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Responses of multiple biomarkers to chloroaniline coexposure in Carassius auratus

Guanghua Lu, Tao Chen

Hohai University, China

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Abstract

2,4,6-Trichloroaniline (2,4,6-TCA) and 2,4-dichloroaniline (2,4-DCA) are widespread environmental pollutants and represent a considerable long-term threat to aquatic and human life. In this study, the in vivo effects of 2,4,6-trichloroaniline and 2,4-dichloroaniline on a set of biomarkers, ethoxyresorufin-O-deethylase (EROD), glutathione-S-transferase (GST), glutathione peroxidase (GPx) and Na+/K+-ATPase, in crucian carp (Carassius auratus) were investigated. Liver EROD and GST activities were significantly increased by 2,4,6-TCA and 2,4-DCA (alone and in combination) after 1, 2, 4, and 7 days of exposure, and obvious dose-response and time-response relationships were observed. Liver GPx and gill Na+/K+-ATP activities were significantly inhibited by the two chemicals (single compounds and in combination) and dose dependence was apparent. The integrated biomarker response (IBR) was calculated by combining multiple biomarkers to single value and used to quantitatively evaluate the toxicological effects of different chemicals. In general, 2,4,6-TCA showed higher IBR values than 2,4-DCA. The joint action of mixture was influenced remarkably by exposure dosages.

Keywords: Chloroanilines; Multiple biomarkers; Carassius auratus; IBR

1. Introduction

The aromatic amines are commonly used in the chemical manufacture of dyes, rubber and textiles and can also originate from gasoline and coal combustion (Palmiotto et al. 2001). Many of these compounds are harmful to the environment and to human health. In which, 2,4-dichloroaniline and 2,4,6-trichloroaniline are widespread environmental pollutants, particularly in wastewater (Kilemade and Mothersill 2000). They represent a considerable long-term threat to aquatic and, ultimately, human life because of their low water solubility and high toxicity (Chen et al. 2007; Lu et al. 2009). Aquatic organisms are typically not exposed to single substances but rather simultaneously to multiple mixtures of chemicals. Assessing the cumulative toxicity of complex chemical mixtures has therefore been an enduring challenge in environmental health research for the past few decades (Monosson 2005).

In the aquatic environment, the exposure of living organisms to xenobiotics leads to interactions between these chemicals and biological systems, and may give rise to biochemical disturbances or/and adaptive responses (Masfaraud et al. 1992). Biochemical biomarkers are increasingly used in ecological risk assessment to identify the incidence of exposure to and effects caused by xenobiotics (Otitoju and Onwurah, 2007). However, most studies evaluated individual biomarker responses without an integrated assessment. The use of a set of biomarkers may be useful to evaluate the various responses to mixtures of pollutants in organisms under stress (Aarab et al. 2004).

The crucian carp (*Carassius auratus*) is distributed widely in freshwaters throughout China and was demonstrated to be a very sensitive species in the study of biotransformation and oxidative stress responses (Lu et al. 2010). This study aims to investigate possible biochemical responses to 2,4,6-TCA, 2,4-DCA and their mixtures, measured as biotransformation phase I enzyme ethoxyresorufin-O-deethylase (EROD) and phase II enzymes glutathione-S-transferase (GST), antioxidant defense enzyme glutathione peroxidase (GPx) and membrane-bound enzyme Na^+/K^+-ATPase in the crucian carp, to study their dose-response and time-response relationships during a 7-day exposure period, and to evaluate integrative toxicological effects of chloroanilines.

2. Materials and methods

Chemicals

2,4,6-Trichloroaniline (98.5% purity) and 2,4-dichloroaniline (98% purity) were obtained from Acros (NJ, USA). Nicotinamide adenine dinucleotide phosphate (NADPH), 3,3'-methylenebis-(4-hydroxycoumarin), 1-chloro-2,4-dinitrobenzene (CDNB), resorufin, ethoxyresorufin and glutathione (GSH) were purchased from Sigma Chemical Company (St. Louis, MO, USA) and the stated purities were >99.9%. Bovine serum albumin was purchased from Shanghai Huixing Biochemistry Reagent Co., Ltd. (Shanghai, China) and the purity was >98%. Coomassie brilliant blue G-250 (Ultra Pure Grade) was purchased from Sinopharm Chemical Reagent Co., Ltd.

