site on the Liuxi River (S0), 5 sites on the Shima River (S1–S5) and 5 sites on the Danshui River (S6–S10) in July 2012 (wet season) and December 2012 (dry season) (Fig. 1). Both S0 and S1 were used as the reference sites as they are located in the upstream of rivers with little human activity. All samples were collected continuously from each of the sites and completed within approximately one week. More detailed information about how to obtain fish and water samples could be found in a previous study (Huang et al., 2016). In addition, some basic parameters about fish and water samples could also be found in Huang et al. (2016).

2.2. Analysis of water samples

The concentrations of eight metals including chromium (Cr), manganese (Mn), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), cadmium (Cd), and lead (Pb) in the surface water samples were measured by using inductively coupled plasma-mass spectrometry (ICP-MS, Agilent 7700, Agilent) (He et al., 2014).

Pesticides and PAHs were extracted from water samples according to our previous methods described by Yang et al. (2010) and Jiang et al. (2015). Sixteen PAHs (SI Table S1) were selected according to the EPA priority control list, while 38 pesticides were selected according to the list of top-use pesticides in Guangdong Province of China (SI Table S2). Quantitative analyses of PAHs and pesticides were performed by gas chromatography–mass spectrometry (GC–MS) using an electron impact ionization source (EI) under selected ion monitoring mode (SIM) (Jiang et al., 2015; Yang et al., 2010).

2.3. Fish morphological analysis

Fish morphological indices including condition factor (CF) and hepatosomatic index (HSI) were analyzed since they have been accepted as integrative indicators of fish condition. The collected mosquitofish were anesthetized in ice, and then total length, body weight and liver weight were measured for a total of 1157 female mosquitofish and 1143 male mosquitofish (Huang et al., 2016). Samples of heads and livers were immediately frozen in liquid nitrogen, and stored at −80 °C for subsequent assays. The condition factor of each fish was calculated as $CF = (W/L^3) \times 100$, where W is body weight (g) and L is total fish length (cm) (Wijeyaratne and Pathiratne, 2006). The hepatosomatic index (HSI) was calculated as $HSI = (LW / W) \times 100$, where W is body weight (g) and LW is liver weight (g) (Corsi et al., 2003).

2.4. Biochemical assays

For MT, liver samples were homogenized in 1:5 (tissue weight: buffer volume) ice-cold 20 mM tris–HCl buffer (pH 8.6, containing 500 mM sucrose, 6 μM leupeptin, 0.5 mM Phenylmethylsulfonyl fluoride, and 0.01% β-mercaptoethanol), then centrifuged at 15,000*g* for 30 min, 4 °C, and the supernatant was used for the MT assay. For EROD and GST analyses, livers were homogenized in 1:5 (tissue weight: buffer volume) ice-cold 100 mM phosphate buffer (pH 7.4, containing 150 mM KCl), then centrifuged at 10,000*g* at 4 °C for 10 min, and the supernatant was used for EROD and GST activity determination. For AChE activity

analysis, the head was homogenized in 1:5 (tissue weight: buffer volume) ice-cold 100 mM phosphate buffer (pH 8.0), then centrifuged at 12,000*g* for 30 min, and the supernatant was used for AChE activity determination. Three replicates of pooled liver or head samples (5 of each sex) per site were performed for the above extractions.

Protein contents of the supernatants for MT, EROD, GST and AChE analyses were measured according to Bradford (1976) adapted in microplate using bovine serum albumin as reference standard. Absorbance of samples was measured at 595 nm.

After acidic ethanol/chloroform fractionation of the tissue homogenate, the levels of MT protein were evaluated using Ellman's reagent by the spectrophotometric assay as described in Viarengo et al. (1997). The amount of MT protein was calculated based on a GSH standard curve and presented as nmol of SH groups per mg of protein (nmol/mg protein).

EROD activity was measured by a microplate kinetic assay according to the method of Eggens and Galgani (1992). The assay was carried out in 96-well plates with excitation at 544 nm and emission at 590 nm. Each well of the plate contained 25 μL liver homogenate supernatant, 300 μL of 2 μM ethoxyresorufin solution and 10 μL of 2 mM NADPH. Fluorescence was measured during 5 min. EROD activity was estimated using resorufin as a standard and expressed as nmol of resorufin formed per mg of protein per min (pmol/min/mg protein).

AChE activity was adapted from Ellman et al. (1961). 50 μL of each sample was mixed with 250 μL of pre-blended solution consisting of 30 mL of 100 mM phosphate buffer (pH 8.0), 1 mL of glutathione (10 mM) and 0.2 mL of 5,50-dithiobis-2-nitrobenzoic acid (DTNB, 75 mM) in clear 96-well plates. The change in absorbance was recorded at 412 nm at 1 min interval for 3 min, and AChE activity was calculated as nmol/min/mg protein.

GST activity was estimated as described in Habig et al. (1974). The reaction mixture consisted of 50 μL of sample, 200 μL of reduced glutathione (1 mM) and 10 μL of 1-chloro-2,4-dinitro-benzene (CDNB, 1 mM). The change in absorbance was recorded at 340 nm during 3 min using a microplate reader. GST activity was expressed as nmol/min/mg protein.

2.5. MT and CYP1A mRNA expression analysis

MT and CYP1A mRNA expression analysis in fish livers was performed as described by Huang et al. (2016). Briefly, total RNA was extracted from each of three replicate pooled liver samples (5 of each sex per pooled sample) at each sampling site using Trizol reagent (Invitrogen). Total RNA was then reverse transcribed into cDNA using ReverTra Ace® qPCR RT Master Mix (Toyobo, Japan) in a 20 μL total volume. Real-time quantitative PCR amplification was performed with the Applied Biosystems ViiA™ 7 Dx (ABI) using the THUNDERBIRD SYBR® qPCR Mix (Toyobo) (Toyobo). Primer sequences for *G. affinis* MT, CYP1A and β-Actin were obtained from Huang et al. (2013). Primer sequences for *G. affinis* ribosomal protein L8 (RPL8) gene were obtained from Huang et al. (2016). Both β-Actin and RPL8 were used as multiple reference genes to normalize MT and CYP1A mRNA expression levels. Fold change in MT and CYP1A mRNA expression levels was determined by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.6. Other chemical and biological parameters

In order to assess if the estrogenic and androgenic effects in mosquitofish were responsive to changes in heavy metals, PAHs and/or pesticides and if biomarkers in this study are affected by (xeno)estrogens and/or androgens, some main data related to hormonal activities and effects from a previous study (Huang et al., 2016) have also been integrated in this study. They included: both calculated E2 equivalent (CEEQ) and calculated DHT equivalent (CDEQ) in water samples were estimated using the addition model; the width ratio of ray 3 and ray 4 (3/4W) were used to assess the anal fin development; the ratio of 16 L/D was used to assess the elongation and the anterior bending of the hemal spine on the 16th vertebrae in mosquitofish (total spine length of the 16th hemal spine (16 L), and the perpendicular distance from the distal tip of the 16th hemal spine to the vertebral column (16D)); vitellogenin (Vtg) was used to assess estrogenic effect; androgen receptors (ARα and ARβ) were to assess androgenic effect.

