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Effects of 2,4-dinitrotoluene exposure on enzyme activity, energy reserves and condition factors in common carp (*Cyprinus carpio*)

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ABSTRACT

In this study relative condition factor (RCF) and hepatosomatic index (HSI) as well as the available energy reserves of common carp (*Cyprinus carpio*) by 2,4-DNT semi-static bioassay were determined and linked to effects of enzymes in liver tissues. Fish were exposed at sublethal concentrations of 2,4-DNT (0.13 μg/L, 0.1, 0.5, 1.0 mg/L) for 7 and 15 d. Based on the results, there was no significant change in all parameters measured in fish exposed to 2,4-DNT at environmental related concentration, but 2,4-DNT stress in fish exposed to higher concentrations reflected the significant changes of physiological and biochemical responses. 2,4-DNT stress resulted in EROD activity induction in the liver, and the levels of EROD activity ranged from 0.39- to 1.83-fold higher than control. For GK, Na^+/K^+-ATPase, and GST, these enzyme activity continued to decline after exposure to 0.1, 0.5 and 1.0 mg/L 2,4-DNT, whereas the trend on GK and Na^+/K^+-ATPase was more obvious than GST. Through principal component analysis, effects by 2,4-DNT-stress in each test group were distinguished. Additionally, indications of a trade-off between metabolic cost of toxicant exposure and processes vital to the survival of the organism were seen at the enzyme activity level as well as on higher levels of biological organization.

1. Introduction

The widespread use of nitroaromatic compounds, especially nitrotoluenes in the manufacturing of munitions, dyes, and other industrial products such as polyurethane foams and pesticides has no doubt contributed to the contamination of the environment [1]. In America, more than ten thousand tons of DNT (dinitrotoluenes) were used to produce polyurethane foams every year [2]. In addition, According to the Environmental Protection Agency's Toxic Release Inventory, production of explosives resulted in the third largest output of waste DNT in the US, with the largest source being production of toluene diisocyanate and methylene diphenyl diisocyanate [3]. In China more than 500 plants, which spread over 27 provinces, produce TNT (trinitrotoluene). In this process, a great number of intermediates, such as mononitrotoluenes (NT) and DNT are generated, which cause pollution of water resources. Among them, 4-NT, 2,4-DNT and 2,6-DNT are priority pollutants in Chinese water body. US Environmental Protection Agency (USEPA) has also ordained: 2,4-DNT and 2,6-DNT are priority pollutants monitored in the environment [4–6]. Moreover, DNT is also one of the several aromatic compounds on USEPA's Draft Drinking Water Contaminant Candidate List [5]. Therefore, this paper selects 2,4-DNT as research objects.

Although 2,4-DNT is mostly found in the environment in relatively low concentrations (around the ng/L range), concentrations up to 0.88 μg/L have been detected in the Songhua River upstream of a petroleum chemistry manufacturing company [7,8]. Songhua River, flowing through Jilin province and Heilongjiang province, is one of the seven largest rivers in North China. Because of its refractory and bioaccumulation property, common carp (*Cyprinus carpio*) as well as other more than 10 freshwater species from different branches of Songhua River have been shown to have 2,4-DNT concentrations in whole fish ranging from <0.22 up to 0.95 mg/kg wet weight [7,9]. The average bioconcentration factors of 2,4-DNT in the fishes were 7.8×10^2 in muscles and 7.8×10^3 in whole fish [7].

Effects of nitrotoluene exposure are mainly studied in mammalian species. DNTs have been reported as the major agent of ecotoxicity, carcinogenesis, urinary tract tumors, reproductive toxicity and hepatic toxicity [10–14]. Animal consumption of 2,4-DNT and 2,6-DNT could ultimately lead to the formation of a carcinogen when metabolized by hepatic enzymes and intestinal microflora [1]. Masami et al. [15] examined the effects of p-nitrotoluene, an endocrine disruptor, on rat behavior and gene expression in rat. Wu

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et al. [16] investigated the effect of TNT on the serum biochemical indicators among workers in a TNT chemical factory. Li analyzed the lens and liver damage caused by TNT in 205 chemical factory workers [17]. In addition, biomarkers of exposure and susceptibility in workers exposed to nitrotoluenes were studied [18–20].

Some studies under laboratory conditions focused on the bioaccumulation of nitrotoluenes in freshwater species, which include evaluation of the bioconcentration factor (BCF) and toxicokinetics analysis by clearance-volume-based model [21,22]. Aquatic biology toxicity and Quantitative Structure Activity Relationship (QSAR) of nitro aromatic compounds were reported in various freshwater species [23–25]. Toxicity effect on northern bobwhite (*Colinus virginianus*), western fence lizard (*Sceloporus occidentalis*), and bullfrog (*Rana catesbeiana*) is investigated [26–30]. Whereas the available energy reserves (proteins, glycogen and lipids) and their linkage to effects of 2,4-DNT-mediated enzyme expression in freshwater fish species has hardly been addressed.

In this study, biochemical methods were utilized to determine that enzyme activity and energy reserves (proteins, glycogen and lipids) in the liver of *C. carpio* by 2,4-DNT subacute exposure in vivo 15 d. The enzymes in liver of juvenile carp include 7-ethoxyresorufin-O-deethylase (EROD), glutathione-S-transferase (GST), adenosine triphosphohydrolase (Na^+/K^+-ATPase), and glucokinase (GK). The affected enzymes (EROD, GK, Na^+/K^+-ATPase, GST) in liver were mainly involved in energy metabolism, biotransformation and stress response. *C. carpio* is one of the most economically important freshwater fish in North China and is widely aquacultured all over Asia, in most parts of Europe and on a small scale in some countries of Africa and Latin America [31,32]. In order to further characterize the biochemical effect pattern of the nitrotoluene chemical in liver tissue of juvenile carp, *C. carpio* was exposed through water for 15 d at concentrations of 0.1, 0.5 and 1.0 mg/L 2,4-DNT. Liver was selected as a target tissue because DNT is known to bioconcentrate and bioaccumulate in liver, a primary target organ in fish, as was demonstrated for common carp [7,21]. The focus of the present study is on the induction and inhibition level of different enzymes (Phase I and Phase II) in the liver tissues of *C. carpio* by 2,4-DNT stress under laboratory conditions. Furthermore, we expected that this fish could be useful models in linking the biochemical and physiological aspects of the ecotoxicology of fish.

