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Chronic toxicity of verapamil on juvenile rainbow trout (*Oncorhynchus mykiss*): Effects on morphological indices, hematological parameters and antioxidant responses

Zhi-Hua Li[a,b,*], Josef Velisek[a], Vladimir Zlabek[a], Roman Grabic[a], Jana Machova[a], Jitka Kolarova[a], Ping Li[a,b], Tomas Randak[a]

[a] University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, 389 25 Vodnany, Czech Republic
[b] Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences, Jingzhou 434000, China

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ABSTRACT

In this study, the toxic effects of verapamil (VRP) were studied on juvenile rainbow trout, *Oncorhynchus mykiss*, by chronic semi-static bioassay. Fish were exposed to sublethal concentrations of VRP (0.5, 27 and 270 μg/L) for 0, 21 and 42 d. Multiple biomarkers were measured, including morphological indices, hematological parameters and antioxidant responses of different tissues (brain, gill, liver, muscle and intestine). Based on the results, there was no significant change in all parameters measured in fish exposed to VRP at environmental related concentration, but VRP-induced stress in fish exposed to higher concentrations reflected the significant changes of physiological and biochemical responses. Through principal component analysis and integrated biomarker response assessment, effects induced by VRP-stress in each test group were distinguished. Additionally, all parameters measured in this study displayed various dependent patterns to VRP concentrations and exposure time using two-way ANOVA statistic analysis. In short, the multiple responses in fish indicated that VRP induced physiological stress and could be used as potential biomarkers for monitoring residual VRP in aquatic environment; but molecular and genetic mechanisms of these physiological responses in fish are not clear and need to be further studied.

1. Introduction

In recent years, chemical pollution from residual pharmaceutical has been increasingly important issue due to its wide presence in the aquatic environment [1,2]. Since metabolic stability is necessary for pharmacological action, they are often resistant to biodegradation and emerging as environmental contaminants. Although several residual pharmaceuticals are unlikely to result in lethal toxicity in aquatic organisms because of low concentrations combined with low toxicity, prolonged exposure may lead to observable toxic effects. Verapamil, a cardiovascular pharmaceutical, is widely prescribed and used for the treatment of supraventricular arrhythmias and coronary heart disease. It has been detected in the groundwater at concentrations of 0.058–0.9 μg/L [3,4]. In addition, the environmental entry concentration of this pharmaceutical by sewage treatment plant (STP) effluent is assumed high as less than 3% on average is predicted to be removed in the STP [5]. Unfortunately, till now, very little information is available for its potential toxic effects on aquatic organisms such as fish.

Environmental stressors can alter the physiological and biochemical parameters in fish, including morphological indices, hematological parameters and antioxidant responses [6]. Condition factor (CF) and hepatosomatic index (HSI) as the most frequent morphological indices have been used in various environmental stress-related studies [7]. And some previous reports have demonstrated that CF and HSI are potential indicators of toxicant effects, providing information on the ability of individual to tolerate chemical pollution or other kind of environmental stress [8]. Besides, blood is known to exhibit pathological changes before the onset of any external symptoms of toxicity. Differential blood cell counts and plasmatic enzymes are effective indicators of environmental stress and provide a general overview of the integrity of the immune system [9]. Therefore, the hematological analysis and biochemical parameters of blood plasma are useful for monitoring the

* Corresponding author at: University of South Bohemia in Ceske Budejovice, Faculty of Fisheries and Protection of Waters, South Bohemian Research Center of Aquaculture and Biodiversity of Hydrocenoses, Zatisi 728/II, 389 25 Vodnany, Czech Republic. Tel.: +420 387 774 622; fax: +420 387 774 634.
E-mail address: zhihuali06@yahoo.com (Z.-H. Li).

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physiological status of fish and used as health indicators in aquatic environment even though they are not routinely used for fish diseases diagnosis [10].

Some toxic contaminants exert cytotoxic effects by the production of reactive oxygen species (ROS), which can induce oxidative damage and maybe a mechanism of toxicity for aquatic organisms living in polluted areas [11]. Antioxidant defenses, present in all aerobic organisms, include antioxidant enzymes and low molecular mass antioxidants whose function is to remove ROS, thus protecting organisms from oxidative stress [12]. But when ROS generation exceeds the capacity of the cellular antioxidants, it will cause oxidative stress and oxidative damages. Fish antioxidant responses are very sensitive to environmental contamination and frequently used in aquatic environmental monitoring.

Due to the presence of a tertiary amino group, VRP is a weak base (pK_a 8.9) and predominantly exists in a protonated form in physiological media and it is a lipophilic drug with an octanol–water coefficient $\log p_{ow}$ of 3.79 for the free base [13,14]. O- and N-demethylation are the two major metabolic pathways for verapamil in humans [13]. Although its mechanism had been speculated earlier, only in recent years there have been attempts to identify conjugation products of verapamil. Walles et al. investigated verapamil metabolism in rat hepatocytes by LC–MS^n and identified 24 phase I and 14 phase II metabolites [15]. Borlak et al. studied the metabolism of verapamil in primary human hepatocytes and human urine by LC–MS^n and LC–NMR (nuclear magnetic resonance) [16]. They found 21 phase I and 16 phase II metabolites. There have been a few studies about experimental ecotoxicological data from *Daphnia magna* and the results of a structure–activity relationship study which both revealed toxic effects at low doses on aquatic organisms [17,18], as well as about the capability of fungi and anaerobic bacteria from rat cecal contents to metabolize verapamil [19,20], however, little is known about toxic effects in fish.

In this study, rainbow trout (*Oncorhynchus mykiss*), a widely used model in aquatic toxicology, was exposed to VRP to determine its chronic effects on biochemical and physiological responses. Morphological indices and hematological parameters were analyzed, as well as the oxidative stress and the enzymatic antioxidant responses in fish tissues.

2. Materials and methods

2.1. Chemicals

Verapamil ((RS)-2-(3,4-dimethoxyphenyl)-5-[2-(3,4-dimethoxyphenyl)ethyl]-(methyl)amin-2-prop-2-ylpentanenitrile) and other chemicals were obtained from Sigma–Aldrich Corporation (USA). The VRP was dissolved in pure distilled water to make a stock solution at a concentration of 0.05 mg/L.

2.2. Fish

Juvenile rainbow trout, weighing 40.43 ± 2.55 g (mean ± S.D.), were obtained from a local commercial hatchery (Husinec, Czech Republic). They were held in aquaria containing 250 L of freshwater continuously aerated to maintain dissolved oxygen values at 7.5–8.0 mg/L. Temperature was 15 ± 1 °C and pH was 7.4 ± 0.2. Photoperiod was a 12:12 light–dark cycle. Fish were acclimatized for 14 d before the beginning of the experiment and were fed commercial fish food (BioMar, Denmark). The fish were starved for 24 h prior to experimentation to avoid prandial effects during the assay.

2.3. Exposure to VRP

A 200 L semi-static system was used in which 40 fish were randomly distributed to each of eight aquaria. The nominal concentrations of VRP used were 0.5 μg/L (E1 group, environmental related concentration), 27 μg/L (E2 group, 1% 96 h LC 50 of VRP in rainbow trout [21]), and 270 μg/L (E3 group, 10% 96 h LC 50 of VRP in rainbow trout). A control group exposed to clean freshwater. Each experimental condition was duplicated. The fish were fed daily with commercial fish pellets at 1% total body weight at a fixed time and the extra food was removed. The exposed solution was renewed each day after 2 h of feeding to maintain the appropriate concentration of VRP and to maintain water quality. The test fish were exposed to VRP for 0, 21 and 42 d.