(Shanghai, China). All other chemicals were of analytical grade and were obtained from Nanjing Sunshine Biotechnology Co., Ltd. (Nanjing, China).

Animals

Juvenile crucian carp weighing 50.6±3.4 g were obtained from Shengliwei Aquatic Farm in Nanjing. The fish were acclimatized for two weeks in dechlorinated municipal water prior to experimentation. Fish were fed with OSI freshwater aquarium pellet food (6% of body weight/day). Feces and uneaten food were removed every day by suction. Fish were not fed for 24 h prior to the experiments and no food was provided during the test period.

Exposure Test

Five treatments were delivered via intraperitoneal injection at dosages from 0.1 to 20 or 100 mg kg^{-1} chloroanilines dissolved in dimethyl sulphoxide (DMSO). Control animals received DMSO only. Several dosages of 2,4,6-TCA/2,4-DCA mixture were also tested according to an equiconcentration ratio of 1:1. A blank control and a solvent control (DMSO) were included in the experimental design. Dose range was based on range finding experiments (no lethal effect at the highest exposure dosage). Fish were weighed before injection to determine the volume of dosage per kilogram body mass of each fish. Masses and dosages were recorded. Fish were kept in groups of twelve in 30-l glass tanks containing dechlorinated municipal water under constant aeration. A 50% water change was performed every other day. Water temperatures ranged from 20 to 22°C with pH 7.0±0.2, and dissolved oxygen of 5.8±0.2 mg l^{-1} during the period of exposure.

Enzyme Assays

Three fish were collected for each treatment and control after 1, 2, 4, and 7 days of exposure. Fish were killed by cervical transection and liver and gill tissues were collected. Tissues were carefully dissected, washed in 0.15 M of cold KCl, weighed, immediately frozen in liquid nitrogen, and stored at –80°C.

Tissue samples were homogenized in nine volumes of cold buffer and centrifuged for 20 min (9,000×g) at 4°C. The supernatants were used as the extract for enzymatic activity determination. EROD activity in liver was quantified at 572 nm using a microplate reader (Lu et al. 2009). The reaction mixture consisted of 140 µl buffer (0.1 M Tris, 0.15 M KCl, pH 8.0), 10 µl of 2 µM 7-ethoxyresorufin and 10 µl extracts. The reaction was initiated at 25°C by the addition of 40 µl of 2.1 mg ml^{-1} nicotinamide adenine dinucleotide phosphate (NADPH). GST activity in liver was determined at 340 nm by the method of Habig and Jakoby (1981) adapted to a microplate reader as described in Frasco and Guilhermino (2002), using 0.03 ml of homogenate and 0.15 ml of the reaction solution (100 µl of 0.1 mM potassium phosphate, 10 µl of 1.0 mM 1-chloro-2,4-dinitrobenzene, 10 µl of 1.0 mM GSH, and 880 µl H_2O). GPx activity in liver was determined by measuring the decrease in absorbance (340 nm) due to the decline in NADPH at 23–25°C (Berntssen et al. 2003).

Na^+/K^+-ATPase activity, expressed as μmol PO_4^{3-} liberated per mg of protein in a gill homogenate, was measured by liberating PO_4^{3-} from a hydrolysis reaction with ATPase, as described previously (Levesque et al. 2003). Each enzymatic activity was determined in triplicate and expressed as units (U) per mg of protein. A U is a picomole (for EROD), nanomole (for GST and GPx) or micromole (for Na^+/K^+-ATPase) of substrate hydrolyzed per minute. Protein concentrations were determined with the Coomassie Protein Assay Kit (Bradford, 1976), with bovine serum albumin as the standard. The measurements were done on a microplate reader at 595 nm.

