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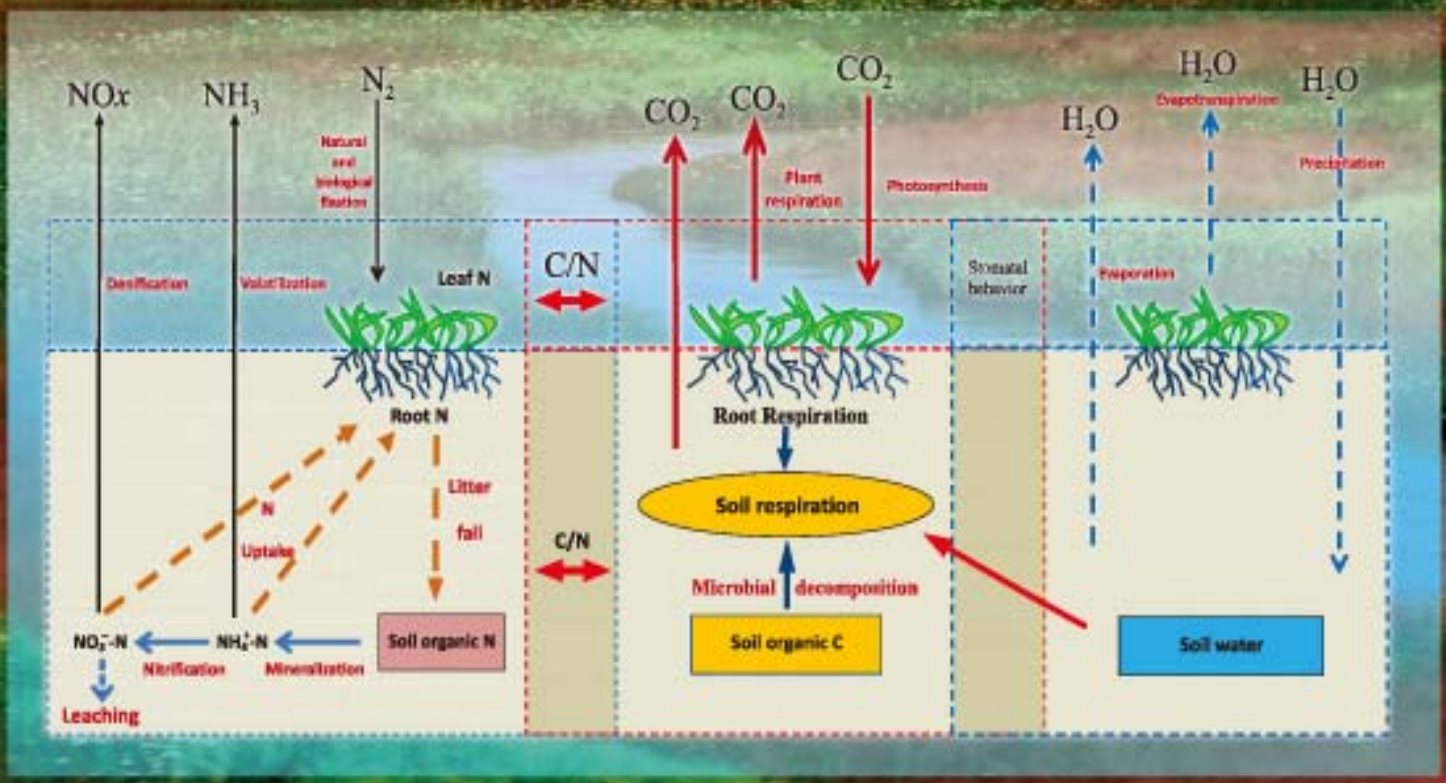

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Integrated biomarkers in wild crucian carp for early warning of water quality in Hun River, North China

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ABSTRACT

Metabolizing enzymes play important roles in the detoxification of various pollutants in aquatic organisms, thereby they can also be used to provide early-warning signals of environmental risks. Real-time quantitative reverse-transcription polymerase chain reaction assays were developed to quantify cytochrome P450 1A (CYP1A), superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), and glutathione-S-transferase (GST) in crucian carp (*Carassius auratus*). The methods were then used to detect the respective mRNA expression levels in liver tissue in wild crucian carp from the Hun River, North China. CYP1A mRNA expression was significantly up-regulated in fish from stations S5, S6, and S8 ($p < 0.05$). SOD mRNA expression was significantly down-regulated in downstream areas relative to fish from upstream sites ($p < 0.05$); GPx and CAT mRNA expression levels were also down-regulated at S9 ($p < 0.05$). In contrast, GST mRNA expression showed no obvious change between fish collected from up- or downstream areas of the river. Finally, an integrated biomarker response was used to evaluate the integrated impact of pollutants in the Hun River and allow better comprehension of the real toxicological risk of these investigated sites.