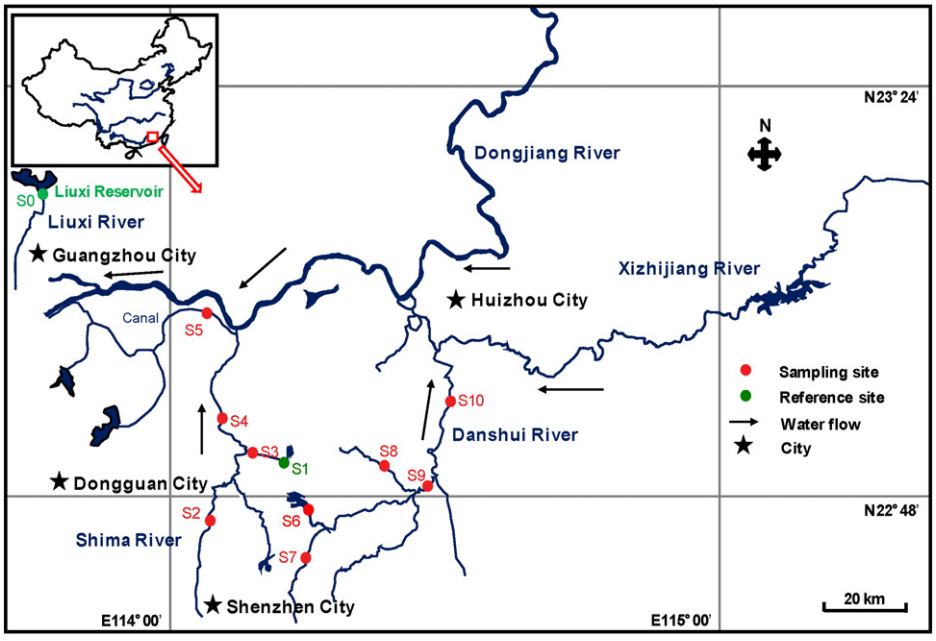

Fig. 1. Map showing location of the sampling sites in the Shima and Danshui Rivers as well as the reference site, South China (Huang et al., 2016). The red circles represent the sampling sites.

2.7. Statistical analysis

Data on morphological, biochemical and transcriptional biomarkers were presented as mean ± standard deviation (SD) in each site. All data were analyzed using SPSS 13.0. The normality of data and the homogeneity of variance were examined with Kolmogorov-Smirnov and Levene's test, respectively. If necessary, data were transformed to meet parametric assumptions. Statistical differences between sampling sites for fish various parameters were determined by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests. Differences were considered statistically significant when $p < 0.05$. Correlations between the changes of various biomarkers in mosquitofish and the environmental levels of various contaminants were analyzed using Pearson correlation and redundancy analysis (RDA). RDA analysis was selected according to an initial detrended correspondence analysis (DCA) using CANOCO 4.5 for Windows. The Monte Carlo Permutation Procedure was used to assess the significance of various contaminants in accounting for the variance of the biological responses.

3. 3. Results

3.1. Chemical contamination in the rivers

The concentrations of metals, PAHs and pesticides detected in surface water samples are summarized in Table 1. The mean concentrations of the eight metals in the river water had the following increasing trend: Cd (0.10 μg/L) < As (1.66 μg/L) < Pb (1.95 μg/L) < Cr (1.74 μg/L) < Cu (8.95 μg/L) < Ni (22.1 μg/L) < Zn (52.1 μg/L) < Mn (176 μg/L). The metals Mn, Zn, Ni and Cu were detected in water samples with maximum concentrations up to 712 μg/L, 163 μg/L, 165 μg/L and 35.2 μg/L, respectively. Among 16 target PAHs, 10 PAHs were detected in water samples, with the sum concentrations ranged from 45.2 ng/L to 1450 ng/L with a mean of 256 ng/L (Table 1). 11 of 38 target pesticides were detected in surface water samples, with the total concentrations varying between not detected and 177 ng/L with a mean of 63.1 ng/L (Table 1). Among the 11 detected pesticides, only three pesticides (fipronil, dichlorvos and butachlor) were found to have their average detection frequencies >40%.

3.2. Morphological, biochemical and transcriptional biomarkers

The results of various biomarkers were shown in Figs. 2–4. Significant changes were observed for CF and HSI parameters in female and male mosquitofish at most sampling sites compared with the reference site (S0) (Fig. 2A–D).

A significant increase in MT content in female and male mosquitofish was showed in some sites, and the increase was more evident in some sites (S7-S9) from the Danshui River than from the Shima River (Fig. 3A and B). The peak MT content in males was observed at site S7 at dry season (Fig. 3A and B). Hepatic EROD activity in female and male mosquitofish was significantly elevated at the majority of sampling sites compared to the reference site (S0) (Fig. 3C and D). The

Table 1
Concentrations of eight heavy metals, detected ten PAHs and eleven pesticides by chemical analysis in the Shima and Danshui Rivers as well as the reference site.