Calculation of the IBR

A method for integrating all the measured biomarker responses into one general "stress index", termed "Integrated Biomarker Response" (IBR; Beliaeff and Burgeot 2002), was applied to evaluate an integrated impact of toxicants. The procedure of IBR calculation is: (1) Calculation of mean and standard deviation (SD) for each biomarker; (2) Standardization of data: $F_i'=(F_i - \text{mean}F)/S$, where F_i' is the standardized value of the biomarker, F_i is the value of each biomarker responses, mean$F$ is the mean value of the biomarker, and S is the standard deviation of the biomarker; (3) Using standardized data, Z was computed as $+F_i'$ in the case of activation and $-F_i'$ in the case of an inhibition, and then the minimum value for each biomarker was obtained and added to Z. Finally the score B was computed as $B=Z + |\text{min}|$, where $B \geq 0$ and |min| is the absolute value. The corresponding IBR value is: $\{[(B_1 \times B_2)/2]+[(B_2 \times B_3)/2]+\cdots[(B_{n-1} \times B_n)/2]+[(B_n \times B_1)/2]\}$.

Statistical analysis

For each biomarker, the data were expressed as mean ± SD. All data from different treatments were checked for normality. Data from different treatments were compared by a one-way analysis of variance (ANOVA) and statistically different treatments were identified by Dunnett's t test. All differences were considered significant at $p<0.05$. Statistical analyses were performed using the SPSS statistical package (ver. 11.5, SPSS Co., Chicago, USA).

3. Results

No mortality occurred during the experiments. In crucian carp exposed to DMSO in the solvent controls, liver EROD, GST, GPx and gill Na^+/K^+-ATPase activities did not differ significantly from those in the water controls. Therefore, enzymatic activities of chemical-exposed fish were compared with those of solvent controls. The in vivo effects of 2,4,6-TCA and 2,4-DCA on EROD are presented in Fig. 1. Lower dosages of 2,4,6-TCA and 2,4-DCA (0.1 mg kg^{-1}) did not induce obvious effects on EROD. EROD activity was significantly increased by exposure to higher dosages tested (p<0.05) and the increasing level of EROD activity matches the dosage increase. However, the highest 2,4-DCA dosage (100 mg kg^{-1}) resulted in a significant decrease of EROD induction as compared with that the exposure of 20 mg kg^{-1} (p<0.05). Regarding time response, most significant EROD increases occurred at 2 d for 2,4,6-TCA and 4 d for 2,4-DCA for most exposure dosages.

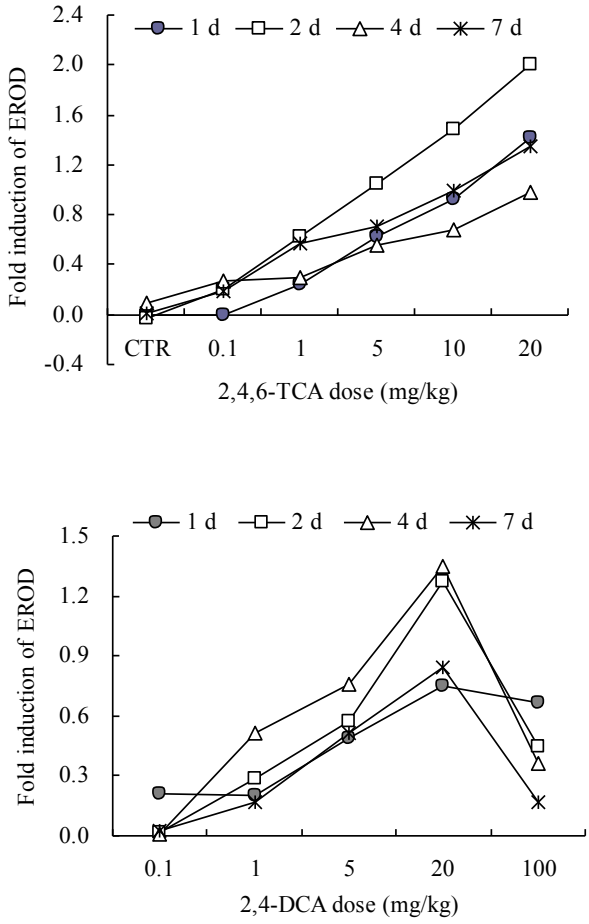

Fig. 1 EROD responses in goldfish exposed to individual compounds (CTR=solvent control)