Responses to detoxication and reduced food intake could result in reduced energy stores which are necessary for the maintenance and survival of the organism. Whereas standard maintenance is an obligatory cost, other processes, such as growth and reproduction, can be suppressed without compromising survival [33]. These indications could be found on biochemical level as well as on higher levels of biological organization. In the perspective of this so-called metabolic cost hypothesis [34], relative condition factor (RCF) and hepatosomatic index (HSI) as well as the available energy reserves (proteins, glycogen and lipids) in liver were determined and linked to effects at enzyme activity level in this study.

2. Materials and methods

2.1. Fish and exposure experiment

Some good condition, active, and healthy Juvenile carp (*C. carpio*), with mean weight 4.81 ± 0.23 g and mean length 6.87 ± 0.26 cm, was sampled from the Songhua Lake aquaculture facility for tests. The fish had been acclimated in laboratory condition for 2 weeks after washing with 0.1% (w/v) NaCl solution to avoid from possibility of infection. The death rate was lower than 1% during domestication.

A 90 L semi-static system was used, in which 16 fish were randomly housed in each test aquarium. The expectant concentrations of 2,4-DNT used were 0.13 μg/L (E1 group, environmental related concentration [7,8]), 0.1 mg/L (E2 group, 0.5% 96 h LC50 of 2,4-DNT in common carp), 0.5 mg/L (E3 group, 2.5% 96 h LC50 of 2,4-DNT in common carp) and 1.0 mg/L (E4 group, 5% 96 h LC50 of 2,4-DNT in common carp). 96 h LC50 is determined by the authors' pre-test.

Exposure concentrations were selected by evaluation of results from a 96 h range finding tests on juvenile carp for 2,4-DNT in our laboratory. The 96 h LC50 is one of the most important parameters in acute toxicity test. Generally the maximum dose for the chronic study is 1/10 of the calculated 96 h LC50 for test compound [29,35]. Previously determined 96 h LC50 was 20.03 mg/L on juvenile carp (95% confidence interval: 16.93–23.83 mg/L) for 2,4-DNT, according to logarithmic concentration-probit line for determination of LC50. This value is lower than 96 h LC50 of 2,4-DNT in developing bullfrog (*R. catesbeiana*) tadpoles at 40.29 mg/L (95%CI: 30.66–52.96 mg/L) [29], which indicates juvenile carp is more susceptive than bullfrog tadpoles to 2,4-DNT. This fact shows that carp could be useful models in linking the biochemical and physiological aspects of early warning biomarker to assess 2,4-DNT stress. In addition, low concentrations such as 0.6135 mg/L and 1.0 mg/L were used as 2,4-DNT exposure concentration to investigate bioaccumulation in juvenile carp [21,22]. Thus, the nominal exposure concentrations for 2,4-DNT were 0, 0.13 μg/L (E1 group, environmental related concentration), 0.1 mg/L (E2 group, 0.5% 96 h LC50), 0.5 mg/L (E3 group, 2.5% 96 h LC50) and 1.0 mg/L (E4 group, 5% 96 h LC50) in the present study.

2,4-DNT was dissolved in acetone with a final concentration less than 0.05%. Two other groups were used as contrast groups, a control group exposed to clean freshwater and a acetone group exposed to the volume of acetone (v/v, 0.05%) used for the highest 2,4-DNT concentration. For each exposure concentration as well as for the controls, three aquaria were used resulting in three full biological replicates for each exposure condition. Mean values and standard deviation (±SD) are presented for each parameter ($n = 6$). The water used during acclimatization and treatment was filtered and aerated. The fish were exposed to a 13 h light/11 h dark cycle and fed daily with commercial fish pellets at 1.5–2.0% total body weight at a fixed time with extra food removed. During the experiment direct sunlight was avoided to limit photolysis of nitrotoluene. The exposed solution was renewed each day after 2 h of feeding to maintain the appropriate concentration of 2,4-DNT and to guarantee water quality. Exposure experiment results with a test period of 14/15 d were used to estimate growth and energy metabolic conditions for rainbow trout [36]. In addition, the results with a test period of 14/15 d can also indicate the biochemistry and physiology response, as well as toxicity effects in crustaceans and rats exposed to contaminants, such as metals and other xenobiotics [37,38]. In this sturdy, the test fish were exposed to 2,4-DNT for 0, 7 and 15 d.

To ensure agreement between nominal and actual 2,4-DNT 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. In this study, the measured concentration of 2,4-DNT in the water samples was 0.12 ± 0.01 μg/L, 0.097 ± 0.007 mg/L, 0.48 ± 0.04 mg/L and 0.96 ± 0.08 mg/L corresponding to the nominal concentration 0.13 μg/L, 0.1, 0.5, 1.0 mg/L.

The test chemical 2,4-DNT obtained from chemical reagent store (Lancaster comp.), was of the highest purity and the best available quality. The toxicant was available commercially in sufficient purity (i.e., 98% or greater), so repurification would not be necessary prior to testing. All the other reagents used were of superpure grade or analytical reagent grade.

2.2. Morphological indices (RCF and HSI)

At the end of each exposure period, 6 fish of every aquarium were randomly sampled. Fork length (up to 1 mm accuracy), body weight and liver weight (up to 0.01 g accuracy) were recorded.

The relative condition factor (RCF) was calculated as an indicator of the general 'well-being' of the fish:

$$K = \frac{W}{\alpha L^b}$$

where W is total body weight (in g) and L is fork length (in cm). The parameters a and b are determined from the length–weight relationship ($W = aL^b$) of all fish at day 0 and was used as a constant factor for all individual fish [39]. This procedure allows comparing the condition of each concentration group as well as the control group at day 0, 7 and 15. Additionally, hepatosomatic index (HSI) was calculated for all fish at day 0, 7 and 15. The HSI is the liver weight as a percentage of the whole body weight (liver weight/body weight × 100).

2.3. Determination analysis of liver 2,4-DNT concentrations

The excised livers of fishes were homogenized with 20–25 volumes of extraction agent (acetone: petroleum = 59:41, v/v) in a glass tissue homogenizer. The homogenate was centrifuged at 900 × *g* for 5 min, cleaned up with an Al_2O_3-column, then dried with a N2-stream before analyzing on HPLC (DOS C-18 reversed-phase column, wavelength 254 nm, pH 7.0, temperature 40 °C, flow rate 0.6 mL/min). The 2,4-DNT concentrations in liver tissue were measured on HPLC, IR, GC–MS [21].