Introduction

It is generally accepted that the physical and chemical attributes of aquatic environments cannot reflect the actual health status of an aquatic ecosystem. Furthermore, monitored water quality variables often do not reflect long-term events that play critical roles in determining ecosystem health. Aquatic organisms, such as fish, are often affected by a variety of stressors in aquatic systems and thus reflect the combined and cumulative effects of these stressors over an extended period of time (Lam and Gray, 2003; van der Oost et al., 2003).

Generally, environmental contaminants entering organisms are metabolized quickly by three sets of cellular proteins or enzymes, called the phase I (transformation) and phase II (conjugation) enzymes, and the phase III (transport) proteins. And so, the most sensitive responses in aquatic organism are alterations in the levels and activities of oxidative stress enzymes following exposure to xenobiotic compounds (Bucheli and Fent, 1995; van der Oost et al., 2003). Moreover, gene transcription levels are useful supplements to protein levels or enzyme activities, as the mRNA levels represent a snapshot of the cell activity at a given time; and in many instances, single gene mRNA expressions can be useful biomarkers of stress in an organism (Fisher et al., 2006). For example, the real-time quantitative polymerase chain reaction for mRNA expression is more sensitive than the detection of protein or enzyme activity (Miller et al., 1999; Cousinou et al., 2000). Therefore, changes in mRNA expression level of these enzymes have been proposed as sensitive biomarkers of exposure that can be used in environmental monitoring programs (Olsvik et al., 2005; Bigot et al., 2010; Nahrgang

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et al., 2010; Albertsson et al., 2012; Chen et al., 2012).

The Hun River is one of the main tributaries of the Liao River and has a drainage area of 11,481 km^2. Recently, large volumes of treated and untreated wastewater have been discharged into the Hun River from surrounding cities, such as Shenyang, Fushun, and Anshan (Wang et al., 2003; Lin et al., 2006; Zhang, 2007). The water quality of the Hun River system has seriously deteriorated, which has had a negative impact on the ecological integrity of the Hun River (Li et al., 2000; Wang et al., 2011). As a representative native species, the selection of crucian carp, *Carassius auratus*, has some advantages as it shows the effects on the feral fauna in the target environment and integrates the effects throughout its life history. Thus crucian carp can be expected to be an ideal bioindicator for monitoring water quality, especially in heavily polluted rivers. In the present study, partial cDNA fragments encoding cytochrome P450 1A (CYP1A), catalase (CAT), glutathione peroxidase (GPx), superoxide dismutase (SOD), and glutathione-S-transferase (GST) in crucian carp were isolated and sequenced. Real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) assays were then developed to quantify their mRNA expression levels in wild crucian carp from the Hun River. Subsequently, an integrated biomarker response (IBR) was calculated using the mRNA expression levels of these biomarkers to identify possible impacts on organisms at an early stage and to establish a warning signal for use in monitoring the quality of the aquatic environment in the Hun River.

1 Materials and methods

1.1 Sampling

Within the study area (**Fig. 1**), site S1 was near the downstream side of the dam of the Dahuofang Reservoir, which is the main source of drinking water for the city of Shenyang. Following the reservoir, the Hun River enters flat lowland territory, then passing through Fushun and Shenyang. After Shenyang, the river enters its lower catchment, which is intensively used for agriculture and oil drilling. Then it joins the Taizi River, which is another tributary of the Liao River, 250 km downstream of the reservoir. Based on our previous investigation, the S1 site experiences almost no impacts from industrial or agriculture activities, and so the water quality is very clean, while S2 was located downstream of Fushun City and was slightly affected by the human activities there. S3 and S4 were near the city of Shenyang and received pollution inputs from various anthropogenic activities, and sites S5, S6, and S7 were located far downstream of Shenyang. S8 was located at the junction of the Hun River and the Taizihe, another main tributary of the Liao River, and S9 was downstream of S8.

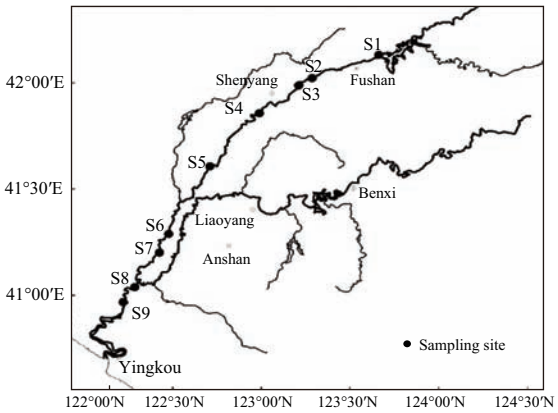

Fig. 1 Sampling sites (S1–S9) for wild crucian carp from the Hun River, northern China.