To ensure agreement between nominal and actual compound concentrations in the aquaria, water samples were analyzed during the experimental period by LC–MS/MS. Water samples were collected from the test aquaria after 1 h and 24 h of renewing the test solutions. The mean concentration of VRP in the water samples was always within 20% of the intended concentration (the measured concentration of VRP in the water samples was 0.47 ± 0.05, 26.18 ± 1.36, and 251.33 ± 19.81 μg/L corresponding to the nominal concentration 0.5, 27, and 270 μg/L).

2.4. Morphological indices

At the end of each exposure period, 12 fish of every aquarium were randomly sampled, and fork length, body weight and liver weight recorded. CF and HSI for each fish was calculated according to previous description [22]:

$$\mathrm{CF} = \frac{b}{f^3} \times 100 \quad (1)$$

$$\mathrm{HSI} = \frac{l}{b} \times 100 \quad (2)$$

where b is the body weight (g); f the fork length (cm); l the liver weight (g).

2.5. Hematological and biochemical blood plasma parameters

Blood samples were taken from each fish by caudal vein-puncture using a syringe heparinized (Heparin inj., Leciva, Czech Republic) at a concentration of 5000 IU heparin sodium salt in 1 ml. An aqueous solution of heparin sodium salt at 0.01 ml per 1 ml blood was used to stabilize the samples. The indices tested included hematocrit (PCV), hemoglobin concentration (Hb), red blood cells (RBC), leukocyte count (Leuko), mean erythrocyte hemoglobin (MCH), mean erythrocyte volume (MCV) and mean color concentration (MCHC). The procedures were based on unified methods for the hematological examination of fish [23].

Blood plasma obtained from cooled centrifuged blood samples (4 °C, 837 × g) was stored at −80 °C until use. Biochemical indices including glucose (GLU), ammonia (NH_3), total proteins (TP), lactate (LAC), creatine kinase (CK), lactate dehydrogenase (LDH), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were determined using a VETTEST 8008 analyzer (IDEXX Laboratories Inc., USA.).

2.6. Antioxidant indices

2.6.1. Tissue sampling

After sampling blood, tissues of each fish were quickly removed in the following order: gill, liver, brain, intestine and white muscle. The samples were immediately frozen and stored at −80 °C. Frozen tissue samples were weighed and homogenized (1:10 w/v)

Table 1
Morphological parameters in rainbow trout chronically exposed to VRP. Control and experimental group (0.5, 27 and 270 μg/L) values are shown in C, E1, E2 and E3, respectively. Data are mean ± S.D., $n = 12$.

Indices	0 day	21 d				42 d			
	C	C	E1	E2	E3	C	E1	E2	E3
BW	40.43 ± 2.55	53.38 ± 1.95**	57.91 ± 5.21**	58.72 ± 4.23**	55.65 ± 2.89**	69.16 ± 5.29**	71.33 ± 4.57**	62.63 ± 5.79**	59.21 ± 6.47**
LW	0.39 ± 0.05	0.52 ± 0.05*	0.54 ± 0.09*	0.57 ± ±0.05**	0.47 ± 0.06	0.63 ± 0.08**	0.62 ± 0.11**	0.51 ± 0.07*	0.48 ± ±0.04
FL	15.90 ± 0.36	17.77 ± 0.81**	18.31 ± 0.74**	18.33 ± 0.71**	18.95 ± 1.42**	19.26 ± 0.39**	19.43 ± 0.45**	19.25 ± 1.03**	19.68 ± 1.12**
CF	1.01 ± 0.09	0.96 ± 0.15	0.94 ± 0.09	0.95 ± 0.09	0.84 ± 0.11*	0.96 ± 0.06	0.97 ± 0.05	0.88 ± 0.12	0.79 ± 0.13*
HSI	0.97 ± 0.10	0.97 ± 0.11	0.95 ± 0.16	0.98 ± 0.09	0.86 ± 0.14	0.91 ± 0.11	0.88 ± 0.20	0.84 ± 0.10	0.81 ± 0.09*

Notes: BW, body weight (g); LW, liver weight (g); FL, fork length (cm); CF, condition factor; HSI, hepatosomatic index.
* Significant difference compared with control value $p < 0.05$.
** Significant difference compared with control value $p < 0.01$.

using an Ultra Turrax homogenizer (Ika, Germany) using 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA. The homogenate was divided into two portions, one for measuring oxidative stress indices, and a second centrifuged at 12,000 × *g* for 30 min at 4 °C to obtain the post-mitochondrial supernatant for antioxidant enzymes analyses.

2.6.2. Biochemical analysis

A 500 μl aliquot of homogenate was mixed with 1 ml of 30% (w/v) TCA and centrifuged for 10 min at 5000 × *g*. The supernatant was used for lipid peroxidation (LPO) assays and the pellet used for carbonyl proteins (CP) assay.

The thiobarbituric acid-reactive substances (TBARS) method described by Lushchak et al. [24] was used to evaluate LPO in fish tissues. CP were detected by reaction with 2,4-dinitrophenylhydrazine (DNPH) according to the method described by Lenz et al. [25]. The values of TBARS and CP were expressed as nanomoles per gram of wet weight tissue.

Total superoxide dismutase (SOD; EC 1.15.1.1) activity was determined by the method of Marklund and Marklund [26]. Glutathione peroxidase (GPx; EC 1.11.1.9) activity was assayed following the rate of nicotinamide adenine dinucleotide phosphate (NADPH) oxidation at 340 nm by the coupled reaction with glutathione reductase [27]. Glutathione reductase (GR; EC 1.6.4.2) activity was determined spectrophotometrically, measuring NADPH oxidation at 340 nm [28]. One unit of GPx or GR activity is defined as the amount of the enzyme that consumes 1 μmol of substrate or generates 1 μmol of product per min; activity was expressed in international units (or milliunits) per mg of protein. Protein levels were estimated spectrophotometrically by the method of Bradford [29] using bovine serum albumin as a standard.

2.7. Integrated biomarker response (IBR)

Biomarkers were combined into one general "stress index" termed IBR [30]. The result is directly dependent on the number of biomarkers (n) in the set and thus, IBR values presented were divided by n as suggested by Broeg and Lehtonen [31]. Results of data standardization procedure needed for IBR calculation were presented in site star plots.

2.8. Statistical assays

All values were expressed as mean ± S.D. and analyzed by SPSS for Win 13.0 software. Analyses of variance (one-way and two-way ANOVA), followed by a Tukey HSD test when significant differences were found, was performed to determine the effect of VRP concentration and exposure time on each parameters. To get a general picture of the trends and groupings in the material, the data from all fish individuals were subjected to multivariate data analyses, to principal component analysis (PCA) using the software Statistic 6.0.

3. Results

3.1. Morphological indices

In this study, significantly increasing ($p < 0.05$) fork length, body and liver weight were observed among the groups, except for liver weight of E3 group after 21 d exposure and above. CF determined in the E3 group was significantly lower after 21 d and above ($p < 0.05$) than that of the control. For HSI, although a decreasing hint was observed in VRP-treated groups, the significantly lower ($p < 0.05$) HSI was observed only in E3 group after 42 d (see Table 1).