Gas chromatography (Shimadzu, Model GC-7AG, with a ^{63}Ni electron capture detector and a CR-3A data processor) was used in chemical analysis. HPLC (Shimadzu LC-6A, with a SPD-6AV ultraviolet detector), IR (Matteion, ALPHA-600, USA), and GC–MS (VGQuattro, England) were used.

2.4. Enzyme indices

2.4.1. EROD

Fish were killed, the livers were immediately removed from each fish by avoiding the gall bladders and were flash-frozen in liquid nitrogen. Microsomes were prepared from the thawed liver tissue by differential centrifugation. Aliquots of microsomes were gassed with nitrogen and stored in liquid nitrogen until the enzyme assays were performed. EROD activity was measured in duplicate in the microsomal fraction by the spectrofluorometric method [40], with slight modifications. The reaction was initiated by the addition of substrate and followed for 2 min in a spectrofluorometer. Finally, a known amount of resorufin was added as an internal standard to the reaction mixture and the increase in fluorescence was recorded. Enzyme activities were calculated using the fluorescence increase caused by the addition of resorufin. Protein concentration was determined by the method of Lowry et al. [41].

2.4.2. GST

All steps in the preparation of cytosol and microsomes were performed at approximately 4 °C. Tissues were thawed briefly and homogenized in 100 mM potassium phosphate (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol and 20% glycerolusing a glass tissue homogenizer. Homogenates were centrifuged twice at 12,000 × *g* for 10 min. The supernatants were then centrifuged at 105,000 × *g* for 1 h to obtain cytosolic and microsomal fractions. Cytosol and microsomes were frozen in liquid nitrogen until use.

GST activity was measured according to the method of Habig et al. [42] using 1-chloro-2,4-dinitro-benzene (chlorodinitrobenzene CDNB) as substrate. The formation of GSH-CDNB conjugate was monitored by the change in absorbance at 340 nm. One unit of glutathione S-transferase activity is the amount of enzyme catalyzing the conjugation of 1 μmol substrate per min. A standard curve was obtained using pure glutathione S-transferase (Sigma G 8642). Protein concentrations in the cytosol were measured by the method of Lowry et al. [41].

2.4.3. Na^+/K^+-ATPase

The common carp liver was dissected from each fish and homogenized in an ice-cold (1–4 °C) solution contained 0.25 mol/dm^3 sucrose, 1 mmol/dm^3 ethylenediaminetetraacetic acid (EDTA), 0.35 mol/dm^3 mannitol (CHO), 1 mg/dm^3 bovine serum albumin (BSA), and 0.01 mol/dm^3 Tris–HCl (pH 7.2). The solution was then separated by centrifuging at 700 × *g* for 10 min followed by 12,500 × *g* for 20 min. The sediments were resuspended in an ice-cold solution containing 0.25 mol/dm^3 sucrose, 1 mmol/dm^3 EDTA, 0.35 mol/dm^3 CHO, and 0.01 mol/dm^3 Tris–HCl (pH 7.2). The fraction (suspension of sediment in the 12,500 × *g* centrifuging) contained mitochondria and nerve ending particles. Each preparation was appropriately diluted and the samples were kept at −20 °C until the Na^+/K^+-ATPase assay. Na^+/K^+-ATPase activities of a heavy microsomal fraction of liver homogenate from *C. carpio* were determined as previously described [43]. Protein concentration was determined by the method of Lowry et al. [41].

2.4.4. Glucokinase (GK)

For measurement of glucokinase activity, a frozen sample of liver from each fish was homogenized (dilution 1/10) in ice-cold buffer (80 mM Tris; 5 mM EDTA; 2 mM dithiothreitol; 1 mM benzamidine; 1 mM 4-(2-aminoethyl) benzenesulphonyl fluoride, pH 7.6). Homogenates were centrifuged at 900 × *g* for 10 min. The GK activities were measured using 100 mM of glucose as described previously [44] at 37 °C by coupling ribulose-5-phosphate formation from glucose-6-phosphate to the reduction of NADP using purified glucose-6-phosphate dehydrogenase (Sigma Chemical Co.) and 6-phosphogluconate dehydrogenase (Sigma Chemical Co.) as coupling enzymes. The assay for measuring GK activity on frozen samples necessitated correction by measuring glucose dehydrogenase (EC 1.1.1.47) activity as described by Tranulis et al. [44]. Glucose dehydrogenase is a moderately active microsomal enzyme in fish liver that can introduce significant bias in GK measurements on frozen tissues [44].

2.5. Available liver energy reserves

The available energy reserves in liver were determined by liver homogenates of 6 fish of each aquarium. The homogenates were analyzed for protein content by the method of Lowry et al. [41]. Liver glycogen content was determined with the anthrone reagent as described by Roe and Dailey [45], using spectrophotometry at a 620 nm wavelength. Total lipids were extracted following Bligh and Dyer [46] and absorbance was measured at 340 nm. Lipid concentrations were calculated with the help of a standard curve of tripalmitine (Sigma Chemical Co.).

2.6. Statistics

All bioassays were carried out in duplicate or triplicate for statistical purpose. The statistical analysis was performed using a one-way ANOVA (Origin 8.0). All data tested were found to conform to assumptions of equal variance and normality, and expressed as mean ± standard deviation. Student t test was applied to study the relationship between the different concentration samples. A $p < 0.05$ was considered to be significant. To get a general picture of the trends and groupings in the material, the data from all fish individuals were subjected to principal component analysis (PCA)