The responses of GST activity exposed to 2,4,6-TCA and 2,4-DCA are shown in Fig. 2. It was similar to EROD response pattern, GST activity was significantly induced by all tested dosages of 2,4,6-TCA and 2,4-DCA with the exception of 0.1 mg kg^{-1} and the fold induction declined at the highest exposure dosage (20 or 100 mg kg^{-1}). Furthermore, most significant induction of GST activity was found after 1 or 2 d exposure to 2,4,6-TCA, after 2 or 7 d to 2,4-DCA.

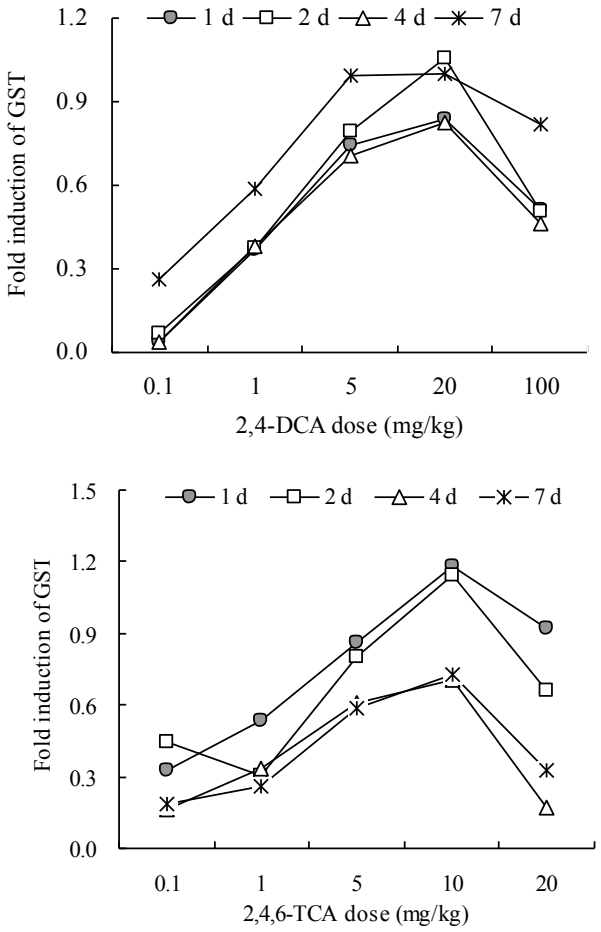

Fig. 2 Liver GST responses in goldfish exposed to individual compounds

Inhibition rates of 2,4,6-TCA and 2,4-DCA on GPx activity during all periods exposure (1, 2, 4 and 7 d) are presented in Fig. 3. 2,4,6-TCA significantly inhibited liver GPx activity at dosages equal to or higher than 1.0 mg kg^{-1} (p<0.05). 2,4-DCA significantly inhibited GPx activity at all test dosages (p<0.05), and dosage dependence was apparent. For both compounds, the most significant GPx inhibition was observed at 20 mg kg^{-1}, however, time dependence was not apparent.