Wild crucian carp were collected from nine sampling sites, S1–S9. At each site, 12–22 fish were collected using purse nets, and portions of their hepatic tissues were quickly frozen in liquid nitrogen for mRNA analysis. Body weight and total length were measured for each individual (**Table 1**). At the same time, water quality parameters were measured *in situ,* including the temperature, total dissolved solids (TDS), and dissolved oxygen (DO), using a portable water quality meter (DR/820, Hach, USA).

1.2 Amplification and sequencing of cDNA fragments for crucian carp metabolizing enzymes

Approximately 20–30 mg of liver tissue was pulverized in liquid nitrogen and extracted for total RNA isolation using Trizol reagent (Invitrogen Ltd., USA) according to the manufacturer's protocol. cDNA was then synthesized following previously described methods (An et al., 2006). A negative control without reverse transcriptase was performed in parallel. The synthesized cDNA was diluted 10-fold with sterilized water and stored at –20°C for subsequent PCR analysis.

Degenerate primers (**Table 2**) were used to amplify the cDNA fragments for CYP1A, CAT, GPx, GST, and SOD following previously described amplification conditions and processes (An et al., 2006). The expected fragments that were produced were purified, cloned, and sequenced. The obtained nucleotide and amino acid sequences were identified by comparison with databases that were available on the National Center for Biotechnology Information (NCBI) website (www.ncbi.nlm.nih.gov) using the BLAST programs. Using a similar strategy, the ribosomal protein L7 (RPL-7) gene in crucian carp was also amplified.

1.3 Quantifying CYP1A, CAT, GPx, GST, and SOD mRNA expression levels

Specific primers for real-time quantitative reverse-transcription polymerase chain reaction (qRT-PCR) were

Table 1 Parameters for wild crucian carp and water quality in the Hun River from sampling in July, 2011

Site	Crucian carp						Water quality		
	Length (cm)	Weight (g)	GSI (%)	*K*	Gender	*n*	Temp. (°C)	DO (mg/L)	TDS (mg/L)
S1	12.37±2.56	52.98±43.55	13.54±5.46	2.44±0.26	Female	12	27.5	5.47	0.25
	10.91±1.77	32.50±18.94	3.76±1.39	2.32±0.49	Male	10			
S2	14.08±2.31	75.17±28.82	24.08±13.24	2.58±0.24	Female	11	24.8	6.16	0.35
	13.17±1.60	58.62±18.76	3.26±1.49	2.48±0.15	Male	7			
S3	13.58±1.86	72.87±31.68	15.19±9.90	2.78±0.27	Female	13	26.5	5.58	0.33
	13.25±1.77	64.44±22.88	1.93±1.09	2.68±0.24	Male	8			
S4	15.00±1.55	97.67±27.40	14.44±10.55	2.84±0.17	Female	11	26.9	8.85	0.34
	14.35±3.25	96.82±77.06	3.46±2.40	2.85±0.38	Male	9			
S5	13.50±1.46	71.94±17.66	11.45±8.22	2.93±0.45	Female	9	29.6	6.28	0.33
	11.92±0.66	47.45±8.83	3.92±1.71	2.78±0.20	Male	7			
S6	10.70±0.36	32.33±5.08	13.24±5.93	2.62±0.21	Female	14	27.0	2.92	0.37
	10.72±1.06	33.87±8.87	1.08±0.62	2.72±0.24	Male	8			
S7	12.32±1.05	52.13±14.49	21.38±17.29	2.74±0.26	Female	10	26.7	2.78	0.41
	11.86±1.41	47.44±21.57	3.06±1.54	2.67±0.31	Male	8			
S8	11.10±0.63	37.14±7.13	7.35±3.84	2.69±0.09	Female	12	26.7	2.35	0.38
	12.03±0.77	49.56±10.20	1.42±0.59	2.82±0.12	Male	7			
S9	11.82±0.62	40.67±5.66	12.21±8.45	2.46±0.22	Female	14	28.2	3.02	0.40
	10.40±0.60	27.20±3.76	2.59±1.59	2.41±0.13	Male	8			

The data of length and weight are expressed as mean ±SD. GSI: gland-somatic index; *K*: condition factor; Temp.: temperature; DO: dissolved oxygen; TDS: total dissolved solids.