3.2. Hematological and biochemical blood plasma profiles

The hematological properties of rainbow trout chronically exposed to VRP are shown in Table 2. Compared to the control, the chronic exposure to VRP resulted in significantly lower ($p < 0.05$) values for PCV and Leuko count after 21 d and above, and significantly lower ($p < 0.05$) levels of Hb content and RBC after 42 d. All others were not significantly different ($p > 0.05$) between groups.

Table 2
Hematological parameters in rainbow trout chronically exposed to VRP. Other information as in Table 1.

Indices	Experimental groups								
	0 day	21 d				42 d			
	C	C	E1	E2	E3	C	E1	E2	E3
PCV	0.41 ± 0.01	0.36 ± 0.05	0.34 ± 0.03	0.35 ± 0.05	0.31 ± 0.04*	0.39 ± 0.07	0.35 ± 0.05	0.32 ± 0.06	0.30 ± 0.06*
Hb	60.43 ± 4.28	57.92 ± 11.87	57.62 ± 6.29	56.90 ± 7.84	53.13 ± 7.23	60.21 ± 10.17	61.24 ± 8.52	59.33 ± 6.91	49.24 ± 5.32*
RBC	1.01 ± 0.05	0.96 ± 0.21	0.91 ± 0.11	0.95 ± 0.13	0.95 ± 0.15	0.93 ± 0.17	0.88 ± 0.15	0.84 ± 0.14	0.79 ± 0.17*
Leuko	34.15 ± 6.20	33.37 ± 9.80	30.66 ± 9.51	30.29 ± 8.38	24.45 ± 4.78*	30.62 ± 6.20	29.16 ± 7.40	26.04 ± 10.01	20.62 ± 7.59*
MCH	63.68 ± 5.12	61.06 ± 9.02	64.14 ± 7.03	60.42 ± 8.87	59.38 ± 7.11	64.74 ± 8.09	66.09 ± 14.86	62.25 ± 11.01	63.15 ± 13.16
MCV	377.11 ± 28.19	383.34 ± 76.98	382.32 ± 48.75	369.61 ± 67.73	317.72 ± 56.77	429.78 ± 66.43	478.56 ± 55.31	453.32 ± 83.68	386.07 ± 84.35
MCHC	168.91 ± 16.23	161.72 ± 19.09	169.06 ± 19.58	165.40 ± 20.28	176.14 ± 15.23	151.60 ± 10.14	155.56 ± 11.75	152.04 ± 19.84	165.32 ± 21.58

Notes: PCV, hematocrit (l/l); Hb, hemoglobin concentration (g/l); RBC, red blood cells (T/l); Leuko, leukocyte count (G/l); MCH, mean erythrocyte hemoglobin (pg); MCV, mean erythrocyte volume (fl); MCHC, mean color concentration (g/l).

Table 3
Biochemical indices of blood plasma in rainbow trout chronically exposed to VRP. Other information as in Table 1.

Indices	Experimental groups								
	0 day	21 d				42 d			
	C	C	E1	E2	E3	C	E1	E2	E3
GLU	5.61 ± 1.02	5.36 ± 0.87	5.35 ± 0.84	6.70 ± 0.76	7.32 ± 1.04**	5.27 ± 0.40	5.53 ± 0.72	7.11 ± 1.16*	8.09 ± 1.01**
NH_3	508.33 ± 39.81	490.41 ± 67.24	505.75 ± 62.24	669.01 ± 67.27**	706.67 ± 80.68**	479.58 ± 71.87	499.25 ± 66.82	730.83 ± 53.40**	764.08 ± 74.34**
TP	27.83 ± 1.47	27.16 ± 2.12	27.08 ± 2.10	26.67 ± 2.19	31.91 ± 2.10*	26.25 ± 2.17	26.67 ± 2.31	26.83 ± 2.69	33.33 ± ± 2.46**
LAC	2.49 ± 0.62	2.51 ± 0.35	2.45 ± 0.33	2.01 ± 0.47	1.55 ± 0.42**	2.56 ± 0.24	2.37 ± 0.15	1.95 ± 0.34*	1.34 ± 0.28**
CK	15.68 ± 0.95	15.67 ± 1.01	15.64 ± 1.07	15.79 ± 0.96	15.88 ± 1.13	15.33 ± 1.17	15.40 ± 0.99	15.75 ± 1.37	15.47 ± 1.19
LDH	17.30 ± 0.99	17.16 ± 0.82	17.02 ± 1.07	19.05 ± 1.02	20.00 ± 1.07**	17.35 ± 1.60	17.52 ± 1.36	21.88 ± 1.89**	24.53 ± 1.90**
ALT	0.40 ± 0.19	0.46 ± 0.18	0.48 ± 0.21	0.77 ± 0.19*	0.94 ± 0.17**	0.52 ± 0.18	0.54 ± ± 0.19	0.97 ± 0.32**	1.13 ± 0.25**
AST	4.28 ± 0.98	4.19 ± 0.58	5.10 ± 0.67	5.61 ± 0.53**	6.06 ± 0.55**	4.66 ± 0.61	5.90 ± 0.38**	6.28 ± 0.88**	6.97 ± 0.58**

Notes: GLU, glucose (mmol/l); NH_3, ammonia (μmol/l); TP, total proteins (g/l); LAC, lactate (mmol/l); CK, creatine kinase (μkat/l); LDH, lactate dehydrogenase (μkat/l); ALT, alanine aminotransferase (μkat/l); AST, aspartate aminotransferase (μkat/l).

The plasma biochemical parameters of the rainbow trout treated with VRP are presented in Table 3. The GLU and LDH activities were significantly higher ($p < 0.05$) in E3 group after 21 d and in E2 and E3 groups after 42 d, but contrarily, the levels of LAC significantly lower ($p < 0.05$) for those groups at the same period. The NH_3 level and two aminotransferases (ALT and AST) increased significantly ($p < 0.05$) in E2 and E3 groups after 21 d and above. The significant higher ($p < 0.05$) TP level was observed only in E3 group after 21 d and above. But during the whole experimental period, there was no significant change ($p > 0.05$) in CK activity among all groups.

3.3. Antioxidant responses

Levels of LPO (expressed by TBARS levels) and CP in the tissues of rainbow trout after chronic exposure to VRP are presented in Figs. 1 and 2. The significantly higher ($p < 0.05$) levels of TBARS were observed in brain and gill of E2 and E3 groups after 21 d and above, in muscle of E2 group after 42 d and E3 group after 21 d and above, as well as in liver and intestine of E3 group after 42 d. For CP, the significant higher levels ($p < 0.05$) were found in brain and gill of E2 group after 42 d and E3 group after 21 d and above, in muscle of E3 group after 21 d and above, in liver of E2 and E3 groups, and in intestine of E3 group after 42 d.

