

## Alteration of parameters of energy metabolism and ATPase enzymatic system in juvenile common carp (*Cyprinus carpio*) chronically exposed to tributyltin

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**ABSTRACT:** The effect of long-term exposure to tributyltin (TBT) on energy metabolism and adenosine triphosphatase (ATPase) enzymatic system in freshwater teleost was investigated. The impact on the parameters of energy metabolism (the content of glucose (Glu) and lactate (LA), and the activities of hexokinase (HK), pyruvate kinase (PK), and lactate dehydrogenase (LDH)) in the muscle tissue, as well as on the ATP enzymatic system (ATP content, Total-ATPase, Na<sup>+</sup>-K<sup>+</sup>-ATPase, and Ca<sup>2+</sup>-Mg<sup>2+</sup>-ATPase) in the gill tissue of common carp was evaluated. Fish were exposed to sublethal concentrations of TBT (75, 0.75, and 7.5 µg/l) for 60 days. Based on the results, long-term exposure to TBT could lead to obvious ATP enzymatic system responses, including the decreased ATP content and Na<sup>+</sup>-K<sup>+</sup>-ATPase activity, and the increased activities of Total-ATPase and Ca<sup>2+</sup>-Mg<sup>2+</sup>-ATPase. Moreover, the parameters of energy metabolism in muscle were also regulated, such as induced indices of the levels of Glu and LA, and the activities of HK and PK, and inhibited indices of LDH activity. Shortly, the measured physiological responses in fish could provide useful information to better understand the mechanisms of TBT-induced bio-toxicity, and could be used as potential biomarkers for monitoring the TBT pollution in the field.

**Keywords:** organotin; fish; physiological indices; chronic toxicity; biomarkers

### INTRODUCTION

Tributyltin (TBT) is used as a biocide in a variety of consumer and industrial products and it enters the aquatic environment mainly via leaching from antifouling paints. The use of TBT as antifouling

agent in paint has been banned for small boats and fishing nets in most countries as it was shown to be toxic to aquatic life and as an endocrine disrupting chemical that causes severe reproductive effects in aquatic organisms (Zhang et al. 2008; Li et al. 2015). But TBT and its metabolites were still

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detectable in many regions. And, it was reported that butyltins, including TBT, were even detected in human blood at concentrations ranging between 50 and 400 nM (Whalen et al. 1999).

Environmental contaminants inducing physiological regulations in organisms are being used as diagnostic tools in assessing potential biological impacts in aquatic systems (Brain and Cedergreen 2009; Li et al. 2011a, b), because they offer a rapid and positive means of monitoring. Fish readily take up lipophilic organic contaminants and possess a variety of cellular mechanisms for protection against the toxic effects of such chemicals (Santos et al. 2010). These processes are energy-demanding and, in theory, should also affect intermediary metabolism of the fish. To supply the energy demand for detoxification and repair processes in fish exposed to environmental contaminants, glucose (Glu) and/or lactate (LA) is mobilized in the fish tissues (Carvalho Cdos and Fernandes 2008). Inside the cells, each metabolic pathway is continuously regulated in order to maintain homeostasis and, in general, few key enzymes control the metabolic flux, such as hexokinase (HK) is at the beginning of the glycolytic sequence and the pyruvate kinase (PK) and lactate dehydrogenase (LDH) are at the terminal sequence of the glycolytic pathway (Polakof et al. 2007b). But there are few studies addressing the impact of TBT on energy metabolism in fish.

Adenosine triphosphatases (ATPases), which are integral parts of active transport mechanisms for cations across the cell membrane, are always influenced by aquatic environmental stress (Li et al. 2009). ATPase can hydrolyze the terminal pyrophosphate bond of ATP to provide the energy for ion-bump to drive the membrane transport of mono- and divalent ions (Cotou et al. 2001). ATPases are responsible for a large part of basic metabolic and physiological activities, which can be taken as a meaningful indicator of cellular activity and forms a useful toxicological tool (Uner et al. 2005; Li et al. 2011c). However, studies of TBT effects on ATP enzymatic system at sub-organism levels are limited.