Table 2 Primers used to amplify crucian carp HSP30, 60, 70, and 90 partial sequences for conventional PCR and SYBR Green real-time PCR assays

Gene/primer		Sequence (5′–3′)	Amplification efficiency (%)
Degenerate primer	CYP1A	F: TC(B)GTGGC(Y)AA(Y)GT(N)ATCTG*	/
		R: CA(S)CG(Y)TTGTG(Y)TTCAT(K)GT	
	CAT	F: GA(R)ATG(K)C(V)CA(Y)TT(Y)GAC(M)G	
		R: AA(S)A(R)(R)(R)AA(R)GACACCTG(R)TG	
	GPx	F: CTGCAA(Y)CAGTT(Y)GG(M)CATC	
		R: GGT(S)AGGAAR(Y)TTCTGCTGTA	
	GST	F: TACTTCAATGGCAGAGG(S)AA(R)ATGGA(R)	
		R: TGGAGGTTTCCTAGCGCTGCC(H)GG(Y)	
	SOD	F: T(W)(Y)GGAGA(Y)AA(Y)AC(V)AA(Y)GG	
		R: CC(M)A(R)(R)TC(R)TC(N)(K)C(Y)TTCTC	
	RPL-7	F: CACAAGGA(R)TA(Y)A(R)GCAG(M)T	
		R: GGTC(Y)TCCCTGTT(K)CC(D)GC	
Real-time PCR primer	CYP1A	F: CGTATCTCGAGGCCTTCATC	99.8
		R: CGACGGATCTTTCCACAGTT	
	CAT	AGCCAAAGTGTTCGAGCATGT	99.6
		TCACCAGCCACAGTGGAAAA	
	GPx	GCCCACCCTCTGTTTGTGTT	99.5
		GGGATCCCCCATCAAGGA	
	GST	AGCAGGTGCCTTTGGTGGA	100.0
		GTCGATCATAGCCCGTTCTTTAA	
	SOD	TCCGCACTACAACCCTCATAATC	99.5
		ACAGGGTCACCATTTTATCCACA	
	RPL-7	GTCTCCGCCAGATCTTCAAC	99.7
		GGCAGTTGTCTGTCAGTGGA	

Nucleotides in parentheses indicate degenerate sites. F: forward primer; R: reverse primer "/" indicates no values. CYP1A: cytochrome p450 1A, CAT: catalase, GPx: glutathione peroxidase, GST: glutathione-S-transferase, SOD: superoxide dismutase, RPL-7: ribosomal protein L7.

designed using the nucleotide sequences obtained above and are listed in **Table 2**; the amplification efficiencies were between 99.00% and 100.00%. The reaction mixture for quantifying the mRNA expression level was used with the instrument's default conditions. Then, CYP1A, CAT, GPx, GST, and SOD mRNA expression levels were normalized to RPL-7 and evaluated in each individual using SYBR green dye (Applied Biosystems, UK) in an ABI Prism Sequence Detection System (7500, Applied Biosystems, USA). All samples were analyzed in duplicate.

Negative controls that contained no template control (NTC) and no reverse transcription control (No RT) were conducted. NTC provides a control for external contamination or other factors that can result in nonspecific increases in the fluorescence signal. The No RT control should show no amplification in the subsequent PCR step because Taq polymerase cannot amplify any RNA templates. The optimal primer quantity was evaluated using a dissolution curve that was obtained after PCR amplification.

To evaluate the integrated impact of pollutants at each site, an integrated biomarker response index (IBR) was calculated for each pair of neighboring enzyme gene expression levels in a given data set; star plots that combined all of the measured biomarker responses were then used to display the biomarker results, as reported by Beliaeff and Burgeot (2002).

1.4 Data analysis

Target gene expression normalized to RPL-7 was quantified using the relative standard curve method. In order to evaluate the differences of gene expression in fish from the Hun River, the fish from S1 were used as the reference samplings. The data analysis was performed with SPSS v12.0 software (SPSS Inc., Chicago, USA). Data normality and homogeneity of variances were evaluated by the Kolmogorov-Smirnov test (K-S test) and Levene's test, respectively. Then one-way analysis of variance (ANOVA) followed by Tukey's test was used for statistical comparisons. The level of statistical significance was set at $p < 0.05$.