The activities of antioxidant enzymes in tissues of rainbow trout are shown in Figs. 3–5. The significantly higher ($p < 0.05$) levels of SOD activity in brain of E2 group after 21 d, in liver of E3 group after 21 d and E2 group after 42 d were observed, when compared to the control. The activities of GR and GPx were significantly induced in liver after chronic exposure at higher concentrations of VRP, except for GR in E2 group after 21 d. All antioxidant enzymes activities (SOD, GR and GPx) were significantly inhibited in brain (except for GR) and gill of E2 group after 42 d and E3 group after 21 d and above, in muscle and intestine of E3 group after 42 d.

3.4. Chemometrics

Two-way ANOVA, using all parameters measured as the dependent variables, and VRP concentrations and exposure time as fixed factors, revealed the significant variation in each parameter with different experimental conditions (Table 4). The VRP concentrations-dependent parameters included CF, HSI, PCV, Leuko, MCH, MCV, MCHC, NH_3, GLU, TP, CK, LAC, LDH, ALT, AST and all antioxidant related parameters (TBARS, CP, SOD, GR and

Table 4
Two-way ANOVA for the effects of VRP concentrations (VRP) and exposure time (time) on parameters measured in rainbow trout chronically exposed to VRP.

Indices	Experimental conditions			Indices	Experimental conditions		
	Time	VRP	Time × VRP		Time	VRP	Time × VRP
CF	0.577	<0.001	0.585	$TBARS_B$	<0.001	<0.001	<0.001
HSI	0.009	0.045	0.357	CP_B	<0.001	<0.001	<0.001
				SOD_B	<0.001	<0.001	<0.001
PCV	<0.001	0.002	0.985	GPx_B	<0.001	<0.001	<0.001
Hb	0.119	0.220	0.938	GR_B	<0.001	<0.001	<0.001
RBC	0.391	0.414	0.930	$TBARS_G$	<0.001	<0.001	<0.001
Leuko	0.006	0.005	0.595	CP_G	<0.001	<0.001	<0.001
MCH	0.003	0.011	0.747	SOD_G	<0.001	<0.001	<0.001
MCV	<0.001	0.003	0.679	GPx_G	<0.001	<0.001	<0.001
MCHC	0.001	0.030	0.980	GR_G	<0.001	<0.001	<0.001
GLU	0.185	<0.001	0.390	$TBARS_L$	0.003	0.012	<0.001
NH_3	0.139	<0.001	0.109	CP_L	<0.001	<0.001	<0.001
TP	0.544	<0.001	0.312	SOD_L	0.042	0.007	<0.001
LAC	0.564	<0.001	0.683	GPx_L	<0.001	<0.001	<0.001
CK	0.493	0.813	0.946	GR_L	<0.001	<0.001	<0.001
LDH	<0.001	<0.001	<0.001	$TBARS_M$	0.013	<0.001	0.054
ALT	0.018	<0.001	0.508	CP_M	0.125	0.006	0.231
AST	<0.001	<0.001	0.666	SOD_M	0.069	0.130	0.329
				GPx_M	0.251	0.187	0.657
				GR_M	0.129	0.388	0.524
				$TBARS_I$	0.138	0.104	0.419
				CP_I	0.072	0.717	0.314
				SOD_I	0.652	0.271	0.306
				GPx_I	0.068	0.152	0.161
				GR_I	0.138	0.212	0.218

Notes: Data were expressed by significant value. B, brain; G, gill; L, liver; M, muscle; I, intestine.

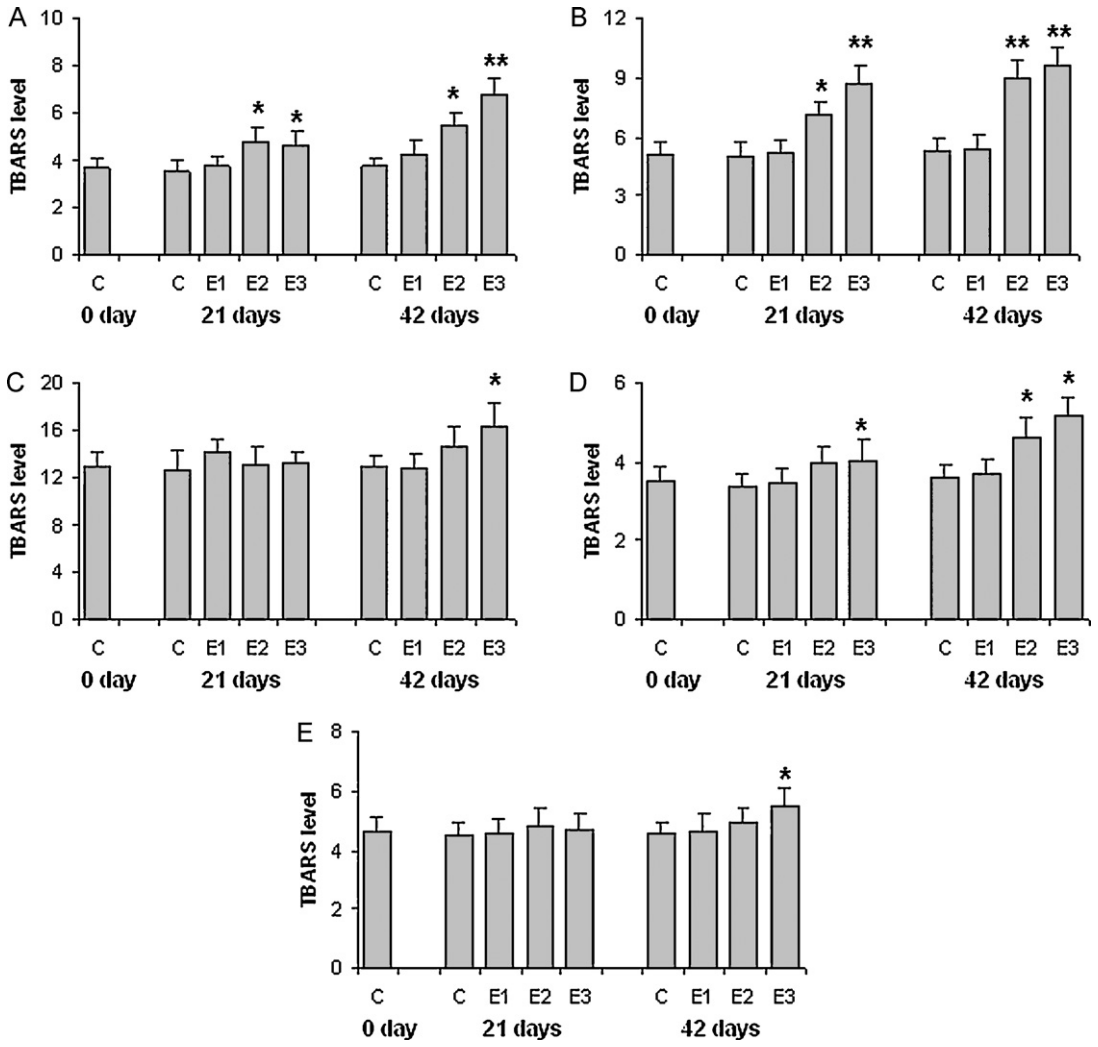

Fig. 1. Levels of thiobarbituric acid reactive substances (TBARS, nmol/gww) in tissues of rainbow trout chronically exposed to VRP. Control and experimental groups (0.5, 27 and 270 μg/L) values are shown in column C, E1, E2 and E3, respectively. A: brain, B: gill, C: liver, D: muscle, E: intestine. Data are mean ± S.D., $n = 12$. Significant differences compared with control value $^{*}p < 0.05$, $^{**}p < 0.01$.