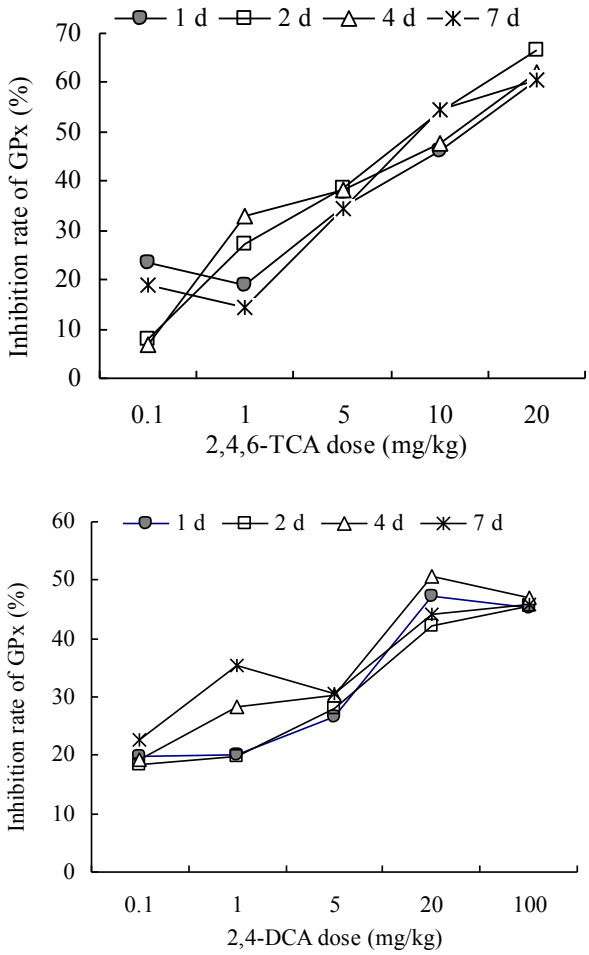

Fig. 3 Liver GPx responses in goldfish exposed to individual compounds

Inhibition rates of 2,4,6-TCA and 2,4-DCA on gill Na^+/K^+-ATP activity during all periods of exposure are presented in Fig. 4. 2,4,6-TCA significantly inhibited Na^+/K^+-ATP activity in all cases (p<0.05) and dosage dependence was apparent. In addition, most significant Na^+/K^+-ATP inhibition occurred at 1 d for all dosages with the exception of the lowest dosage. Low dosages of 2,4-DCA (0.1 and 1.0 mg kg^{-1}) did not significantly inhibit Na^+/K^+-ATP activity after 1 d and 2 d of exposure. However, Na^+/K^+-ATP activity was significantly inhibited by exposure to higher dosages of 2,4-DCA at the four exposure periods (p<0.05). Regarding time response, most significant Na^+/K^+-ATPase inhibition occurred at 2 d for higher dosages of 2,4-DCA exposure (≥5 mg kg^{-1}) and at 4 or 7 d for lower dosages (0.1 and 1.0 mg kg^{-1}).