Compounds	Reference sites				Shima River				Danshui River			
	Range	Mean	Median	Freq	Range	Mean	Median	Freq	Range	Mean	Median	Freq
Heavy metals (μg/L)												
Cr	<0.47–2.37	1.18	1.18	75%	0.83–3.75	2.43	2.26	100	0.67–3.43	1.62	1.46	100
Mn	11.9–81.6	36.6	26.4	100	75.9–343	184	178	100	19.4–712	306	262	100
Ni	0.43–2.20	0.92	0.53	100	6.96–39.4	20.3	18.5	100	1.14–165	45.2	23.3	100
Cu	0.71–26.7	7.73	1.73	100	2.81–16.4	6.03	4.23	100	1.08–35.2	13.1	10.5	100
Zn	25.2–53.9	35.1	30.6	100	20.5–163	69.4	62.9	100	14.2–108	51.8	43.0	100
As	0.40–0.72	0.52	0.48	100	1.00–2.41	1.63	1.74	100	1.07–4.51	2.82	2.80	100
Cd	0.02–0.06	0.04	0.04	100	0.02–0.11	0.06	0.07	100	0.04–0.63	0.19	0.10	100
Pb	<0.67–3.05	1.62	1.72	75%	0.84–2.99	1.90	1.94	100	<0.67–8.45	2.34	1.77	90
∑Heavy metals	40.1–121	83.6	86.8	100	188–533	285	281	100	56.4–785	423	409	100
PAHs (ng/L)												
Naphthalene	9.16–26.2	16.7	15.7	100	ND–22.3	12.8	11.8	88	1.90–82.6	17.5	11.4	100
Acenaphthylene	ND			0	ND			0	ND–35.9	3.59	0	10
Acenaphthene	ND			0	ND			0	ND–1043	104	0	10
Fluorene	17.4–41.4	24.8	20.3	100	20.5–44.4	32.7	33.4	100	8.00–102	27.2	17.8	100
Phenanthrene	34.1–156	77.3	59.7	100	33.2–106	76.4	81.7	100	23.7–135	57.8	51.1	100
Anthracene	ND–156.2	48.2	18.3	50	ND–108	70.5	83.5	88	ND–75.3	23.9	0	40
Fluoranthene	6.37–80.8	26.2	8.8	100	8.18–25.6	17.7	16.5	100	ND–29.1	10.5	10.8	80
Pyrene	8.63–36.5	22.8	22.9	100	14.0–44.6	30.8	32.1	100	ND–35.1	21.7	23.2	90
Benz(a)anthracene	ND			0	ND			0	ND–29.3	7.32	0	30
Chrysene	7.04–27.2	19.0	21.0	100	ND–29.4	12.6	9.76	63	ND–26.2	5.10	0	20
∑PAHs	127–511	235	151	100	125–341	253	261	100	45.2–1450	279	162	100
Pesticides (ng/L)												
Isoprocarb	ND			0	ND			0	ND–44.3	5.89	0	20
Atrazine	ND			0	ND–100	21.5	0	38	ND–98.2	15.4	0	40
Alachlor	ND			0	ND–12.8	3.03	0	25	ND			0
Metolachor	ND			0	ND			0	ND–24.8	2.48	0	10
Fipronil	ND–10.3	2.58	0	25	10.6–33.9	19.1	13.8	100	ND–15.4	8.74	10.6	80
Butachlor	ND–35.2	8.79	0	25	ND–33.3	9.60	0	38	ND–26.2	10.1	9.11	60
Procymidone	ND			0	ND–16.3	3.66	0	25	ND			0
Phoxim	ND			0	ND				ND–2.83	0.28	0	10
Dichlorvos	ND–20.6	5.1	0	25	ND–34.9	21.3	23.6	88	ND–24.4	11.8	13.2	80
Isofenphosmethyl	ND			0	ND			0	ND–9.68	0.97	0	10
Chlorpyrifos	ND–94.3	23.6	0	25	ND			0	ND–121	15.5	0	20
∑Pesticides	ND–105	40.1	27.9	75	10.6–167	78	57	100	ND–177	71.2	67.6	90

Remark: Mean value, median value and frequency are calculated based on those with concentrations higher than the limit of quantification. Freq, the detection frequency. ND, not determined.

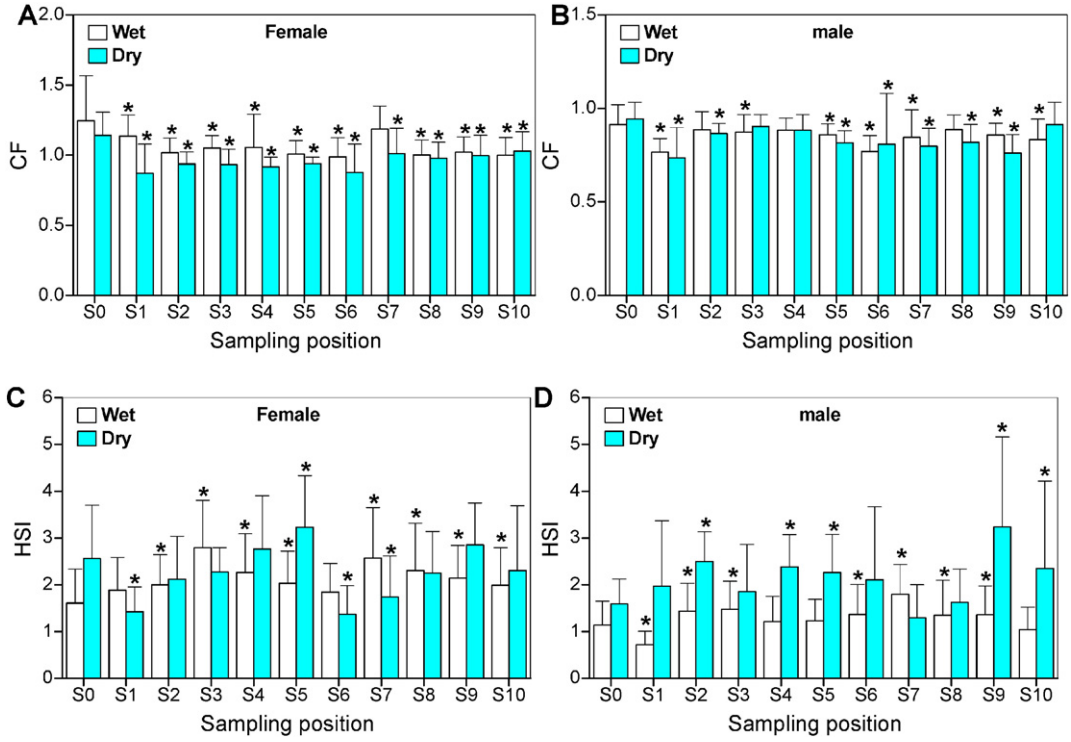

Fig. 2. Three morphological parameters (CF and HSI) in male and female mosquitofish from the Shima River and Danshui River as well as the reference site. Error bars represent the standard deviations of the measured values. Significant differences between the sampling sites and reference site (S0) are indicated by an asterisk ($p < 0.05$).

AChE activity of mosquitofish brain from the majority of sampling sites was inhibited when compared with that from the reference site (S0) (Fig. 3E and F). The GST activity was generally enhanced at most sampling sites relative to the reference site (S0) (Fig. 3G and H). A higher GST activity was detected in females than in males at all sampling sites except for site S10 at wet season and site S5 at dry season.

In contrast to MT protein, significant induction in MT mRNA expression was detected at the majority of sampling sites when compared to the reference site (S0) (Fig. 4A and B). In addition, significant inhibition in MT mRNA expression in females was also observed at sites S1, S2 or S3 (Fig. 3A and B). MT mRNA expression in fish collected from the Shima River in the dry season was significantly greater compared to that in the wet season (Fig. 4A and B). CYP1A mRNA expression in female and male mosquitofish was significantly induced at some sampling sites relative to the reference site (S0), and induction of CYP1A mRNA expression was more evident in the dry season than in the wet season at most sampling sites (Fig. 3C and D).

3.3. Correlation between chemical concentrations and fish biomarkers

Pearson correlation analysis showed that only a few contaminants in water samples have correlations with CF or HSI parameters (SI Tables S3, S4 and S5). Pearson correlation analysis also indicated the increases in MT content and mRNA expression level were correlated significantly with the presence of target metals in the water of the Shima River and Danshui River (SI Table S3). In addition, Pearson correlation analysis showed that EROD activity was correlated to CYP1A mRNA expression level but not to the sum concentration of PAHs in the river water (SI Table S4). No significant correlation between AChE activity and the sum concentration of pesticides was found, but AChE activity had significantly negative correlation with the aqueous concentrations of fipronil (SI Table S5). GST activity was not correlated with the concentrations of metals, PAHs and pesticides in water samples (SI Tables S3, S4 and S5). More importantly, Pearson correlation analysis showed that both the sum concentration of metals and the sum concentration of pesticides have significant correlations with some biological parameters in mosquitofish related to the estrogenic and androgenic effects (Vtg, AR, 3/4 W and 16 L/D in females or males).