2 Results

2.1 Fragments of CAT, GPx, GST, and SOD in crucian carp

Using the degenerate PCR primers, fragments of 853, 368, 277, 557, and 233 nucleotide base sequences that corresponded to 122, 92, 185, and 77 amino acid sequences for CYP1A, CAT, GPx, GST, and SOD, respectively, were obtained. Each obtained sequence was confirmed by BLAST (http://blast. ncbi. nlm. nih. gov/) and the fragments of deduced amino acid sequences were conserved. Gene sequence data were deposited in GenBank with the following accession numbers: JQ776513, JQ776514, JQ776515, JQ776516, and JQ776518 for CAT, CYP1A, GPx, GST, and SOD, respectively. Using a similar strategy, a 4505-bp RPL-7 fragment that corresponded to a 150-amino acid sequence was isolated, sequenced, and deposited in GenBank with the accession number JQ776517.

Multiple sequences were aligned using the CLUSTALW algorithm and then compared with corresponding genes from other organisms, especially members of the Cyprinidae family. According to the identity matrix, crucian carp CYP1A showed 94%, 92%, 91%, 86%, and 86% identity with common carp (BAB39379), goldfish (ABF60890), rare minnow (ABV01348), zebrafish (AAQ97766), and Atlantic salmon (ACN11220), respectively (**Fig. 2**). CAT showed 100%, 100%, 99%, and 97% identity with silver carp (ADJ67807), grass carp (ACL99859), common carp (ADK26528) and zebrafish (AAH51626), respectively. GPx showed 100%, 97%, 95%, 95%, 93%, and 92% identity with goldfish (ABJ09418), common carp (ADK26519), grass carp (ACF39780), bighead carp (ACO53608), silver carp (ABU84810), and zebrafish (AAO86703), respectively. GST showed 99%, 91%, 87%, 85%, and 82% identity with goldfish (ABW37113), common carp (ABD67507), bighead carp (ABK96973), silver carp (ABK96975), and fathead minnow (ABV30909), respectively. SOD showed 81%, 77%, 77%, 77%, 77%, and 73% identity with rainbow trout, zebrafish, grass carp and Atlantic salmon, respectively.

2.2 CYP1A, CAT, GPx, GST, and SOD mRNA expression in wild crucian carp from the Hun River

qRT-PCR methods were developed to quantify CYP1A, CAT, GPx, GST, and SOD mRNA expression levels efficiently and then performed with wild crucian carp from the Hun River (**Fig. 3**). Compared with mRNA expression in fish from S1 (1.00), the reference site, CYP1A mRNA expression levels at S5, S6, and S8 showed significant up-regulation ($p < 0.05$), with expression levels that were 10.43-fold, 7.03-fold, and 7.53-fold higher relative to S1, respectively. SOD mRNA expression levels at sites S3–S9 were significantly down-regulated ($p < 0.05$), showing levels that were 0.00043-fold to 0.0016-fold relative to S1, while it did not change significantly at S2 (1.13-fold) relative to that at S1 ($p > 0.05$). For the CAT gene, mRNA expression was strongly down-regulated, with expression levels being 0.38-fold at S3 and 0.42-fold at S9 ($p < 0.05$); slight down-regulation was shown at other sites ($p > 0.05$). Similar results were observed with GPx mRNA expression. GPx mRNA expression was obviously down-regulated at S9 (0.27-fold) ($p < 0.05$) but no notable differences were observed at other sites ($p > 0.05$). Moreover, there was no significant difference in GST mRNA expression between fish from up- or downstream sites ($p > 0.05$).