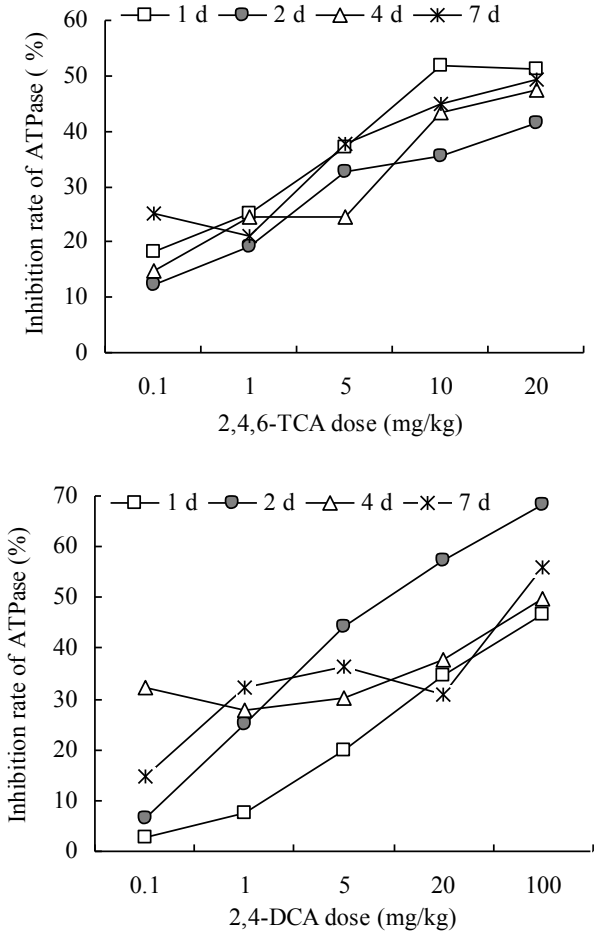

Fig. 4 Gill Na^+/K^+-ATP responses in goldfish exposed to individual compounds

The changes of enzymatic activities after 2 d of exposure to 2,4,6-TCA/2,4-DCA mixture are presented in Fig. 5. The mixture significantly changed the activities of the four enzymes at all test dosages except at 0.1 mg kg^{-1} on EROD activity (p<0.05). Induction of EROD activity was elevated with increased mixture dosages, with the exception of slight reduction at 5 mg kg^{-1}. The dose–response curve of the mixture on GST was similar to those of individual chemicals, GST induction increased continuously corresponding to the dosages, and the fold induction declined significantly at the highest exposure dosage. Percentage inhibition of GPx activity increased continuously corresponding to the exposure dosages and exhibited a good dose–response relationship. Na^+/K^+-ATP activity was significantly decreased by all the dosages of mixture, however, dose dependence was not as apparent as that of individual chemicals.

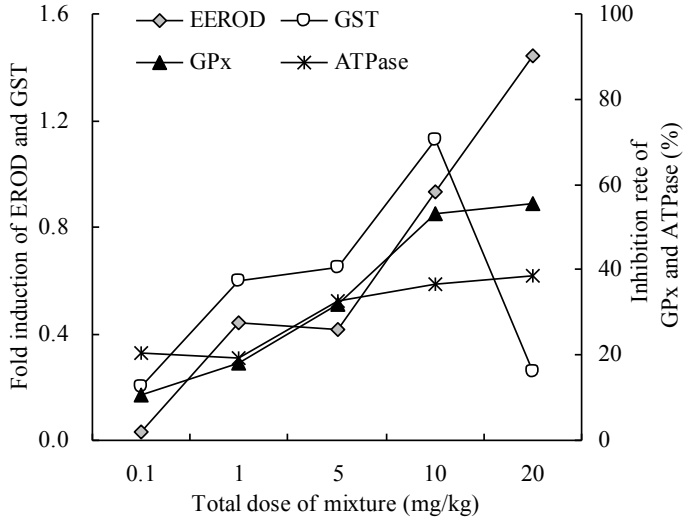

Fig. 5 Biomarker responses in goldfish exposed to 2,4,6-TCA/2,4-DCA mixture

Standardization was carried out on EROD, GST, GPx and Na^+/K^+-ATP activities obtained from each chemical and their mixture. The integrated biomarker response (IBR) values were computed and given in Fig. 6. As the exposure dosages of both chemicals and their mixture increased, the IBR values tended to increase. IBR values of 2,4,6-TCA were always higher than those of 2,4-DCA. However, IBR displayed different manners of joint action dependent to mixture dosages, and it seemed to be synergistic effect at 1 mg kg^{-1}, additive effect at 5 mg kg^{-1} and antagonistic effect at 20 mg kg^{-1}.