The results of RDA for chemicals and biomarkers are displayed in the triplot diagram (Fig. 5). Single PAHs and pesticides were not included in the RDA analysis because some of them had high linear correlation with the sum concentration of PAHs or pesticides and showed high variance inflation factors (VIF > 10). The VIFs of the chemicals chosen for RDA were reasonably low (from 2.0 to 9.1). The first ordination RDA axis was mainly correlated to As, Ni and Mn and explained 48.6% of the variation in various biomarkers (64.1% of their relation to chemicals). MT protein content was strongly positively correlated with Mn, Ni and As concentrations. The second ordination axis, which was associated with Zn, Cr, Pb and the sum concentration of PAHs, accounted for 10.5% of the variation (13.8% of their relation to chemicals). The sum concentration of pesticides seems to have a negative influence in AChE activity in mosquitofish. In addition, the sum concentration of PAHs was not correlated with CYP1A mRNA expression and EROD activity. CYP1A mRNA expression and EROD activity in females and males seem to be not influenced by the sum concentration of PAHs. RDA analysis showed that both heavy metals and the sum concentration of pesticides have significantly correlated with some biological parameters in mosquitofish related to the estrogenic and androgenic effects.

4. Discussion

The results from this study showed toxic effects in mosquitofish from the two urban rivers. There was an obvious seasonal variation of contaminant load in the river water and biological effects in mosquitofish, which may be due to the difference of precipitation, surface runoff, municipal wastewater discharges or other factors between seasons.

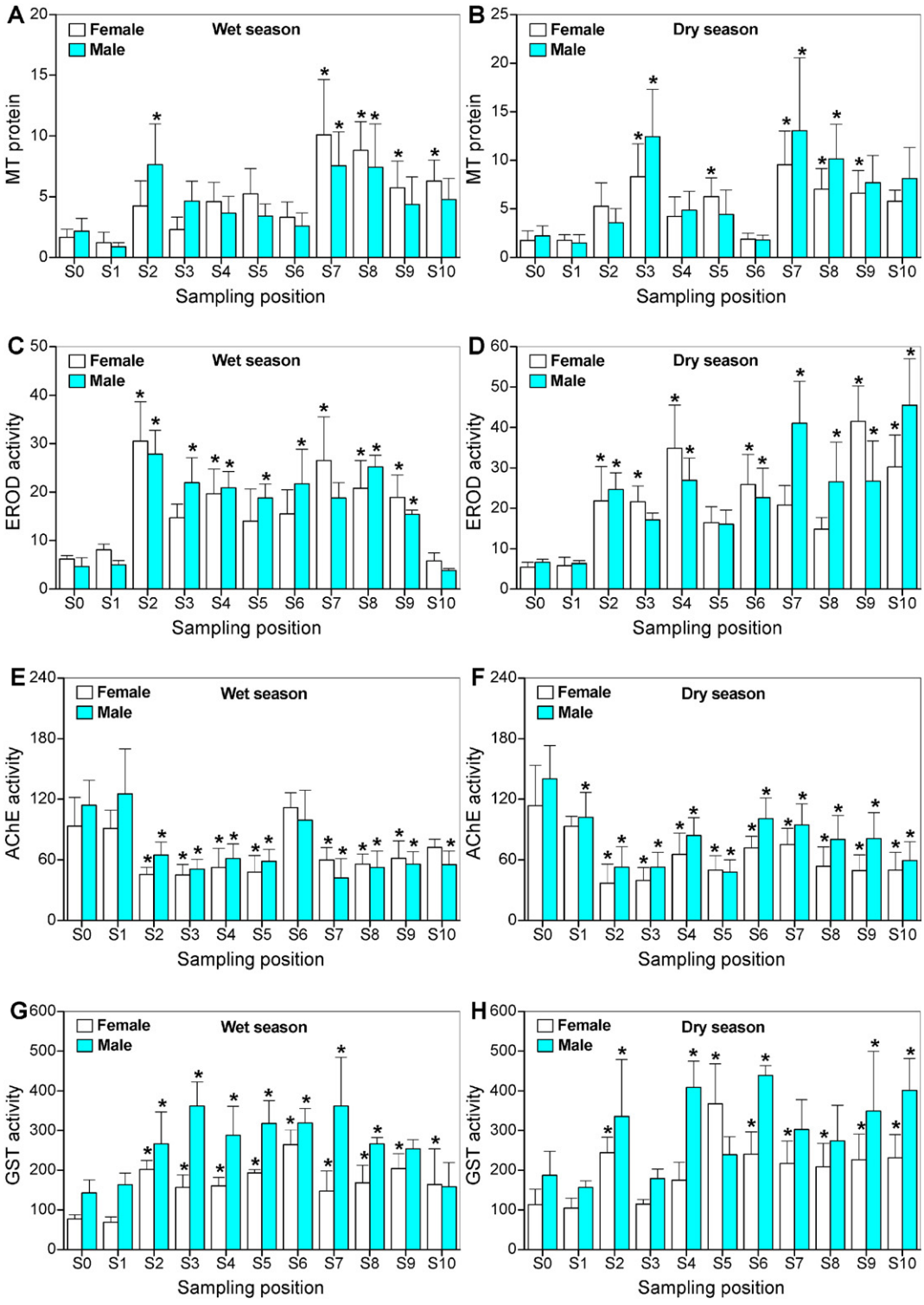

Fig. 3. MT protein (nmol/mg protein), EROD activity (pmol/min/mg protein), AChE activity (nmol/min/mg protein) and GST activity (nmol/min/mg protein) in male and female mosquitofish from the Shima River and Danshui River as well as the reference site. Error bars represent the standard deviations of the measured values. Significant differences between the sampling sites and reference site (S0) are indicated by an asterisk ($p < 0.05$).

Various types of biomarkers have been used for assessing the toxic effect of contaminants in environmental risk assessment (Linde-Arias et al., 2008). Toxic effects at higher organization levels can be analyzed with parameters involving whole tissues or the whole organism (Olivares et al., 2010). Previous studies have highlighted the usefulness of integrating a set of different biomarkers when assessing the biological effects in polluted environments since a single biomarker may not reflect the health status of a sentinel species (Frenzilli et al., 2008; Linde-Arias et al., 2008). Prior to death or overt sickness, organisms may respond to stress by changing biological responses. Understanding these changes of biological responses may provide an early warning of later, much more serious consequences (Linde-Arias et al., 2008).