GPx) in brain, gill and liver, as well as oxidative stress (TBARS and CP) indices in muscle. The exposure time-dependent parameters included HSI, PCV, Leuko, MCH, MCV, MCHC, LDH, ALT, AST and all antioxidant related parameters (TBARS, CP, SOD, GR and GPx) in brain, gill and liver, as well as TBARS in muscle. The parameters affected significantly by interactions between VRP concentrations and exposure time included LDH and all antioxidant related parameters (TBARS, CP, SOD, GR and GPx) in brain, gill and liver.

Based on the bilinear decomposition of the original data, the PCA method is used to transform a multivariate data array into a new data set, in which the new variables are orthonormal and explain maximum. In this present study, a data matrix was constructed with 42 analyzed biomarkers as independent variables and 108 sampled individuals as group variables. Based on these parameters analyzed in the present study, different groups with 73.15% of total accumulated variance were distinguished (Fig. 6). The individuals in the same area had the similar biochemical responses in fish. After data statistics, E2 group after 42 d and E3 group after 21 d and above were strongly influenced by VRP-stress.

3.5. Integrated biomarker response (IBR)

IBR index showed E3-42 d as the most affected group under VRP-induced stress. IBR values ranged from 0.13 in C-0 d up to 3.68 in E3-42 d (Fig. 7). According to this index, the rank of the most affected group could be ordered as: E3-42 d > E2-42 d > E3-21 d > E2-21 d > E1-42 d > E1-21 d > C-42 d > C-21 d > C-0 d.

4. Discussion

The worldwide occurrence of residual pharmaceuticals in aquatic environments requires environmental risk assessment procedures to monitor their effects on fish and other aquatic organisms. Laboratory studies of biochemical responses in fish exposed to pharmaceuticals can help to elucidate the mechanism, and provide information on the impact of these chemicals on fish.

4.1. Morphological indices

Morphological indices, especially CF and HSI, have been proposed as an "exposure index" to environmental contaminants [32]. Condition factor, which assumes that heavier fish of a given length is in better condition, is able to indicate fish fitness under stress of pollution as metabolic trade-off is required to deal with detoxification and the energy available for growth may thus be reduced [33]. Some reports have demonstrated that CF declined in fish exposed to environmental pollutants [34,35]. HSI reflects the relative liver size and is linked to the hepatic enzyme activity for detoxification of compounds, indicating exposure to pollutants [7,36,37]. Increases

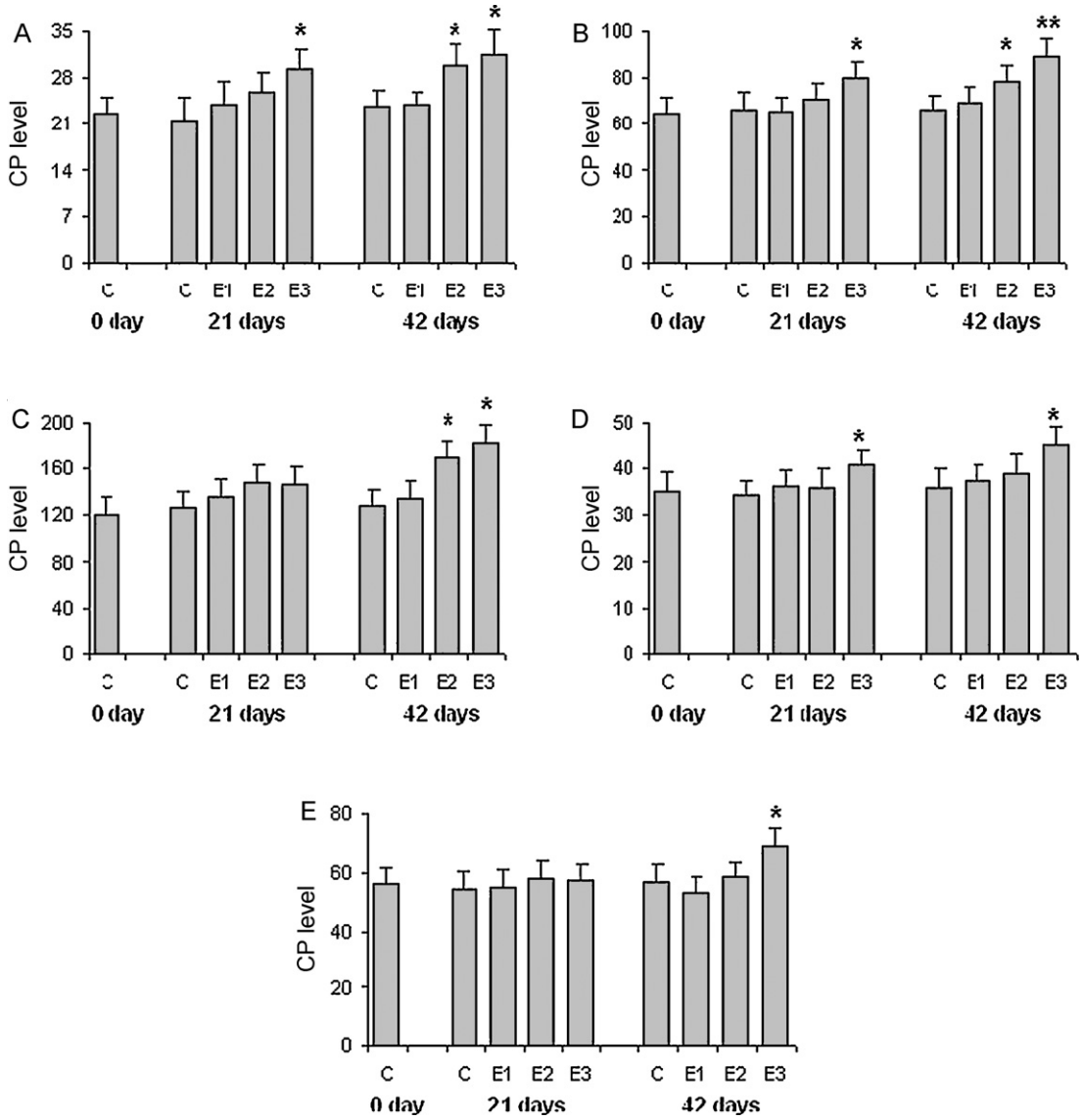

Fig. 2. Levels of carbonyl proteins (CP, nmol/gww) in tissues of rainbow trout chronically exposed to VRP. Other information as in Fig. 1.

in HSI are normally associated with enhanced detoxification activities in response to the presence of toxic compounds [38–40]. In this study, significant lower CF and HSI were observed in the VRP treated group with the highest concentration (E3 group), indicating a decrease in fish growth and overall condition caused by direct metabolic effect on fish.

4.2. Hematological and biochemical blood plasma parameters

Hematological and biochemical profiles of blood can provide important information about the internal environment of the organism. The evaluation of hematological and biochemical characteristics in fish has become an important means of understanding normal and pathological processes and toxicological impacts [41,42]. Our results showed that VRP at various concentrations exerted certain influence on several blood indices in fish.