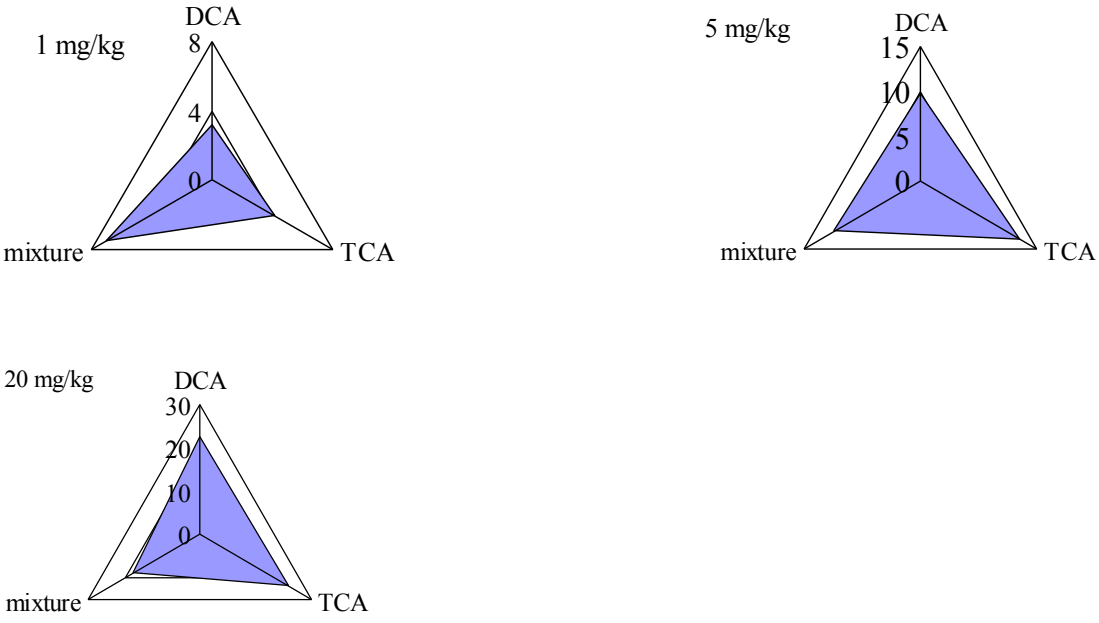

Fig. 6 IBR values of chloroanilines at different exposure dosages at 2 d

4. Discussion

P450 induction is primarily due to the transcriptional activation of the gene, but can also be caused by post-transcriptional regulation or post-translational regulation (Werlinder et al. 2001; Sadar and Andersson 2001). The mechanism by which cells recognize inducers and transmit information to genes is well understood in the case of the members of subfamily CYP1A, which are induced by polycyclic aromatic hydrocarbons (PAHs) and their halogenated forms (Tuvikene 1995). CYP1A induction potency of a chemical has been related to its binding affinity to the Ah receptor (Mekenyan et al. 1996). The CYP1A induction measured either by immunodetection or through its catalytic activity is probably the best-studied biomarker (Bucheli and Font 1995). Hence, EROD activity has been widely used as a biomarker for fish exposure to substances that bind to the aryl hydrocarbon (Ah) receptor (Teles et al. 2005). EROD and GST are considered related biomarkers. The metabolites formed by phase I biotransformation activity are conjugated via phase II enzymes (e.g., GST) before excretion. GST may play an important role in detoxifying strong electrophiles with toxic, mutagenic and carcinogenic properties. It can catalyze the conjugation of the tripeptide glutathione with the xenobiotic in phase II of the biotransformation process and promote its elimination from the organism (Richardson et al. 2008).

The results in this study demonstrate that both chemicals (alone and in combination) resulted in a significant increase in hepatic EROD and GST activities in crucian carp. This is consistent with previous findings in same or different species of fish treated by various aromatic compounds. For example, both naphthalene (NAP) and β-naphthoflavone (BNF) revealed to be strong biotransformation (phase I) inducers (Pacheco and Santos 2002). Hepatic GST activity in *Carassius auratus* was slightly elevated by 2,4-dichlorophenol exposure at 0.01 and 0.05 mg l^{-1} (Zhang et al. 2004). Benzo(a)pyrene displayed strong hepatic EROD and GST induction potency in *Sparus aurata* (Banni et al. 2008). Phenanthrene was found to induce a concentration-dependant formation of the enzyme EROD in the tilapia, *Oreochromis mossambicus* (Shailaja and Classy 2003).