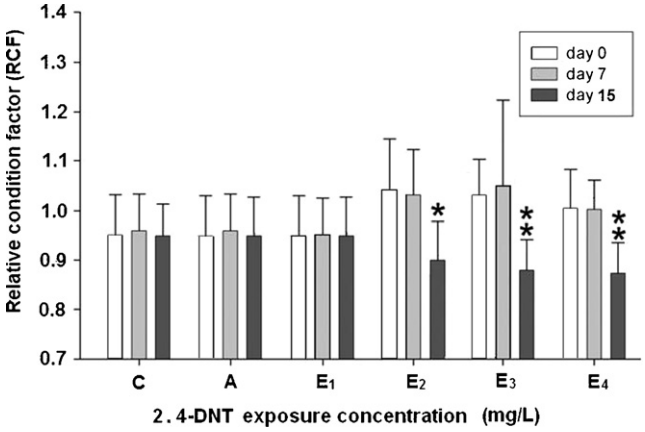

Fig. 1. Relative condition factor (RCF) in the control groups and exposed groups after 0, 7 and 15 d. After 7 d, no significant effects ($p > 0.05$) were seen. After 15 d the RCF significantly decreased ($p < 0.05$, $p < 0.01$) in all fish of E2, E3, and E4 group exposed to 2,4-DNT and this effect increased with increasing exposure concentration. All values are means ± SD ($n = 6$). Error bars are SD $^{*}p < 0.05$, $^{**}p < 0.01$.

by SPSS 17.0. In addition, PCA was used to define the most important parameters, which could be used as key factors for individual variations.

3. Results

3.1. Morphological indices

After 7 d of exposure, RCF of the fish in all treatment groups was not significantly different from the RCF at day 0 ($p > 0.05$) in this study. After 15 d the RFC dropped significantly in all fish of E2, E3, and E4 group exposed to 2,4-DNT ($p < 0.05$, $p < 0.01$) and this effect increased as 2,4-DNT water exposure concentration added (Fig. 1). As shown in Fig. 2, the HSI was significantly lower in fish treated with 0.5 and 1.0 mg/L 2,4-DNT than in the control fish at 7 d and 15 d exposure time points ($p < 0.05$, $p < 0.01$).

3.2. Liver 2,4-DNT concentrations

The 15 d water exposure experiment resulted in hepatic mean 2,4-DNT concentrations of 4.58 ± 0.86 μg/g, 21.68 ± 0.84 μg/g and 40.37 ± 1.52 μg/g wet weight (mean ± SD) for the exposure groups of, respectively, 0.1, 0.5 and 1.0 mg/L. We can observe that 2,4-DNT accumulative concentrations in liver of *C. carpio* increased as 2,4-DNT water exposure concentration added.

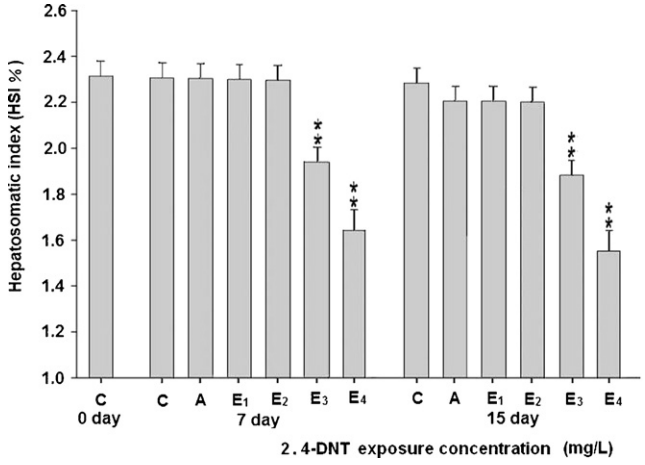

Fig. 2. Hepatosomatic index (HSI) in the control groups and exposed groups after 0, 7 and 15 d. The HSI was significantly lower ($p < 0.05$, $p < 0.01$) in fish treated with 0.5 and 1.0 mg/L 2,4-DNT than in unexposed fish at 7 d and 15 d. Mean values and standard deviation (±SD) are presented for each parameter ($n = 6$). Asterisks (*) and (**), above a bar mean, indicates a value that was significantly different from control ($p < 0.05$, $p < 0.01$).

3.3. Different activity of EROD, GST, Na^+/K^+-ATPase, and GK in response to 2,4-DNT

The 2,4-DNT exposure experiments were aimed at assessing induction or repression of EROD, GST, Na^+/K^+-ATPase, and GK in the different exposure groups sampled after 7 d and 15 d stress. Experiments data show that induction of EROD and repression of GK, Na^+/K^+-ATPase, and GST in liver of treated *C. carpio* were obvious compared with control (Table 1 and Fig. 3).

The results generated by solvent control are presented in Table 1 and Fig. 3. The change of enzyme activity generated by solvent control is very small and can be ignored. There is no significant difference between the enzyme activity of acetone solvent control and that of control ($p > 0.05$), indicating that acetone control has little effects on the results and the enzyme activity of EROD, GST, Na^+/K^+-ATPase, and GK in liver of *C. carpio* is completely generated by chemical tested.

The EROD activity levels increased as 2,4-DNT water exposure concentration added (Table 1 and Fig. 3). Significant differences on EROD activity induction were detected between control and treatment groups (0.1, 0.5 and 1.0 mg/L) sampled after 7 d and 15 d ($p < 0.05$, $p < 0.01$). All 2,4-DNT treatment concentrations except for E1 group resulted in EROD activity induction in the liver, and the levels of EROD activity ranged from 0.39- to 1.83-fold higher than control levels. Maximum induction of EROD activity in carp was recorded in the highest treatment group (E4-15 d).

As shown in Table 1 and Fig. 3, the results demonstrated that enzyme activity levels of GK, Na^+/K^+-ATPase, and GST in liver of juvenile carp declined continuously at 0.1, 0.5 and 1.0 mg/L 2,4-DNT exposure, whereas decline trend on GK and Na^+/K^+-ATPase was more obvious than GST. The maximum repression of GK activity in liver of treated *C. carpio* was recorded (Table 1 and Fig. 3), and the strongest inhibition percent (83.3%) for GK was found at the highest treatment group (E4-15 d). In contrast, enzyme activity levels of GST did not change significantly after 0.1 mg/L 2,4-DNT exposure ($p > 0.05$), but declined GST expression was seen after 0.5 mg/L and 1.0 mg/L 2,4-DNT exposure.

3.4. Effects at higher levels of biological organization

Fig. 4 showed the energy reserves content (glycogen, protein and lipid) in the control group (C), solvent control group (A) and exposed groups (E1, E2, E3, E4). Compared to the control, the exposure of 1.0 mg/L 2,4-DNT after 15 d resulted in significantly lower ($p < 0.05$) values for lipid. In contrast to lipid content, the glycogen content in liver of juvenile carp exposed to 1.0 mg/L was more significantly lower than in the control group ($p < 0.05$, $p < 0.01$) after 7 d and 15 d. Significant differences were not detected in the protein contents between control and all treatment samples after 7 d and 15 d ($p > 0.05$).