In this study, the main hematological response of rainbow trout to the chronic effect of VRP was significantly lower PCV, Hb concentration, RBC count and Leuko count after exposure at the highest VRP concentration (E3 group). Decreases in Hb concentration, RBC count and PCV levels may be indicators of anemia. Changes of differential leukocyte count are recognized as sensitive indicators of environmental stress [9]. There was no prominent change in MCV, MCH and MCHC in all groups, indicating that VRP-induced anemia in fish is characterized as normocytic type [43,44]. Additionally, the changes of hematological parameters can be interpreted as compensatory responses that improve the O_2 carrying capacity to maintain the gas transfer and also indicate a change in water blood barrier for gas exchange in gill lamellae [45].

Long-term exposure to higher concentrations of VRP resulted in increase in levels of GLU, NH_3, TP and LAC, and activities of LDH, ALT and AST. An enhanced energy demand caused by short-term pyrethroid stress stimulates the activity of glutamate dehydrogenase which induces glutamate fission to ammonia and α-ketoglutaric acid utilized in the TCA cycle [46]. In our study, VRP showed elevated levels of plasma ammonia concentration, indicating that detoxifying mechanisms were unable to convert the toxic ammonia to less harmful substances. Moreover, significantly higher levels of blood glucose were observed in exposed fish possibly due to metabolic stress [6]. The significantly lower level of LAC in plasma of fish exposed to the highest concentration of VRP, indicates a decrease in the glycolytic process due to the lower metabolic rate. However, the increasing plasma protein content may indicate physiological adaption to overcome stress situation.

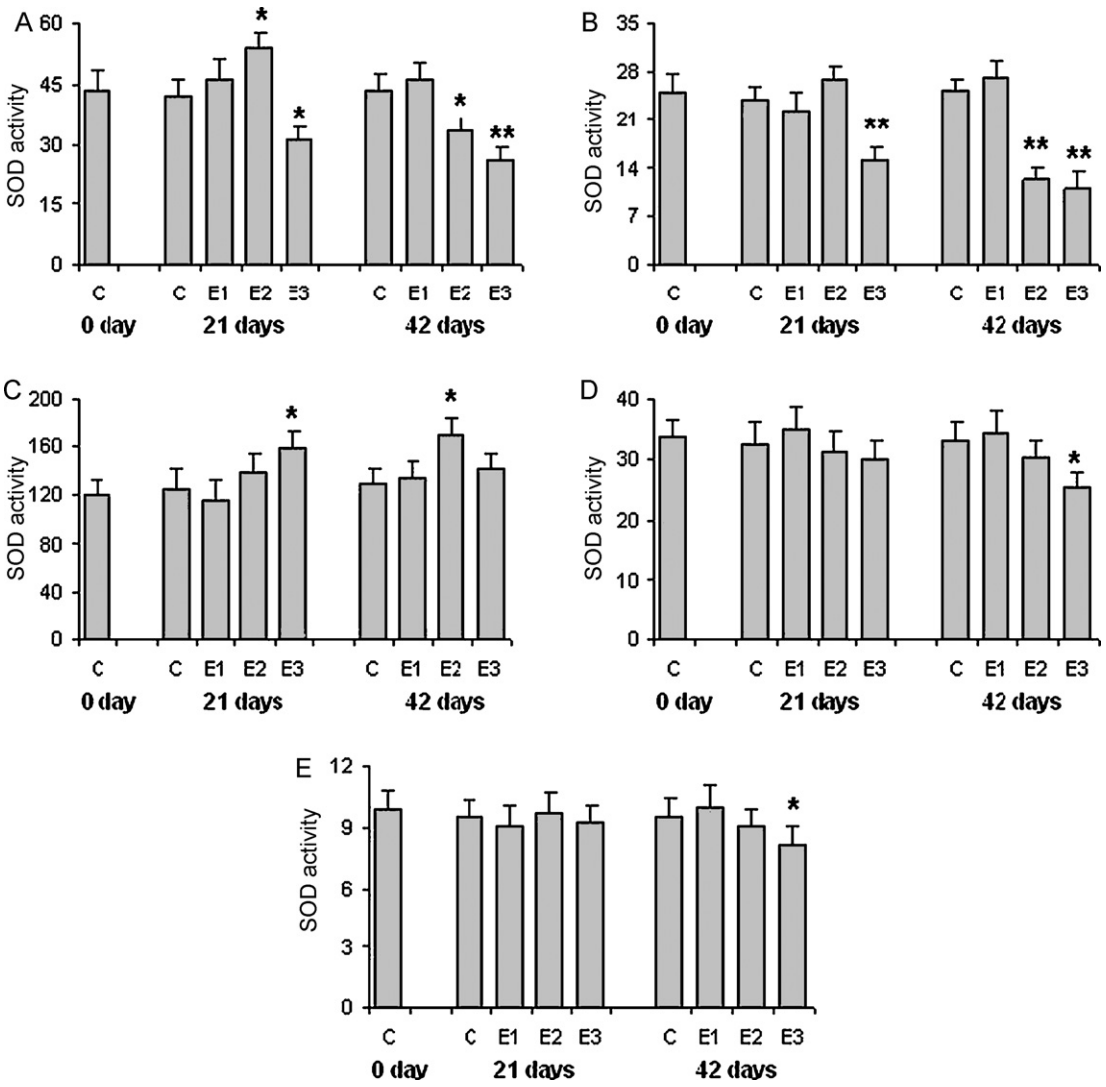

Fig. 3. Superoxide dismutase (SOD, U/mg protein) activity in tissues of rainbow trout chronically exposed to VRP. Other information as in Fig. 1.

Activities of plasma enzymes LDH and the transaminases (ALT and AST) are relevant stress indicators [47]. A significant increase in those enzymes indicates stress-based tissue impairment [48]. LDH is a tetrameric enzyme recognized as a potential marker for assessing the toxicity of a chemical. The elevated levels of LDH in the haemolymph might be due to the release of isozymes from the destroyed tissues [49], while the increased activities of transaminases (ALT and AST) indicate amplified transamination processes. An increase in transamination occurs with amino acid input into the TCA cycle to cope with the energy crisis during pesticide stress [50]. It has been suggested that in general, stress induces elevation of the transamination pathway [51], therefore, it is likely to have contributed to toxic stress induced by VRP and increased transaminase activities in the present study.

4.3. Antioxidant indices

Several studies have demonstrated that environmental pollution induces the production of reactive oxygen species, which may be scavenged by the antioxidant defense system. Oxidative stress will occur if the activities of the antioxidant defense systems decrease or ROS production increases [12].

LPO has been reported to be a major contributor to the loss of cell function under oxidative stress [52] and has usually been indicated by TBARS in fish [53]. Additional, ROS directly attack protein and catalyzed the formation of carbonyl [54]. The formation of CP is non-reversible, causing conformational changes, decreased catalytic activity in enzymes and ultimately resulting, owing to increased susceptibility to protease action, in breakdown of proteins by proteases [55]. Our results showed that chronic exposure to VRP led to oxidative stress, with higher LPO and CP in all tissues, especially in gill and brain, when compared to the control group. The present results suggest that ROS-induced oxidative damage can be one of the major toxic effects of VRP.