Morphological parameters such as CF and HSI could be used to assess the health status of fish and the toxic effects of pollutants in field

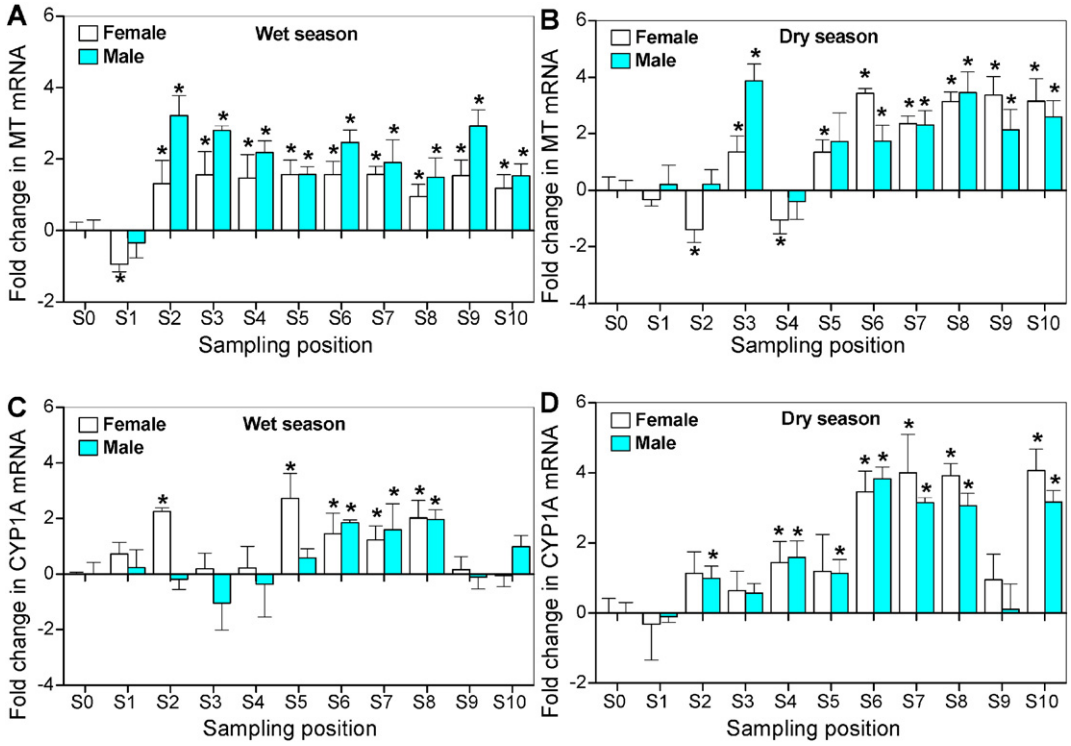

Fig. 4. Transcriptional responses (MT mRNA and CYP1A mRNA) in male and female mosquitofish from the Shima River and Danshui River as well as the reference site. Error bars represent the standard deviations of the measured values. The mRNA expression of each target gene was compared to that in the reference site (S0). Significant differences between the sampling sites and reference site (S0) are indicated by an asterisk ($p < 0.05$).

research (Barhoumi et al., 2014). In the present study, a significant decrease was found in CF of mosquitofish at the majority of sampling sites, which may be due to impaired the food consumption of mosquitofish or due to toxicant stresses. Linde-Arias et al. (2008) reported a similar result that fish from the most polluted sampling site had the lowest CF value. Previous studies reported variable HSI results, i.e. increase (Corsi et al., 2003; de la Torre et al., 2007), no change (Barhoumi et al., 2014), and decrease (Napierska et al., 2009) in fish from contaminated sites. Increased HSI values in mosquitofish in the present study may be the result of either hypertrophy (increase in cell size) or hyperplasia (increase in cell number) (van der Oost et al., 2003). The changes in CF and HSI suggest there may be some contaminants related effects on fish health. However, these parameters are likely influenced by non-pollutant factors and do not give information of specific responses to contaminants, but they may serve as initial screening biomarkers to indicate toxic effects of contaminants in fish.

Biochemical and gene expression biomarkers are useful tools to detect possible and specific environmental impacts (Olivares et al., 2010). The induction of MT protein and mRNA expression in this study was likely associated with the presence of metals in the two contaminated rivers (Houde et al., 2013). This study also suggests that MT may play an important role in metal detoxification in mosquitofish, as has been shown in other fish species. Metals Mn, Cu, and Cd showed better correlations with MT protein than with MT mRNA expression. One reasonable interpretation of the present result might be MT protein represent the total content of all MT isoform protein, whereas MT mRNA expression is only a MT isoform gene transcriptional level. Two MT isoform genes have been identified in fish (Bargelloni et al., 1999). MT genes in mosquitofish might have different isoforms, which exhibit different mRNA expression patterns in response to metals. In a previous study, MT mRNA expression levels in mosquitofish showed different expression patterns in response to different metals (Huang et al., 2014). To the best of our knowledge, no study has investigated the existence of different types of MT genes in mosquitofish. In addition, in this study, Mn appeared to be one primary metal responsible for MT mRNA and protein induction, which was also supported by previous laboratory and field-based studies (Falfushynska et al., 2011; Gehringer et al., 2013; Podrug and Raspor, 2009).

Both EROD activity and CYP1A mRNA expression have been used as a specific biomarker for exposure to AhR agonists in fish (Corsi et al., 2003; Quiró et al., 2007). In the present study, the increases of both EROD activity and CYP1A mRNA expression suggest the presence of AhR agonists in the Shima River and Danshui River. However, no significant correlation was found between the total PAHs and the levels of EROD activity and CYP1A mRNA expression. Therefore, PAHs did not well account for the enhanced EROD activity and CYP1A mRNA expression in mosquitofish from the two rivers. One possible explanation could be due to the presence of other persistent organic pollutants (POPs) such as organochlorine pesticides (OCPs) and polychlorinated bisphenyls (PCBs) (Otter et al., 2012; Ying et al., 2012). Unfortunately, those contaminants were not measured in this study. However, our previous study reported the concentration ranges of 45.7 to 1743 ng/L for OCPs and 0.051 to 4.32 ng/L for PCBs in the investigated Dongjiang River basin (Ying et al., 2012). In addition, the effects of metals or steroid hormones on EROD activity and CYP1A mRNA expression cannot be ignored, where mosquitofish are exposed to complex mixtures including metals and steroid hormones in the two rivers (Huang et al., 2014, Huang et al., 2013; Huang et al., 2012; Lavado et al., 2006; Ying et al., 2012). It is noted in the present study that the correlations between EROD activity and CYP1A mRNA expression appear to be sex specific, which might be due to sex-related differences in P450 activity. In some previous studies, sex-related differences in P450 activity during the reproductive cycle have been reported in other fish (Andersson, 1990; Gray et al., 1991). Moreover, some previous studies showed a good correlation between EROD activity and CYP1A mRNA expression in fish (Quiró et al., 2007; Valdehita et al., 2012), but others also