3.5. Chemometrics

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 variations [35]. In this study, we constructed a data matrix with parameters analyzed as independent variables and 78 sampled individuals as grouping variables. All the parameters measured were distinguished on the ordination plots corresponded to the first (52.13%) and second (23.52%) principle components

Table 1
Activity level of EROD, GST, Na^+/K^+-ATPase, GK in liver of *C. carpio* exposed to 2,4-DNT for 7 d. In laboratory exposure experiments, fish were kept at control (C) and solvent control (A), or subjected to a 2,4-DNT stress at 0.13 μg/L (E1 group), 0.1 mg/L (E2 group), 0.5 mg/L (E3 group) and 1.0 mg/L (E4 group) concentrations. Mean values and standard deviation (±SD) are presented for each parameter ($n = 6$). Asterisks "*" and "**" represent $p < 0.05$ and $p < 0.01$, respectively.

Enzyme	Activity	Experimental groups						
		0 d	7 d					
		C	C	A	E1	E2	E3	E4
EROD	pmol/min/mg protein	4.62 ± 0.15	4.60 ± 0.16	4.45 ± 0.18	4.48 ± 1.20	5.28 ± 1.27**	7.12 ± 2.11**	12.61 ± 1.51**
	Percent of control (%)			96.7	97.4	114.8	154.8	274.1
	Induction percent (%)					14.8	54.8	174.1
GST	nmol/min/ug protein	1237.7 ± 70.1	1236.5 ± 71.4	1237.6 ± 70.9	1238.6 ± 75.2	1200.9 ± 83.7	899.9 ± 79.0**	780.7 ± 76.1**
	percent of control(%)			100.1	100.2	97.1	72.8	63.1
	inhibitation percent(%)					2.9	27.2	36.9
Na^+/K^+-ATPase	nmol pi/mg protein/h	78.48 ± 2.32	78.42 ± 4.95	79.82 ± 8.01	79.87 ± 3.54	60.24 ± 4.23**	22.38 ± 2.13**	28.46 ± 2.81**
	Percent of control(%)			101.8	101.8	76.8	28.5	36.3
	Inhabitation percent (%)					23.2	71.5	63.7
GK	mU/mg protein	8.53 ± 0.87	8.46 ± 0.71	8.36 ± 0.72	8.42 ± 0.73	4.42 ± 0.82**	2.38 ± 0.75**	2.28 ± 0.83**
	Percent of control (%)			98.8	99.5	52.2	28.1	27.0
	Inhabitation percent (%)					47.8	71.9	73.0

* $p < 0.05$.
** $p < 0.01$.

(Fig. 5), which showed the relationship of all the parameters: (a) enzymes activities of GST, ATPase and GK were positively correlated with morphological index HSI while EROD was negatively correlated with HSI; (b) morphological index RCF was positively correlated with energy reserves content (lipid and protein); (c) to a certain extent, glycogen content was positively correlated with morphological indices (HSI, RCF).

Based on these parameters analyzed in present study, different groups with 75.65% of total accumulated variance were distinguished (Fig. 6). The individuals in the same area had the similar biochemical responses in juvenile carp. Results of data statistics indicate that E2 group and E3 group after 7 d and above were strongly influenced by 2,4-DNT-stress.

4. Discussion

In recent years, the interest in the effects of nitrotoluene chemicals on the environment has increased due to its wide spread and unknown associated risks. However, the available energy reserves (proteins, glycogen and lipids) and their linkage to effects of 2,4-DNT-mediated enzyme activity expression level in freshwater fish species have received little attention. Biochemical and physiology