Oxidative substances in cells may cause an elevation of antioxidant enzymes as a defense mechanism. Brain, as the central nervous system, is poor in antioxidant defense system, because it is protected by the blood–brain barrier defending the brain from the pass of toxins, but also limiting the movement of the antioxidant [56,57]. In the present study, activity of SOD in fish brain of E2 group was significantly higher after 21 d exposure, probably due to a response to toxicant stress and serves to neutralize the impact of increased ROS generation. However, we also found that all antioxidant enzymes activities in fish brain were strongly inhibited with prolonged exposure to higher concentration of VRP, which could be due to the flux of superoxide radicals, resulting in increased H_2O_2 in the cell [58,59]. It is well known that oxidative stress is caused by the formation of reactive oxygen species (ROS), e.g., hydrogen per-

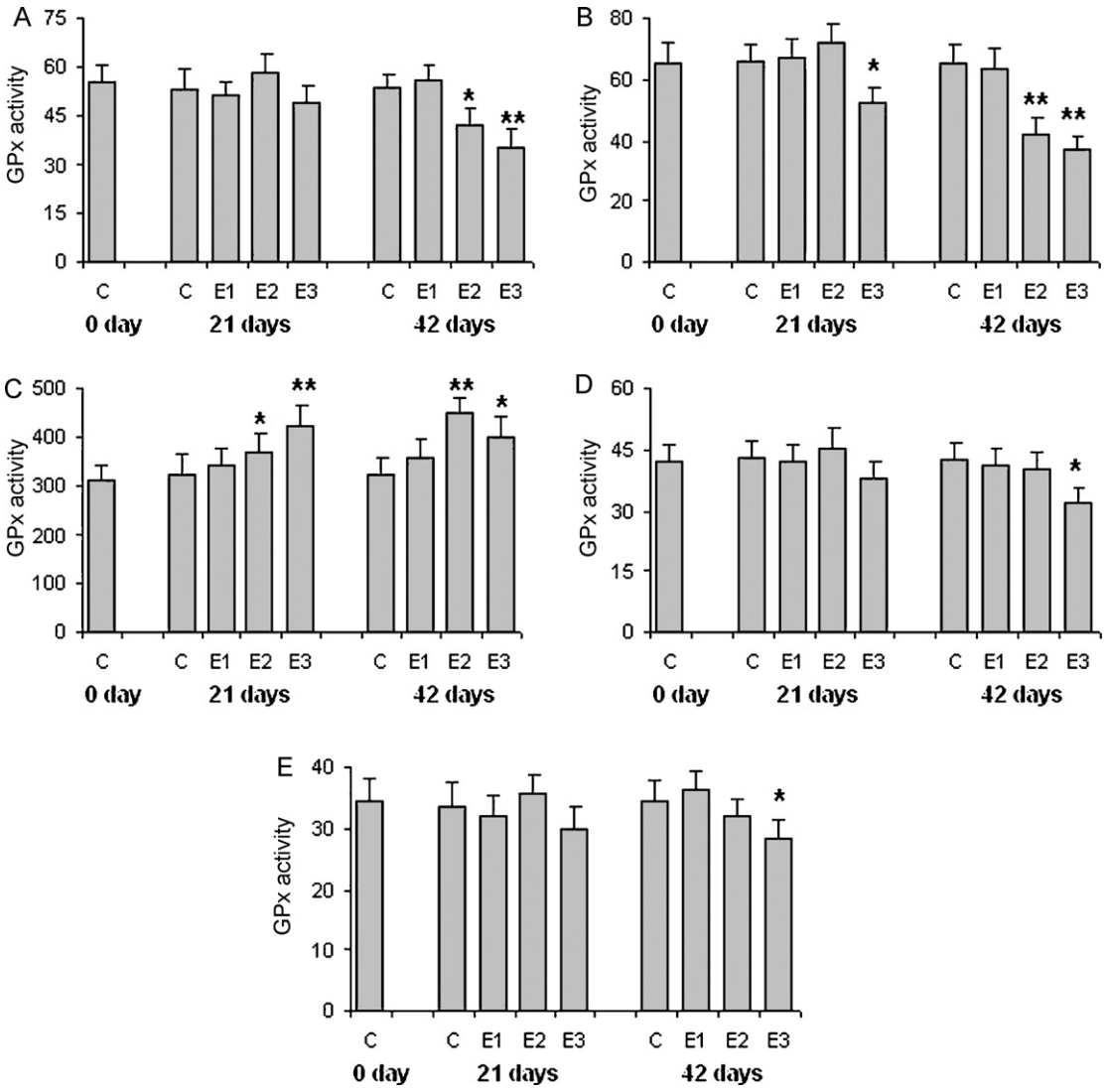

Fig. 4. Glutathione peroxidase (GPx, mU/mg protein) activity in tissues of rainbow trout chronically exposed to VRP. Other information as in Fig. 1.

oxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$), and superoxide anion radical (O_2^-), mainly as byproducts of oxidative metabolism [58,60,61]. In general, ROS are toxic to cells because of their propensity to cause macromolecular damage. Although H_2O_2 is a mild oxidant, being the least reactive of ROS, all aerobic cells are equipped with various H_2O_2-eliminating enzymes because H_2O_2 is readily converted to the highly reactive hydroxyl radical via the Fenton reaction [59,62]. All antioxidant activities were strongly inhibited in gill of fish chronically exposed to higher concentrations of VRP, leading to accumulation of oxidative substances, suggesting inadequate compensation for the presence of environmental pollution [63]. The observation, together with some previous results [64], further demonstrated that gill, the first organ which contact with environmental pollutants, becomes the prime target to toxic chemicals because of not only its large surface area facilitates greater toxicant interaction but also its weak detoxification system [65].

Antioxidant defenses are typically developed preferentially in liver as a result of the central role of this organ in detoxifying xenobiotics and processing metabolic products for degradation [24]. In this study, after long-term exposure, all hepatic antioxidant enzymes (except SOD in E3 group 42 d, which was also induced, but not significantly) activities were significantly induced in E2 and E3 groups compared with the control, which are the adaptive responses to the oxidative stress. All enzymatic antioxidant activities of E3 group showed a decreasing trend after 42 d exposure, but still higher than control group, which indicates the accumulation of ROS but not enough to make enzymes poisoned. The muscle analyzed in the present study was white muscle, which provides burst power output for short-term intensive swimming [66]. Intestine is an important organ in charge of digestive and absorbable function, although it is not mainly responsible for detoxification. Both white muscle and intestine have a low content of mitochondria and low intensity of oxidative metabolism; hence, it is not surprising that the activities of all antioxidant enzymes in fish white muscle and intestine were not significantly induced under VRP-stress [7,67,68]. All antioxidant enzymes activities in muscle and intestine of E3 group showed a significantly inhibited after 42 d exposure, indicating the serious damage caused by accumulation of ROS in these tissues.