The induction effects of the mixture on EROD and GST activities were consistent with those of the corresponding individual exposures, suggesting that the two chemicals when in mixture induce an additive effect on fish toxicity. In addition, EROD induced by 2,4-DCA and GST induced by 2,4,6-TCA and 2,4-DCA (alone and in combination) exhibited bell shaped dose-response curves. Bell-shaped curves have been reported on EROD and GST induction for in vivo or in vitro systems after exposure to PAHs (Bosveld et al. 2002; Lu et al. 2009). Although the mechanism that results in decreased EROD or GST induction has not been completely defined, it is likely that high concentrations of the inducer inhibit or inactivate the induced enzyme (Voorman and Aust 1987).

GPx catalyzes the reduction of both hydrogen peroxide and lipid peroxides, and a reduced GPx activity could indicate that its antioxidant capacity was surpassed by the amount of hydroperoxide products of lipid peroxidation (Monteiro et al. 2006). GPx

catalyzes the reduction of both hydrogen peroxide and lipid peroxides and is considered an efficient protective enzyme against lipid peroxidation (Winston and Di Giulio 1991). GPx activity may be increased due to increased production and enzyme-inducing effect of H_2O_2 derived from O_2^-. Low activity of GP_X in different tissues of exposed fish demonstrates inefficiency of these organs in neutralizing the impact of peroxides (Fatima et al. 2000). The dose–response curve of the mixture on GPx inhibition was similar to those of individual chemicals and an additive effect appeared to exist.

ATPases play important roles in intracellular functions and in all types of physiological activity. Gill Na^+/K^+-ATP is a membrane-bound enzyme that catalyzes the active Na^+ and K^+ transport into the animal, providing a driving force in the gill epithelium (de la Torre et al. 2007). Although ATPase activity is used as a sensitive indicator of heavy-metal toxicity, there is evidence that organic pollutants can inhibit ATPase activity in concentration-based experiments (Reddy et al. 1992; Dutraa et al., 2008). The Na^+/K^+-ATP activity appeared to be noticeably inhibited exposed to 2,4,6-TCA and 2,4-DCA (alone and in combination) which would suggest the animals most likely suffered disruption, as regards ion regulation. It is possible that the toxic organic compounds reacted with the membrane bound ATPases and brought about an alteration in the active transport mechanism (Lakshmi et al. 1990). The inhibition level of the mixture on Na^+/K^+-ATP activity was lower than that observed in individual exposures on the whole. It is suggested that a slight antagonistic effect existed between 2,4,6-TCA and 2,4-DCA with regard to Na^+/K^+-ATP activity inhibition.

IBR index was calculated by combining different biomarkers to single value, which can be used to describe the toxically-induced stress level of populations in different areas. This method has been previously used as a useful tool for environmental risk assessment (Damiens et al. 2007; Lu et al. 2010; Pereira et al. 2010). Recently, IBR was also used to identify the toxicological effects of organic pollutants toward fish including perfluorinated organic compounds (Kim et al. 2010), fungicide (Li et al. 2011a) and verapamil (Li et al. 2011b). Given that the IBR is an indicator of environmental stress, 2,4,6-TCA appeared to be more stressful than 2,4-DCA toward the crucian carp on the whole in this study. The results demonstrated that the IBR might be a useful tool for quantification of various biomarker responses induced by toxic chemicals toward fish.

5. Conclusions

The present study investigated the biological effects of two chloroanilines on crucian carp. 2,4,6-TCA and 2,4-DCA (alone and in combination) significantly induced liver EROD and GST activities and inhibited liver GPx and gill Na^+/K^+-ATP activities, and both dose dependence and time dependence were apparent. The results suggest that those biomarkers should be addressed in ecological risk assessments of chloroanilines in fish. In addition, IBR method was found to be useful for quantitative assessment of the toxicological effects of chlorinated anilines.

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