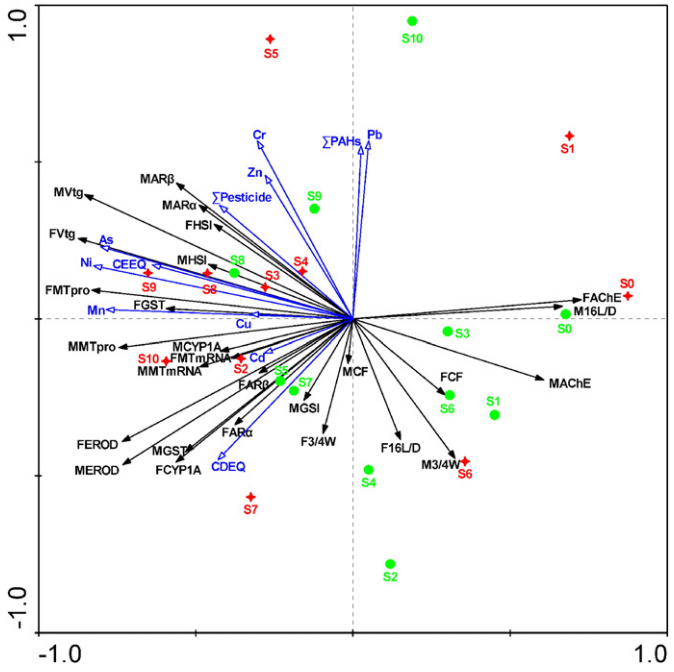

Fig. 5. Redundancy analysis (RDA) ordination diagram (triplot) showing samples (green circles for wet season, red stars for dry season), explanatory variables (blue hollow arrows), and response variables (black solid arrows). First axis is horizontal, second axis is vertical. F and M in front of biomarkers in mosquitofish represent female and male, respectively; MT pro represents MT protein content; CEEQ and CDEQ represent the calculated EEQ and DEQ values, respectively; 3/4 W represents the width ratio of ray 3 and ray 4 (3/4W); 16L/D represents the ratio of total spine length of the 16th hemal spine (16L) and perpendicular distance from the distal tip of the 16th hemal spine to the vertebral column (16D); Vtg represents vitellogenin; ARα and ARβ represent androgen receptor α and androgen receptor β, respectively. The angles among arrows denote the degree of correlation between the individual variables, and the smaller the angle, the larger the correlation. In addition, positively correlated variables are shown as arrows pointing in the same direction, negatively correlated variables pointing in opposite directions. Data (CEEQ, CDEQ, 3/4W, 16L/D, Vtg, ARα, and ARβ) could be obtained from a previous study (Huang et al., 2016).

reported a poor correlation between EROD activity and CYP1A mRNA expression (Della Torre et al., 2010; Kammann et al., 2008; Karaca et al., 2014).

Inhibition of AChE activity in this study can impair cholinergic nerve impulses, which will result in tremors, convulsion and finally the death of the aquatic organism (Fulton and Key, 2001; Oliva et al., 2012). But also, lower AChE activity level, it can interfere with olfaction, and make it more difficult for fish to detect prey and avoid predators (Tierney et al., 2010). Pesticides were widely detected in the Shima River and Danshui River. Moreover, RDA analysis also showed negative correlations with the total concentrations of pesticides in water samples. However, by Pearson correlation analysis, only fipronil and isoprocarb in the river water showed a significant negative correlation with AChE activity in mosquitofish whereas recognized anticholinergic pesticides or carbamates often did not. Fipronil is a broad-spectrum insecticide that disrupts the insect central nervous system by blocking GABA-gated chloride channels and glutamate-gated chloride channels, resulting in central nervous system toxicity (Ying and Kookana, 2002). Moreover, some studies have reported that the inhibition of AChE activity of fish was less specific, and it is a more general biomarker of exposure to neurotoxic contaminants including heavy metals (Corsi et al., 2003, Frasco et al., 2005; Khan et al., 2015). In the present study, the concentrations of Ni, Mn, and As in the two rivers had significant negative correlations to the AChE activity in mosquitofish. A similar result was also observed in a previous study, where the AChE activity in muscle of senegal sole from an estuary was correlated with Pb, Cd and Cu concentrations in water (Oliva et al., 2012). The inhibitory effect of heavy metals on the AChE activity could be interpreted as changes in the enzyme due to binding metals with a functional active AChE site or other location such as the surface of the enzyme (de la Torre et al., 2007; Oliva et al., 2012).

Induced EROD activity and CYP1A mRNA expression could be further supported by the increase of GST activity (Otter et al., 2012). GST is a phase II enzyme, which plays an important role in conjugation of xenobiotics or their metabolites. The alteration in hepatic GST activity in mosquitofish in the present study suggested the possibility of better protection against the toxicity of organic pollutants or their toxic or carcinogenic metabolites in mosquitofish. Many of chemicals such as metals, POPs and pesticides can increase generation of reactive oxygen species and GST induction may be a means of protecting the exposed fish against their toxicity. However, the induction of GST activity in mosquitofish did not show significant correlations with metal, PAHs and pesticides in the present study. This can be explained by the mixture effects in the river water as no single group is responsible for the increased GST activity.

In a previous study, mosquitofish at the same study sites experienced strong estrogenic and androgenic effects, which may be associated with estrogens and androgens in the two rivers, respectively (Huang et al., 2016). However, further analysis also showed that heavy metals and pesticides may have strong estrogenic effect in mosquitofish collected from the Shima River and Danshui River. It is not surprising for estrogenic effects in mosquitofish of heavy metals and pesticides, because a great deal of research has showed that several heavy metals and pesticides have estrogenic effects as reviewed by Iavicoli et al. (2009) and Iavicoli et al. (2009), respectively. In addition, an increase, decrease, or no change in AR mRNA expression levels has been observed in wild mosquitofish from the same study sites (Huang et al., 2016). It is surprising that AR mRNA expression levels have significantly positive correlations with heavy metals and pesticides, but they showed the weak correlations with androgens (Huang et al., 2016). To date, there are also not literatures concerning androgenic effects of heavy metals and pesticides. It is still unclear whether these transcription-level effects of ARs in mosquitofish resulted in masculinization of mosquitofish observed in the two rivers, but heavy metals and pesticides might play a significant role in disrupting AR signaling of mosquitofish. The influence of heavy metals and pesticides should be taken into consideration in field studies, where the estrogenic and/or androgenic effects were assessed in aquatic animals.

5. Conclusions

The results showed significant alterations in the selected morphological, biochemical and transcriptional biomarkers in mosquitofish from the two urban rivers impacted by municipal wastewaters, which may eventually influence the health of mosquitofish population in the rivers. Specific biomarkers (MT protein and mRNA expression, AChE activity, EROD activity and CYP1A mRNA expression) were generally linked to the presence of specific groups of chemicals, i.e. metals, pesticides and POPs in the rivers; however, the complexity of chemical contaminants makes it difficult to well associate biological effects to particular pollutants. Nonetheless, the present study combined with a previous study about significant endocrine disrupting effects in mosquitofish from the same sampling sites in the Shima River and Danshui River (Huang et al., 2016), denote the extremely poor water quality at the majority of sampling sites, and indicate mosquitofish can be conveniently used for the assessment of river environmental quality.