4.4. Integrated biomarker response

In order to compare the overall stress of VRP on fish blood system, the IBR index was applied, that provides a simple tool for a general description of the "health status" of organism, combining the different biomarker signals [30,69]. Its usefulness was

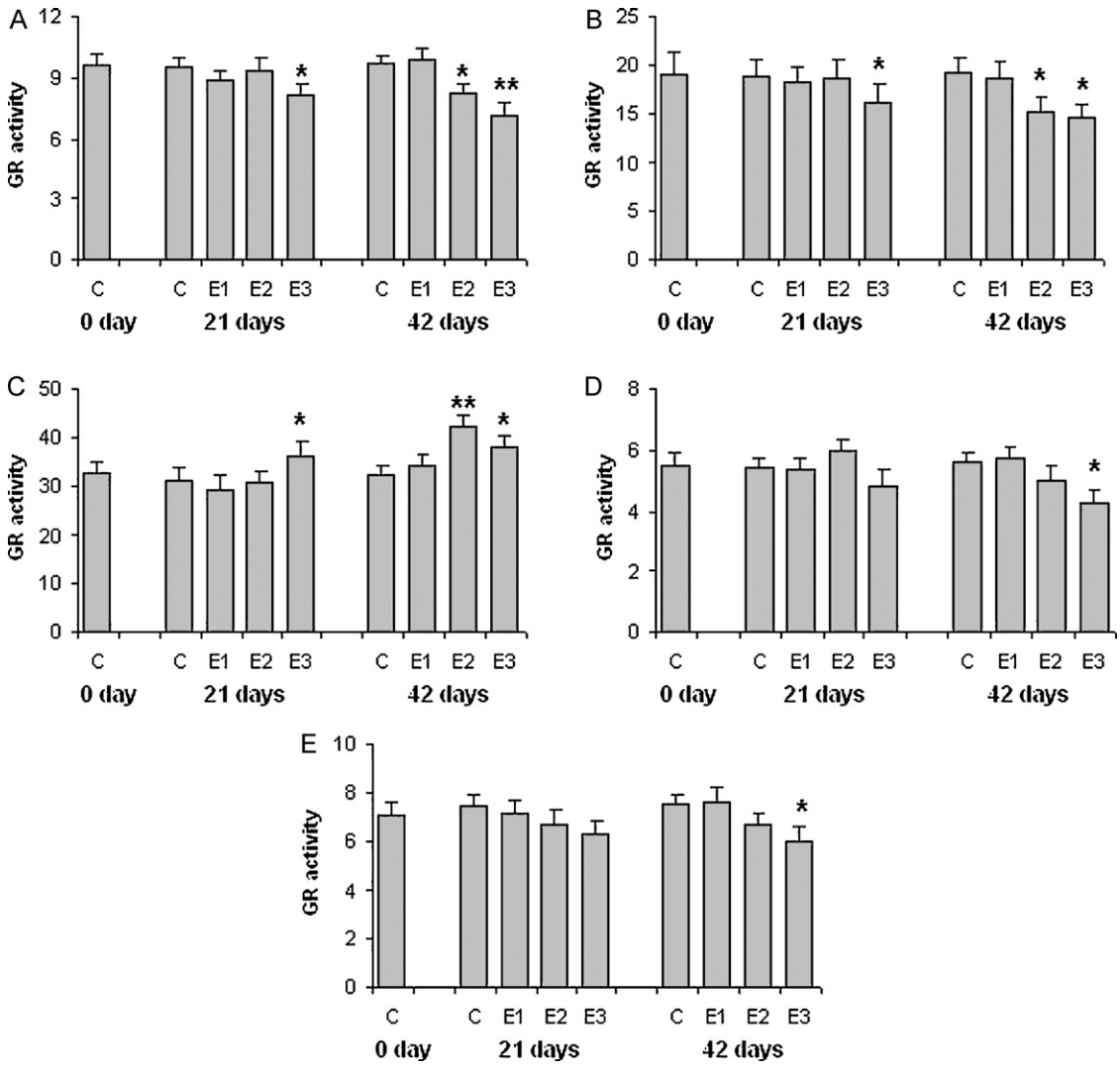

Fig. 5. Glutathione reductase (GR, mU/mg protein) activity in tissues of rainbow trout chronically exposed to VRP. Other information as in Fig. 1.

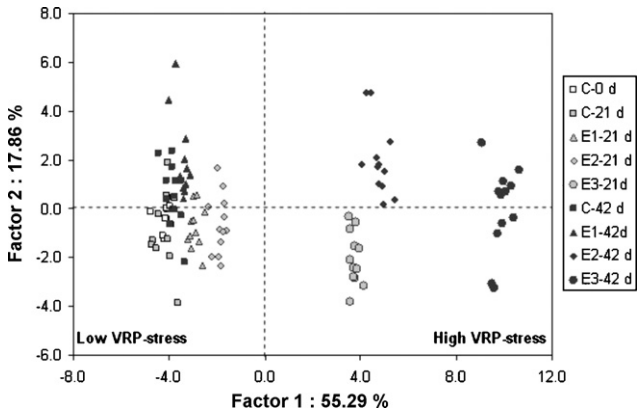

Fig. 6. Individual variations in all parameters measured in rainbow trout chronically exposed to VRP using PCA.

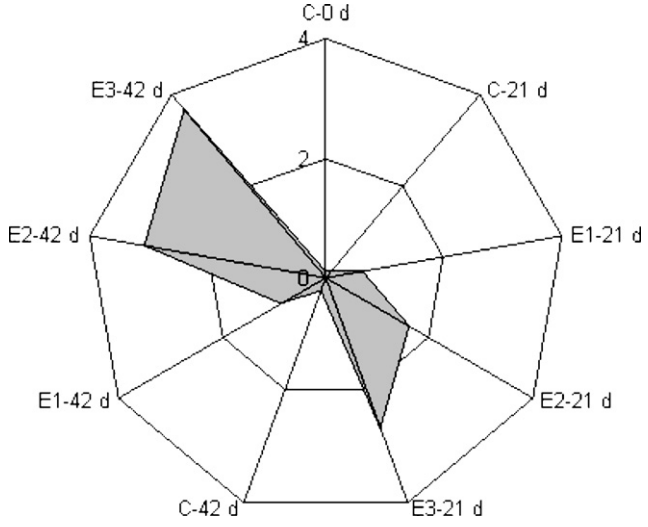

Fig. 7. Integrated biomarker response (IBR) of all parameters measured in rainbow trout chronically exposed to VRP.

previously demonstrated in environmental studies, regardless of the considerable variability in the biomarker sets used, contamination profiles and species [31,70]. IBR results suggest that the VRP-induced stress has become more serious with the increasing concentrations and extending the exposure time and E3-30 d was the most affected group, which is consistent with the analysis for all samples by PCA method.

5. Conclusion

In summary, long term exposure of VRP caused both morphological indices and other biochemical parameters effects in rain-

bow trout, including hematological parameters and antioxidant responses in different tissues. In this study, there was no significant change in all parameters measured in fish exposed to VRP at environmental concentration (E1 group), indicating the adaptive responses to environmental stress. Based on the obtained data, the trout fish, *O. mykiss*, has enough tolerances to VRP-induced changes in surrounding condition. With increasing VRP concentration and prolonging the exposure period, the health status of fish was affected seriously. All parameters measured in this study displayed various dependent manners to VRP concentrations and exposure time, possibly due to different molecular and genetic mechanisms, which need to be investigated in the future. And the reversible possibility of the effects induced by VRP and the potential physiological modulation after longer term (>42 d) exposure to VRP should be given more attention in future studies. According to results of this present study, the biomarkers measured could provide useful information for evaluating the physiological effects of VRP on rainbow trout, but the application of these findings will need more detailed laboratory study before they can be established as special indicators for monitoring residual VRP in aquatic environment.

Acknowledgements

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