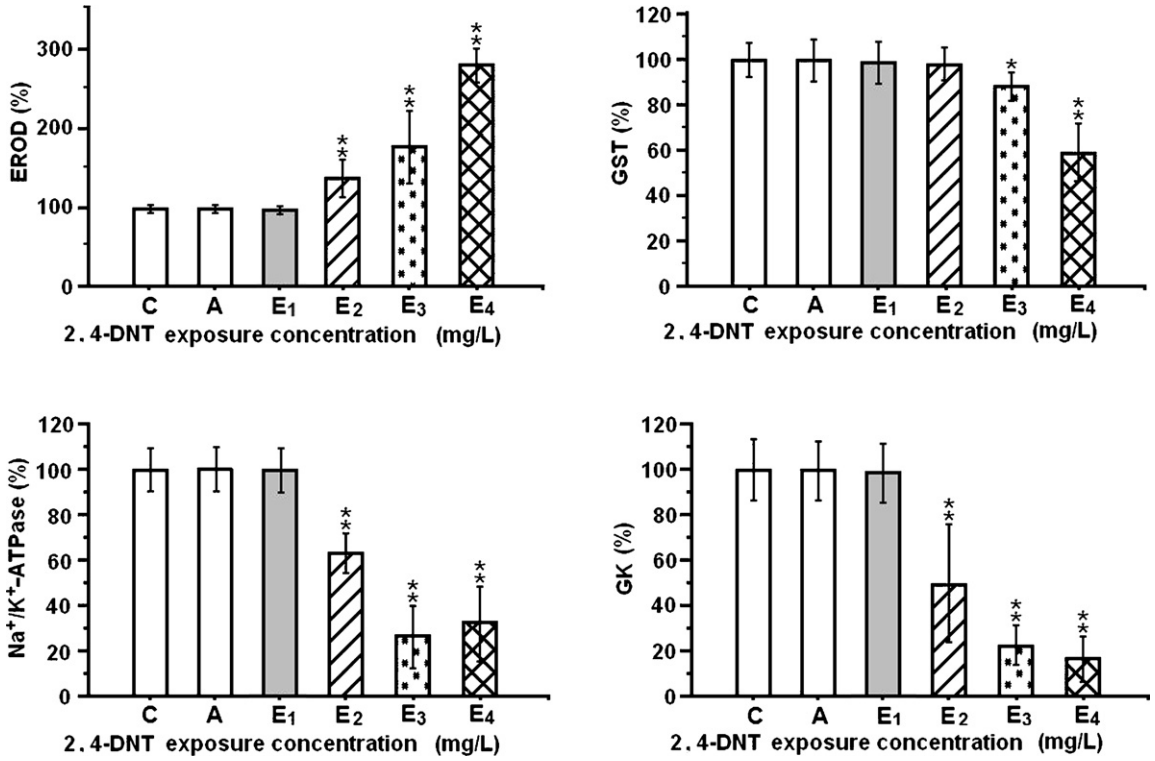

Fig. 3. Enzyme activity level in liver of juvenile carp following 2,4-DNT exposure for 15 d. Control, solvent control and experimental groups (0.13 μg/L, 0.1 mg/L, 0.5 mg/L and 1.0 mg/L) values are shown in column C, A, E1, E2, E3 and E4, respectively. The results are expressed as percent of control and all values are means ± SD ($n = 6$). Significant differences from control ($p < 0.05$, $p < 0.01$) are denoted by '*' and '**', respectively. Error bars indicate standard deviations.

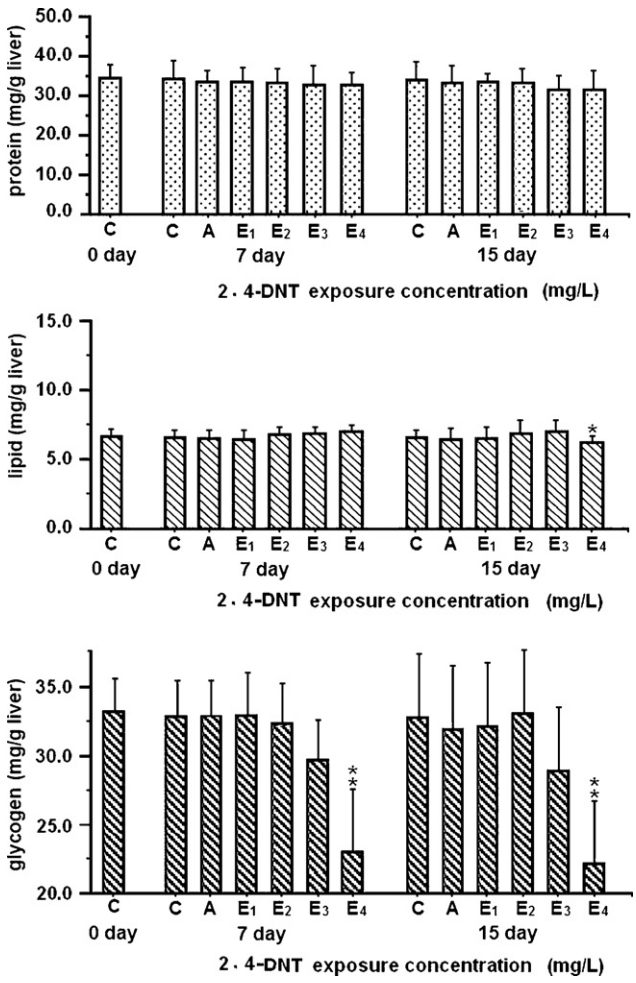

Fig. 4. Energy reserves (protein, lipid and glycogen) in the control group (C), solvent control group (A) and exposed groups (E1, E2, E3, E4) after 0, 7 and 15 d of exposure. Asterisks (*) and (**) represent $p < 0.05$ and $p < 0.01$, respectively. All values are means ± SD ($n = 6$). Error bars indicate standard deviations.

effects of nitrotoluene exposure are mainly studied in mammalian model species [10–13,15,47]. No information was available on induction or repression of 2,4-DNT-mediated Phase I and Phase II enzyme in fish species so far. The present study used biochemical technology to investigate and analyze the induction or repression of 2,4-DNT-mediated Phase I and Phase II enzyme in liver of juvenile

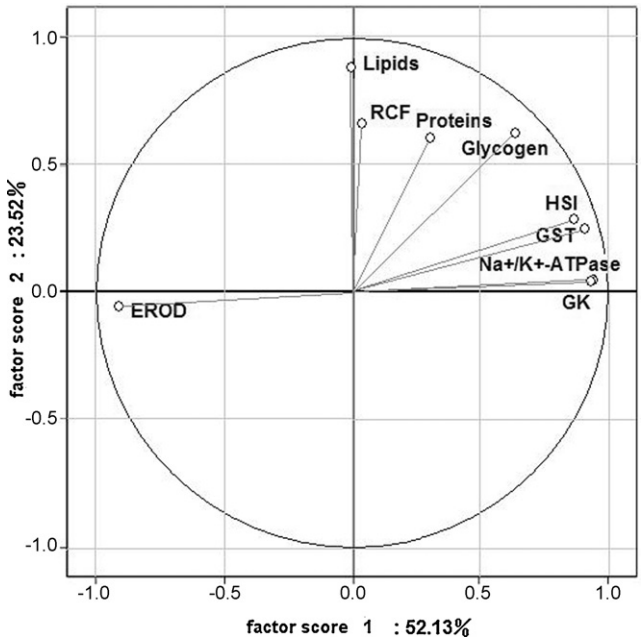

Fig. 5. Ordination diagram of PCA of parameters analyzed in juvenile carp exposure to 2,4-DNT after 0, 7 and 15 d.

carp. The relative condition factor (RCF), hepatosomatic index (HSI) as well as the available energy reserves (glycogen and lipid) were altered after 2,4-DNT exposure. Additionally, indications of a trade-off between metabolic cost of toxicant exposure and processes vital to the survival of the organism were seen, at enzyme activity level as well as on higher levels of biological organization. Laboratory studies of biochemical responses in fish exposed to nitrotoluenes can help to elucidate the mechanism, and provide information on the impact of these chemicals on fish.