Acknowledgement

The authors would like to acknowledge the financial support from the National Natural Science Foundation of China (U1133005, 41201522, 41273119, and U1401235). This is a Contribution No. IS-2226 from GIG CAS.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2016.04.127.

References

Andersson, T., 1990. Sex differences in cytochrome P-450-dependent xenobiotic and steroid metabolism in the mature rainbow trout kidney. J. Endocrinol. 126, 9–16.

Bargelloni, L., Scudiero, R., Parisi, E., Carginale, V., Capasso, C., Patarnello, T., 1999. Metallothioneins in antarctic fish: evidence for independent duplication and gene conversion. Mol. Biol. Evol. 16, 885–897.

Barhoumi, B., Clérandeau, C., Gourves, P., Menach, K.L., Megdiche, Y.E., Peluhet, L., Budzinski, H., Baudrimont, M., Driss, M.R., Cachot, J., 2014. Pollution biomonitoring in the Bizerte lagoon (Tunisia), using combined chemical and biomarker analyses in grass goby, *Zosterisessor ophiocephalus* (Teleostei, Gobiidae). Mar. Environ. Res. 101, 184–195.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Chen, Z.F., Ying, G.G., Liu, Y.S., Zhang, Q.Q., Zhao, J.L., Liu, S.S., Chen, J., Peng, F.J., Lai, H.J., Pan, C.G., 2014. Triclosan as a surrogate for household biocides: an investigation into biocides in aquatic environments of a highly urbanized region. Water Res. 58, 269–279.

Corsi, I., Mariottini, M., Sensini, C., Lancini, L., Focardi, S., 2003. Cytochrome P450, acetylcholinesterase and gonadal histology for evaluating contaminant exposure levels in fishes from a highly eutrophic brackish ecosystem: the Orbetello lagoon, Italy. Mar. Pollut. Bull. 46, 203–212.

de la Torre, F.R., Salibián, A., Ferrari, L., 2007. Assessment of the pollution impact on biomarkers of effect of a freshwater fish. Chemosphere 68, 1582–1590.

Della Torre, C., Corsi, I., Nardi, F., Perra, G., Tomasino, M.P., Focardi, S., 2010. Transcriptional and post-transcriptional response of drug-metabolizing enzymes to PAHs contamination in red mullet (*Mullus barbatus*, Linnaeus, 1758): a field study. Mar. Environ. Res. 70, 95–101.

Eggens, M.L., Galgani, F., 1992. Ethoxyresorufin-*O*-deethylase (EROD) activity in flatfish: fast determination with a fluorescence plate-reader. Mar. Environ. Res. 33, 213–221.

Ellman, G.L., Courtney, K.D., Andreas Jr., V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 82–88.

Falfushynska, H.I., Gnatyshyna, L.L., Stoliar, O.B., Nam, Y.K., 2011. Various responses to copper and manganese exposure of *Carassius auratus* gibelio. Comp Biochem Physiol C 154, 242–253.

Fernandes, D., Porte, C., Bebianno, M.J., 2007. Chemical residues and biochemical responses in wild and cultured European sea bass (*Dicentrarchus labrax* L.). Environ. Res. 103, 247–256.

Frasco, M.F., Fournier, D., Carvalho, F., Guilhermino, L., 2005. Do metals inhibit acetyl cholinesterase (AchE)? Implementation of assay conditions for the use of AchE activity as a biomarker of metal toxicity. Biomarkers 10, 360–375.

Frenzilli, G., Falleni, A., Scarcelli, V., Del Barga, I., Pellegrini, S., Savarino, G., Mariotti, V., Benedetti, M., Fattorini, D., Regoli, F., Nigro, M., 2008. Cellular responses in the cyprinid *Leuciscus cephalus* from a contaminated freshwater ecosystem. Aquat. Toxicol. 89, 188–196.

Fulton, M.H., Key, P.B., 2001. Acetylcholinesterase inhibition in estuarine fish and invertebrates as an indicator of organophosphorous insecticide exposure and effects. Environ. Toxicol. Chem. 20, 37–45.

Gagnon, C., Gagné, F., Turcotte, P., Saulnier, I., Blaise, C., Salazar, M.H., Salazar, S.M., 2006. Exposure of caged mussels to metals in a primary-treated municipal wastewater plume. Chemosphere 62, 998–1010.

Gehringer, D.B., Finkelstein, M.E., Coale, K.H., Stephenson, M., Geller, J.B., 2013. Assessing mercury exposure and biomarkers in largemouth bass (*Micropterus salmoides*) from a contaminated river system in California. Arch. Environ. Contam. Toxicol. 64, 484–493.

Gray, E.S., Woodin, B.R., Stegeman, J.J., 1991. Sex differences in hepatic monooxygenases in winter flounder (*Pseudopleuronectes americanus*) and scup (*Stenotomus chrysops*) and regulation of P450 forms by estradiol. J Exp Zool 259, 330–342.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione-*S*-transferases. The first enzymatic step in mercapturic acid formation. J Biol Chem 249, 7130–7139.

Hansen, B.H., Rømma, S., Garmo, Ø.A., Olsvik, P.A., Andersen, R.A., 2006. Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels. Comp Biochem Physiol C 143, 263–274.

He, L.Y., Liu, Y.S., Su, H.C., Zhao, J.L., Liu, S.S., Chen, J., Liu, W.L., Ying, G.G., 2014. Dissemination of antibiotic resistance genes in representative broiler feedlots environments: identification of indicator ARGs and correlations with environmental variables. Environ Sci Technol 48, 13120–13129.

Holeton, C., Chambers, P.A., Laura, Grace L., 2011. Wastewater release and its impacts on Canadian waters. Can. J. Fish. Aquat. Sci. 68, 1836–1859.

Houde, M., Douville, M., Despatie, S.P., De Silva, A.O., Spencer, C., 2013. Induction of gene responses in St. Lawrence River northern pike (*Esox lucius*) environmentally exposed to perfluorinated compounds. Chemosphere 92, 1195–1200.

Huang, G.Y., Liu, Y.S., Chen, X.W., Liang, Y.Q., Liu, S.S., Yang, Y.Y., Hu, L.X., Shi, W.J., Tian, F., Zhao, J.L., Chen, J., Ying, G.G., 2016. Feminization and masculinization of western mosquitofish (*Gambusia affinis*) observed in rivers impacted by municipal wastewaters. Sci Rep 6, 20884.

Huang, G.Y., Ying, G.G., Liang, Y.Q., Liu, Y.S., Liu, S.S., 2013. Effects of steroid hormones on reproduction- and detoxification-related gene expression in adult male mosquitofish, *Gambusia affinis*. Comp Biochem Physiol C 158, 36–43.

Huang, G.Y., Ying, G.G., Liang, Y.Q., Liu, S.S., Liu, Y.S., 2014. Expression patterns of metallothionein, cytochrome P450 1A and vitellogenin genes in western mosquitofish (*Gambusia affinis*) in response to heavy metals. Ecotox Environ Safe 105, 97–102.