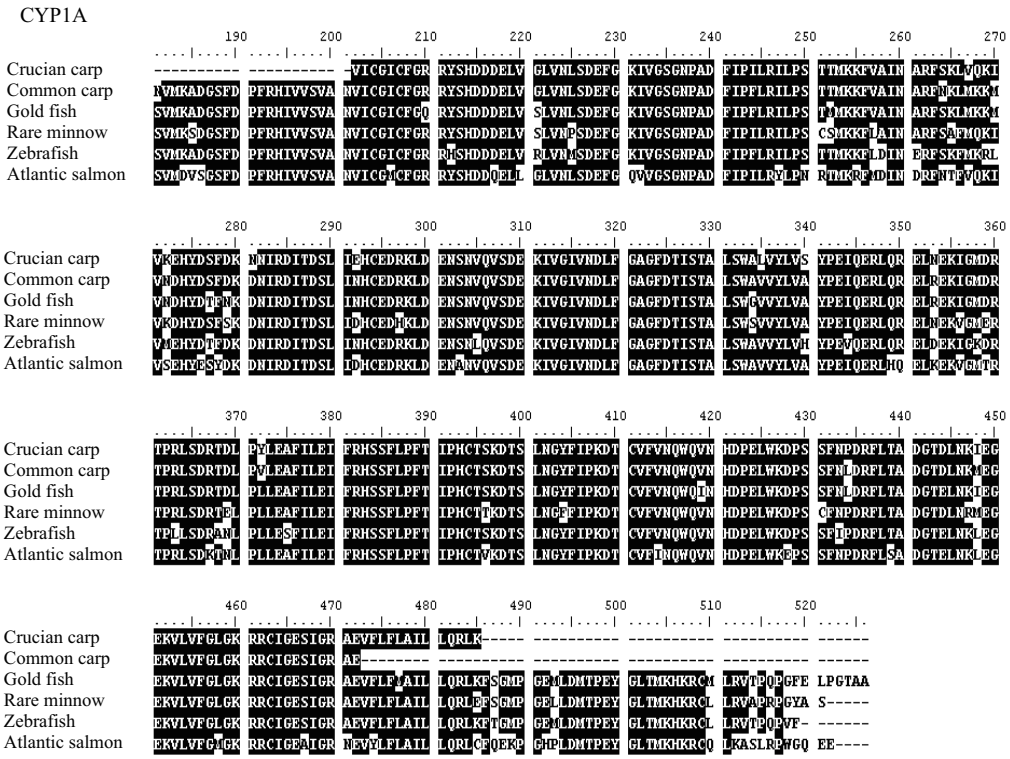

Fig. 2 Comparison between deduced CYP1A, CAT, GPx, GST, and SOD amino acid sequences for crucian carp and sequences that were available in GenBank. Amino acids in black indicate sequence homology among species.

2.3 IBR in wild crucian carp from the Hun River

To evaluate integrated effects in organisms from exposure to various environmental stressors, CYP1A, CAT, GPx, GST, and SOD mRNA expression data for wild crucian carp from different sites in the Hun River were standardized for each site and then an IBR index was calculated and presented as a star plot (**Fig. 4**). Lower IBR values were found at S2 (0.65) and S1 (4.91), whereas the highest IBR values were obtained at S3 (2995.32) and S4 (2501.92). Comparing the IBR values throughout the Hun River, which reflected complex biological responses to stressors, the water quality was ranked as S3 < S4 < S8 < S6 < S5 < S9 < S7 < S1 < S2.

3 Discussion

This study was performed using a suite of early warning biomarkers, mainly metabolizing enzymes, in wild crucian carp from the Hun River. The results showed that CYP1A mRNA expression was up-regulated in some investigation sites and CAT, SOD, and GPx mRNA expression levels were down-regulated. These findings expand the information that is available for evaluating ecological risk in the Hun River and developing an early-warning signal for water quality management.

Several studies have demonstrated that enhancements in CYP1A mRNA expression in organisms following exposure to chemicals or contaminants can be used as a biomarker for environmental monitoring and toxicological testing (Miller et al., 1999; Rees et al., 2003; Rees and Li, 2004). Significant increases in CYP1A mRNA expression were observed in wild crucian carp from sites S5, S6, and S8 relative to S1, the reference site. Previous studies have reported that not only classical AhR ligands, such as PAHs and PCBs, but also chemical classes, including heterocyclic compounds such as the more polar amines, can induce CYP1A mRNA expression in laboratory (Brack and Schirmer, 2003; Navas et al., 2003) and field cases (Fisher et al., 2006; Brack et al., 2000; An et al., 2011). In some studies, CYP1A mRNA expression in wild or caged fish reflected different levels of PCB pollution among rivers (Rees and Li, 2004; Brammell et al., 2010). Thus, the primary results of the present study suggest that fish at sites S5, S6, and S8 experienced higher exposure to xenobiotics than did individuals at other sites. To our knowledge, CYP1A serves to increase the solubility of hydrophobic molecules through reduction reactions that involve an oxygen molecule (Bucheli and Fent, 1995); this allows these reactive chemical intermediates to be metabolized by SOD, CAT, and GST. The enzymatic activities or mRNA expression levels of SOD, CAT, and GST would increase after CYP1A activity or mRNA