4.1. Morphological indices

Integrative measures such as relative condition factor (RCF) and hepatosomatic index (HSI) can provide valuable information concerning the overall effect of pollutants on individual fish [48]. RCF and HSI have been proposed as “exposure index” to environmental contaminants [49]. Some reports have demonstrated that RCF declined in fish exposed to heavy metals [50], polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) [51]. HSI reflects the relative liver size and is linked to the hepatic

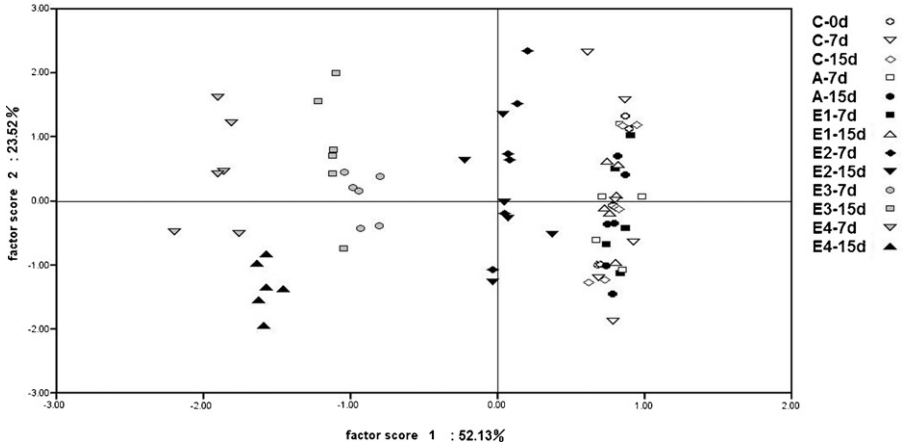

Fig. 6. Individual variations in all parameters measured in juvenile carp exposed to 2,4-DNT after 0, 7 and 15 d using PCA.

enzyme activity for detoxification of compounds, indicating exposure to pollutants [51,52]. Our data show that enzymes activities of GST, ATPase, GK and EROD are closely correlated with morphological index HSI. The results are consistent with the analysis for all samples by PCA method. We think HSI is an index that is commonly used to evaluate fish condition and can provide information on metabolic activity in liver. In present study, significant lower RCF and HSI are observed in the 2,4-DNT-treated group (E2, E3, E4 group) (Figs. 1 and 2).

4.2. Different activity of EROD, GST, Na^+/K^+-ATPase, and GK in response to 2,4-DNT

4.2.1. EROD and GST

Hepatic ethoxyresorufin-O-deethylase (EROD), a widely used biomarker, is a highly sensitive indicator of contaminant uptake in fish, providing evidence of receptor-mediated induction of cytochrome P450-dependent mono-oxygenases (the CYP1A subfamily specifically) by xenobiotic chemicals [53]. The cytochromes P450 (CYPs) are heme-thiolate proteins that are involved in the synthesis and/or degradation of many endogenous compounds. It is well recognized that, among the cytochromes P450, cytochrome P4501A and its associated enzyme EROD plays an important role in the bioactivation [54]. Although the expression of CYP1A is constitutively low, it is highly inducible in animal tissue by environmental contaminants, and has become an established biomarker for environmental pollution. In the previous work, hepatic EROD activity has been reported to be induced in fish by exposure to environmental organic pollutants such as polychlorinated biphenyls (PCBs), dioxins/dibenzofurans (PCDDs/PCDFs) [55]. But it was not known whether this detectable induction of EROD activity resulted from 2,4-DNT water exposure of common carp in laboratory condition. Moreover, it appears to be more important to measure induction at a sublethal level of 2,4-DNT exposure, in order to provide early warning and avoid complications and interferences of this compound under lethal concentrations. In this study, experiment data show that 2,4-DNT had the ability to activate EROD expression, and enzyme activity levels of EROD induction do not exceed 3-fold of control at 0.1, 0.5 and 1.0 mg/L 2,4-DNT stress condition, indicating that 2,4-DNT had toxic effects following binding to the aryl hydrocarbon receptor (AhR) involved in the activation of this detoxifying enzyme [53]. We can conclude that 2,4-DNT is catalyzed by CYP1A enzyme activity, EROD activity level in liver continued to increase after exposure to strong 2,4-DNT concentration. A clear pollution-related inductive response of EROD activities was observed in liver of common carp. The CYP1A-catalyzed activity of 7-ethoxyresorufin O-deethylase (EROD), traditionally measured in fish liver, is one of the major parameters used to monitor aquatic environment [55].

Glutathione S-transferase (GST) catalyses the conjugation of reduced glutathione to electrophilic centers on a wide variety of substrates and is therefore important in the detoxification of endogenous compounds, as well as the metabolism of xenobiotics [56]. In mammals GST activity expression of Phase II enzyme are often used serve as an indication to additional cytotoxicity from xenobiotics. However, Phase II enzymes in fishes have not been characterized nearly as extensively as cytochrome P450s [34]. Many Phase I enzymes which oxidize environmental procarcinogens to more hydrophilic metabolites, and Phase II enzymes which catalyze the conjugation of electrophilic metabolites with carrier molecules such as glutathione and glucuronic acid, are conserved between fish and mammals. In this study, the inductive response of EROD activities could indicate that the biotransformation of Phase I is affected in the 2,4-DNT exposed fish, whereas activities of GST of Phase II enzymes were inhibited in the liver of 2,4-DNT exposed common carp. Studies of GST expression in fish have provided mixed results (elevation or repression of GST activity levels) in terms of tissue inducibility in response to contaminant exposure [57]. In present experiment, GST is significantly inhibited at 0.5 and 1.0 mg/L 2,4-DNT stress (E3, E4 group) and it is possible that the normal redox state is disturbed resulting in oxidative stress through the production of peroxides and free radicals [58]. Similarly, no induction of hepatic GST activity was observed in English sole treated with B[a]P, Aroclor 1254, or an organic extract from contaminated Puget Sound sediments [57]. Nor was GST activity induced in rainbow trout or Atlantic cod (*Gadus morhua*) treated with a relatively high dose of 2,3,7,8-TCDD [57]. We can conclude that 2,4-DNT is not catalyzed by GST enzyme activity, the inhibition of GST activities could indicate that the biotransformation and metabolism of Phase II is affected in the 2,4-DNT exposed fish.