Huang, G.Y., Ying, G.G., Liu, S., Fang, Y.X., 2012. Regulation of reproduction- and biomarker-related gene expression by sex steroids in the livers and ovaries of adult female western mosquitofish (*Gambusia affinis*). Comp Biochem Physiol A 162, 36–43.

Iavicoli, I., Fontana, L., Bergamaschi, A., 2009. The effects of metals as endocrine disruptors. J Toxicol Environ Heal B 12, 206–223.

Jiang, Y.X., Liu, Y.S., Ying, G.G., Wang, H.W., Liang, Y.Q., Chen, X.W., 2015. A new tool for assessing sediment quality based on the Weight of Evidence approach and grey TOPSIS. Sci. Total Environ. 537, 369–376.

Kammann, U., Lang, T., Berkau, A.J., Klempt, M., 2008. Biological effect monitoring in dab (*Limanda limanda*) using gene transcript of CYP1A1 or EROD–a comparison. Environ. Sci. Pollut. Res. 15, 600–605.

Karaca, M., Varıs, L., Korkmaz, K., Özaydın, O., Perççin, F., Orhan, H., 2014. Organochlorine pesticides and antioxidant enzymes are inversely correlated with liver enzyme gene expression in *Cyprinus carpio*. Toxicol. Lett. 230, 198–207.

Khan, S.A., Liu, X., Li, H., Fan, W., Shan, B.R., Li, J., Zhang, L., Chen, S., Khan, S.B., 2015. Organ-specific antioxidant defenses and FT-IR spectroscopy of muscles in Crucian carp (*Carassius auratus* gibelio) exposed to environmental Pb^{2+}. Turk. J. Biol. 39, 427–437.

Lavado, R., Ureña, R., Martin-Skilton, R., Torreblanca, A., Ramo, J., Raldúa, D., Porte, C., 2006. The combined use of chemical and biochemical markers to assess water quality along the Ebro River. Environ. Pollut. 139, 330–339.

Linde-Arias, A.R., Inácio, A.F., Alburquerque, C., Freire, M.M., Moreira, J.C., 2008. Biomarkers in an invasive fish species, *Oreochromis niloticus*, to assess the effects of pollution in a highly degraded Brazilian River. Sci. Total Environ. 399, 186–192.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25, 402–408.

Maes, G.E., Raeymaekers, J.A.M., Hellemans, B., Geeraerts, C., Parmentier, K., De Temmerman, L., Volckaert, F.A.M., Belpaire, C., 2013. Gene transcription reflects poor health status of resident European eel chronically exposed to environmental pollutants. Aquat. Toxicol. 126, 242–255.

Mierzejewski, J., Haney, D.C., van den Hurk, P., 2014. Biomarker responses in sunfish species and largemouth bass from the Saluda River, South Carolina. Ecotox Environ Safe 110, 8–15.

Napierska, D., Baršienė, J., Mulkiewicz, E., Podolska, M., Rybakovas, A., 2009. Biomarker responses in flounder *Platichthys flesus* from the polish coastal area of the Baltic Sea and applications in biomonitoring. Ecotoxicology 18, 846–859.

Oliva, M., Perales, J.A., Gravato, C., Guilhermino, L., Galindo-Riaño, M.D., 2012. Biomarkers responses in muscle of Senegal sole (*Solea senegalensis*) from a heavy metals and PAHs polluted estuary. Mar. Pollut. Bull. 64, 2097–2108.

Olivares, A., Quirós, L., Pelayo, S., Navarro, A., Bosch, C., Grimalt, J.O., Fabregat, M., Faria, M., Benejam, L., Benito, J., Solé, M., Barata, C., Piña, B., 2010. Integrated biological and chemical analysis of organochlorine compound pollution and of its biological effects in a riverine system downstream the discharge point. Sci. Total Environ. 408, 5592–5599.

Otter, R.R., Schreiber, E.A., van den Hurk, P., Klaine, S.J., 2012. Assessment of heavy metal and PAH exposure in largemouth bass (*Micropterus salmoides*) in the Reedy River watershed, South Carolina, USA: a multi-season assessment of metallothionein and bile fluorescence. Environ. Toxicol. Chem. 31, 2763–2770.

Pan, C.G., Ying, G.G., Liu, Y.S., Zhang, Q.Q., Chen, Z.F., Peng, F.J., Huang, G.Y., 2014. Contamination profiles of perfluoroalkyl substances in five typical rivers of the Pearl River Delta region, South China. Chemosphere 114, 16–25.

Podrug, M., Raspor, B., 2009. Seasonal variation of the metal (Zn, Fe, Mn) and metallothionein concentrations in the liver cytosol of the European chub (*Squalius cephalus* L.). Environ. Monit. Assess. 157, 1–10.

Quiró, L., Piña, B., Solé, M., Blasco, J., López, M.Á., Riva, M.C., Barceló, D., Raldúa, D., 2007. Environmental monitoring by gene expression biomarkers in *Barbus graellsii*: laboratory and field studies. Chemosphere 67, 1144–1154.

Roberts, A.P., Oris, J.T., Burton, J.R., Williamh, G.A., Illiam, H., Clements, W., 2005. Gene expression in caged fish as a first-tier indicator of contaminant exposure in streams. Environ. Toxicol. Chem. 24, 3092–3098.

Tierney, K.B., Baldwin, D.H., Hara, T.J., Ross, PS, Scholz, N.L., Kennedy, C.J., 2010. Olfactory toxicity in fishes. Aquat. Toxicol. 96, 2–26.

Valdehita, A., Fernández-Cruz, M.L., Torrent, F., Sericano, J.L., Navas, J.M., 2012. Differences in the induction of cyp1A and related genes in cultured rainbow trout *Oncorhynchus mykiss*. Additional considerations for the use of EROD activity as a biomarker. J Fish Biol 81, 270–287.

van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

Viarengo, A., Ponzano, E., Dondero, F., Fabbri, R., 1997. A simple spectrophotometric method for metallothionein evaluation in marine organisms: an application to Mediterranean and Antarctic molluscs. Mar. Environ. Res. 44, 69–84.

Wijeyaratne, W.M.D.N., Pathiratne, A., 2006. Acetylcholinesterase inhibition and gill lesions in *Rasbora caverii*, an indigenous fish inhabiting rice field associated waterbodies in Sri Lanka. Ecotoxicology 15, 609–619.

Yang, X.B., Ying, G.G., Kookana, R.S., 2010. Rapid multiresidue determination for currently used pesticides in agricultural drainage waters and soils using gas chromatography–mass spectrometry. J Environ Sci Heal B 45, 152–161.

Ying, G.G., Kookana, R., 2002. Laboratory and field studies on the degradation of fipronil in a soil. Aust. J. Soil Res. 40, 1095–1102.

Ying, G.G., Peng, P.A., Zhao, J.L., Ren, M.Z., Chen, H.M., Wei, D.B., Li, B.G., Song, J.Z., 2012. Watershed Ecological Risk Assessment of Chemicals-Dongjiang River Basin as an Example (in Chinese). Science Press, Beijing.