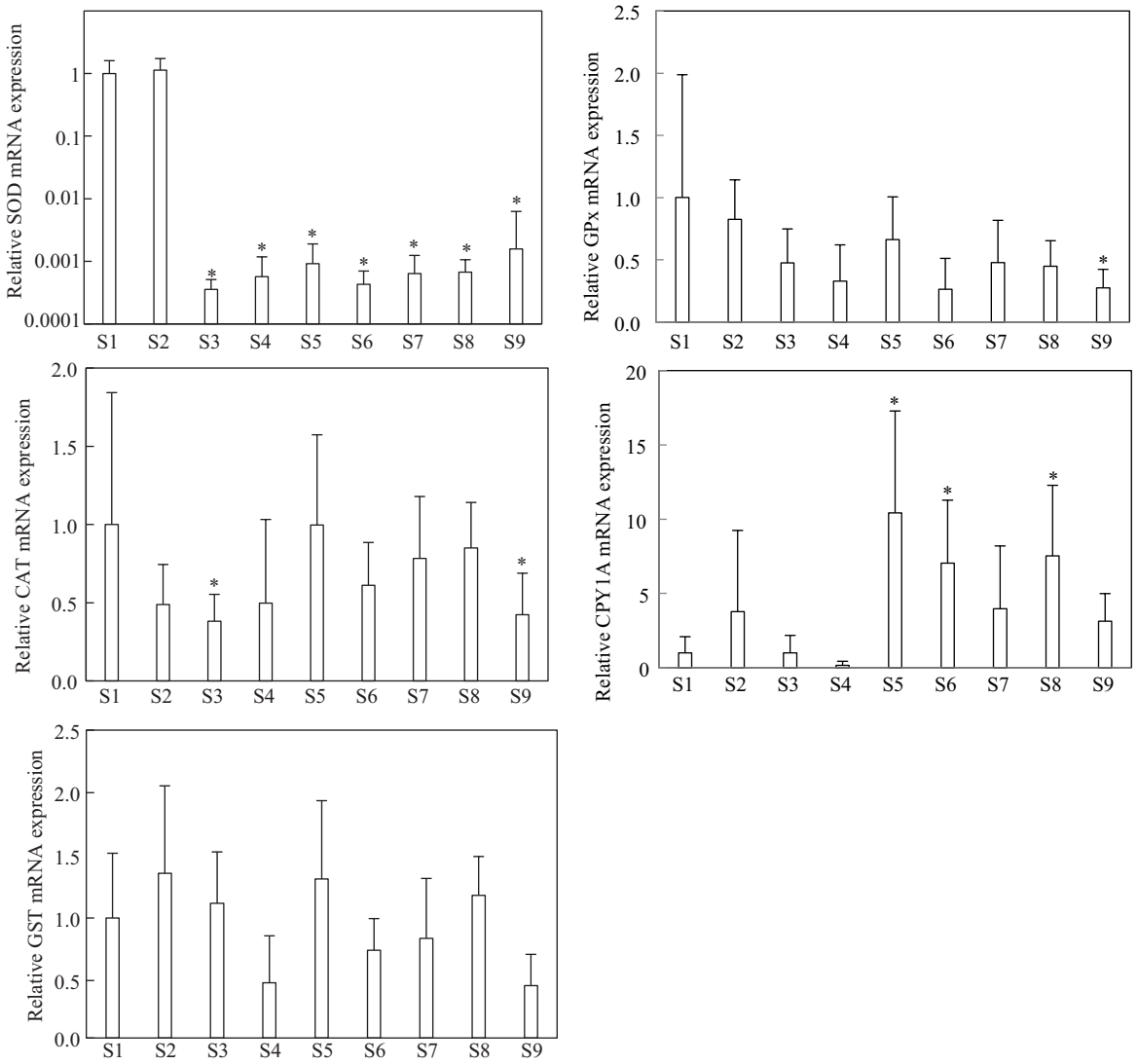

Fig. 3 CYP1A, SOD, CAT, GST, and GPx mRNA relative expression levels normalized to RPL-7 in wild crucian carp from the Hun River (n = 10 for each site). * indicate significant differences ($p < 0.05$) between S1 and other sites. Error bars represent standard deviations.

expression is induced in an organism because of exposure to various contaminants, as has been demonstrated in earlier studies (Pickett and Lu, 1989; van der Oost et al., 1998). Interestingly, however, the SOD, CAT, GST, and GPx mRNA expression levels, especially SOD, were down-regulated in fish from downstream sites in the Hun River relative to the expression levels in fish from S1 (**Fig. 3**), which was contrary to the anticipated results. A similar pattern was also found in carp from the Kalamazoo River in Michigan, USA, in which CYP1A mRNA activity was increased but SOD oxidative stress genes were not affected (Fisher et al., 2006), implying that known and unknown contaminations might be present and do harm to aquatic organisms simultaneously. It should be noted that, under normal physiological conditions, biological defense systems can be induced by a slight oxidative stress as a compensatory response, so that reactive oxygen species can be eliminated effectively to prevent oxidative damage (Di Giulio et al., 1989; Livingstone, 2001). However, severe oxidative stress will suppress the activity levels of enzymes and lead to higher levels of oxidative damage when the increase in the production of reactive oxygen species exceeds the scavenging capacity (Halliwell and Gutteridge, 2007).