4.2.2. GK and ATPase

Hepatic glucokinase (GK, EC 2.7.1.2) is key enzymes playing major roles in the regulation of glycolytic and gluconeogenic pathways [59]. GK catalyses the phosphorylation of glucose to glucose-6-phosphate and thus plays a key role in hepatic glucose utilization [60]. In this study, experiment results demonstrated that 2,4-DNT exposure rendered declining enzyme activity levels on GK in liver, and that enzyme activity of GK continued to decrease after exposure to strong 2,4-DNT concentrations. The maximum repression of GK activity in liver of treated *C. carpio* was recorded (Table 1 and Fig. 3), and the strongest inhibition percent (83.3%) for GK was found at the highest treatment group (1.0 mg/L). Moreover, the glycogen content was significantly lower ($p < 0.05$, $p < 0.01$) in juvenile carp exposed to 1.0 mg/L than in unexposed fish, indicating metabolic stress effect. The lower HSI in the exposed fish could be related to the lower glycogen content. These effects might be due to the high energetic cost of the toxicant exposure, together with the possible reduction of dietary energy supplies [34]. GK is the first step of both glycogen synthesis and glycolysis and plays for this reason a vital role in glucose uptake by the liver and in modulating the expression of an entire network of hepatic glucose-responsive genes [60]. As in mammals, an increase of hepatic GK activity with the increase of dietary carbohydrate was reported in several fish species [61–63]. Although this GK parameter study was not previously reported as indicator of stress in 2,4-DNT exposure, this assay was a sensitive measure of nitrotoluene-induced stress in 7 d and 15 d exposures. According to results of the present study, the glycogen content and GK activity measured in liver exposed to 2,4-DNT could provide useful information for evaluating the hepatic glucose utilization and stress effect on common carp.

Data reported indicated that ATPase (adenosine triphosphohydrolase, EC 3.6.1.3) system, a environmental biomarker, could be used as a target for the toxic action of a variety of toxicants to organism [64,65]. ATPase is the protein inlaid on membrane. Active center of enzyme is ASP (aspartic acid), whose side chain has a nucleophilic effect on ATP, making it phosphorylated. In this study, activity of Na^+/K^+-ATPase (E2, E3, E4 group) was significantly lower ($p < 0.05$, $p < 0.01$) than control group after 7 d and 15 d exposure to 2,4-DNT, indicating that 2,4-DNT affects the oxidative phosphorylation. Hall and Kier pointed out the nitro-group of nitroaromatics had two biochemical reactions in the cells of target organs in fish [66]. The density of the electron cloud of the benzene cycle may be decreased by nitro-group electrons, or the nitro-group runs away because other groups activate it [23]. We suppose that the NO_2 staying with CH_3 and NO_2 in 2,4-DNT may attract electro cloud on benzene cycle. The positive part of benzene cycle attracts the carboxy group of active center of enzyme. This additive reaction inhibited nucleophilic effect. The above mentioned studies demonstrated inhibition of 2,4-DNT on Na^+/K^+-ATPase, indicating that 2,4-DNT affects the oxidative phosphorylation although the physiological relevance could not be determined. However, currently

available data is insufficient to unambiguously identify whether these effects are caused by energy depletion of this chemical.

4.3. Energy reserves (glycogen, protein and lipid)

Organisms stressed by toxicant exposure have less energy available for physiological activity, and therefore indications of trade-offs between the metabolic cost of toxicant exposure and processes vital to the survival of the organism were seen [34]. In absence of compensatory processes, stressful environmental conditions would be expected to negatively impact growth, energy storage and reproduction [33]. Principle of energy allocation and cost of tolerance [67] on heavy-metal exposure have been reported in common carp (*C. carpio*) [68], crayfish (*Procambarus acutus*) [33], Diptera (*Chironomus riparius*) [69], and plant (*Agrostis capillaris*) [70].

Before changes in condition occur, changes in biochemical composition should become apparent. The use of energy stores (glycogen and fat) might be initiated and protein synthesis might decrease [68]. In this study, these indications on the enzyme activity level as well as on higher levels of biological organization were found after 2,4-DNT exposure. The RFC dropped significantly in all fish exposed to 2,4-DNT (E2, E3, E4 group) after 15 d. The available energy reserves (glycogen and fat) followed the same trend as the RCF at day 15. Moreover, experiment results demonstrated 2,4-DNT exposure rendered declining enzyme activity levels on GK in liver. At enzyme activity level, as well as at higher levels of biological organization, there is strong evidence of inhibition of glycogen synthesis and an increase in glycogen breakdown as a result of 2,4-DNT exposure. This effect could be explained by the high energetic cost of the toxicant exposure, together with the possible reduction of dietary energy supplies. In all organisms, energy is used in processes for standard maintenance before it is allocated to other processes [33]. Whereas standard maintenance is an obligatory cost, other processes, such as growth and reproduction, can be suppressed without compromising survival [68].

As we know, an inhibition of any step in the oxidative phosphorylation could result in mitochondrial dysfunction and a lack of ATP generation. Thus, energy under the form of ATP is reduced while the toxicant exposure demands more energy [34]. Reaction of the body by breaking glycogen reserves is a consequence to fulfill the high energetic needs. 2,4-DNT causes inhibition of Na^+/K^+-ATPase, GK and energy reserves depletion, energy under the form of ATP and glycogen production is possibly reduced. Since RFC reflects the well-being of fish, a decline in RFC is directly linked with a decrease in energetic reserves of fish. The decline in RCF can be caused by a reduced food intake and increased metabolic expenditure for detoxification and maintenance of the normal body functions [33]. To fulfill the energetic needs for processes vital to the survival of the organism, a serious glycogen breakdown of the body can be observed. A 15-d exposure of fish to 2,4-DNT has a remarkable effect on the fitness of the fish.

5. Conclusions

Based on the results, there was no significant change in all parameters measured in *C. carpio* exposed to 2,4-DNT at environmental related concentration, but 2,4-DNT-stress effect in common carp exposed to higher concentrations reflected the significant changes of physiological and biochemical responses. The focus of the present study is on the enzyme molecular targets of 2,4-DNT underlying the effects on higher levels of biological organization. The pattern of EROD, GST, Na^+/K^+-ATPase, and GK enzyme expression in liver of common carp may have a close relationship with the physiological tolerance and stress response to 2,4-DNT. The affected enzymes were mainly involved in energy metabolism, biotransformation and stress response. In the perspective of this so-called metabolic cost hypothesis, relative condition factor (RCF) and hepatosomatic index (HSI) as well as the available energy reserves of common carp (*C. carpio*) by 2,4-DNT exposure were determined and linked to effects of enzymes activity expression in liver tissues. Our results support the prediction that increases in energy expenditure negatively affects growth process which is vital to the survival of an organism.

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