Biomarkers are commonly used as tools to predict exposure and contaminant-induced health effects in organisms, and they are helpful for understanding potential risks of environmental contamination (van der Oost et al., 2003). However, it is difficult to quantify actual pollution status and the risk of environmental pollution in ecosystems based on individual biomarker responses that can represent different biological endpoints, as in the above results. Therefore, it is more rational to use a battery of biomarkers to monitor the water quality and evaluate the ecotoxicological risk, an approach that has been applied in the field in recent years (Banni et al., 2005; Broeg and

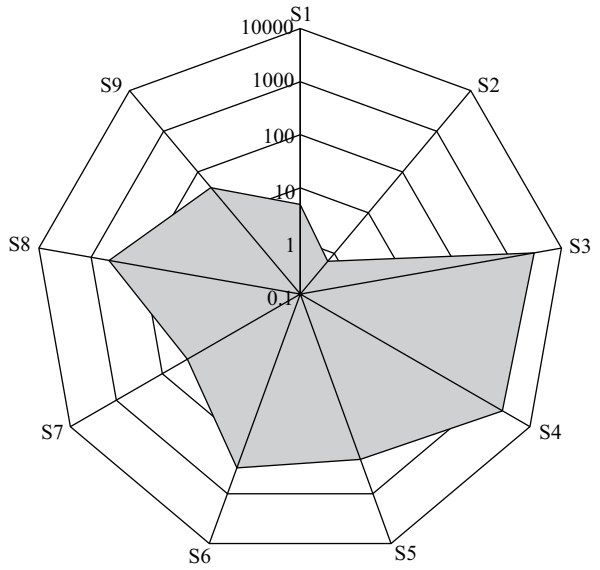

Fig. 4 Star plots of integrated biomarker responses (IBR) in wild crucian carp from sites S1–S9 in the Hun River, northern China.

Lehtonen, 2006; Ji et al., 2010; Tsangaris et al., 2010). The IBR method normalizes individual biomarkers to a single value that can provide comprehensive information about the biological effects of pollution in organisms (Beliaeff and Burgeot, 2002), and it can also serve as a useful tool for environmental management by ranking values of pollution status in the field. Using this method, the highest IBR values were observed at sites S3 (2995.32) and S4 (2501.91), and the lowest values were found at sites S2 (0.65) and S1 (4.91), although the IBR value at S1 was slightly higher than that at S2. The ranking of the IBR values in the present study indicated that higher pollution levels and higher alterations in biological responses were present at sites S3 and S4, and moderate alterations in biological response were observed at sites S5–S9. Moreover, the previous studies showed that the IBR values were in good agreement with PAH and OCP contents in the sediments (Ji et al., 2010), organochlorine compounds in tissues (Broeg and Lehtonen, 2006), and copper and PCB concentrations in muscles (Damiens et al., 2007). Moreover, in previous studies carried out in S3 and S4, Yang et al. (2011) also detected high levels of various environmental pollutants including perfluorinated compounds (PFCs), polycyclic aromatic hydrocarbons (PAHs), and organochlorine pesticides (OCPs) in water and sediment collected near the sites S3 and S4, and high estrogenic activities for the water (ND–0.82) and sediment (0.28–4.76) and six estrogenic compounds (4-nonylphenols (4-NP), 4-*t*-octylphenol (4-*t*-OP), bisphenol-A (BPA), estrone (E1), estradiol (E2) and triclosan (TCS)) were detected in these sites (Wang et al., 2011). All of these contaminations and other unidentified substances might cause serious deterioration in water quality. Thus, the present results of the ranking of IBR suggest that anthropogenic discharges from Shenyang city threatened the integrity of the aquatic ecosystem in the Hun River, although it was very difficult to relate the observed effects to specific contaminants. Of course, more studies will be conducted in the future to determine seasonal variation in IBR relative to possible pollutants in sediments and fish.

4 Conclusions

The present study showed that CYP1A, SOD, CAT, and GPx mRNA expression levels in wild crucian carp varied between downstream and upstream sites in the Hun River. IBR values that were calculated from these integrated biomarker responses reflected changes in biological responses throughout the Hun River, from upstream to downstream. To our knowledge, the present work is the first report on the use of IBR based on a battery of biomarker responses in fish from a local area of Hun River, and will be helpful in controlling the pollution in the river using biomonitoring programs in future.

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