Therefore, the specific objective of the present study was to examine whether or how TBT treatment affects energy metabolism and ATP enzymatic system in fish, using juvenile common carp (*Cyprinus carpio*) as a model. In this context, this study investigated the effect of TBT on the param-

eters of energy metabolism (Glu, LA, LDH, HK, and PK) in muscle tissue, as well as on the ATP enzymatic system (ATP content, Total-ATPase,  $\text{Na}^+$ - $\text{K}^+$ -ATPase, and  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -ATPase) in gill tissue of fish exposed to TBT for 60 days.

## MATERIAL AND METHODS

**Chemicals.** TBT (90%) was purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). Suitable amounts of this compound were directly weighed into a brown bottle and dissolved in 50 ml acetone (ACT)-water (1 : 1) to form a concentration level stability. This stock solution was sealed and stored at 4°C until used. Working standard solution (100 µg/ml) was freshly prepared by diluting the stock solution with deionized water before use.

**Fish.** Juvenile common carp ( $9.65 \pm 0.13$  cm,  $22 \pm 1.8$  g, 4 months after hatching) were obtained from a local hatchery (Jingzhou, China) and were raised in a flow-through system with dechlorinated tap water (pH  $7.4 \pm 0.2$ ; hardness  $42.5 \pm 1.3$   $\text{CaCO}_3/\text{l}$ ) at a constant temperature ( $20 \pm 1^\circ\text{C}$ ) with a photoperiod of 12 h light : 12 h darkness. Fish were acclimatized for 14 days before the beginning of the experiment and fed commercial fish food (Tongwei, China). Waste and residue were removed daily and the test equipment and chambers (100 l) were cleaned once a week. Fish were fasted for 24 h prior to experimentation to avoid prandial effects during the assay. All procedures and animal handling were in accordance with the guidelines approved by the Chinese Association for Laboratory Animal Sciences. The study was approved by the animal ethics committee of the Yangtze River Fisheries Research Institute, Chinese Academy of Fishery Sciences.

**Exposure to TBT.** A 100 l semi-static system was used, in which 20 juvenile common carp were randomly distributed to each of ten aquaria. The nominal concentrations of TBT used were 75 ng/l (E1 group, according to environmental concentration), 0.75 µg/l (E2 group, 1% 96 h-LC50 (lethal concentration after 16 h)), and 7.5 µg/l (E3 group, 10% 96 h-LC50). Based on the unpublished data of our studies, the 96 h-LC50 of TBT for common carp is 75 µg/l. TBT was dissolved in ACT with a final concentration lower than 0.01%. Two other groups were used for comparisons: a control group exposed to clean freshwater and an ACT group exposed to the volume of ACT (v/v, 0.01%) used for the highest TBT concentration. Each experi-

mental 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. 80% of the exposed solution was renewed each day after 2 h of feeding to maintain the appropriate concentration of TBT and ACT and to maintain water quality. The test equipment was cleaned every 7 days. The test fish were exposed to TBT for 60 days. At the end of exposure period, three randomly selected fish from each aquarium were sedated. The tissues (gill and muscle) were quickly removed on ice, immediately frozen, and stored at  $-80^{\circ}\text{C}$  until analysis.

To ensure the agreement between nominal and actual compound concentrations in the aquaria, water samples were analyzed during the experimental period by liquid chromatograph-mass spectrometer/mass spectrometer. Water samples were collected from the test aquaria after 1 h and 24 h of renewing the test solutions. The mean concentration of TBT in the water samples was always within 20% of the intended concentration.

**Biochemical parameters measurement.** Frozen samples for the analysis of enzyme activities were defrosted and homogenized on ice with 10 volumes of cold 0.86% physiological saline. The gill homogenate was centrifuged at 3000 rpm at  $4^{\circ}\text{C}$  for 10 min, and the supernatant was used to evaluate the enzyme activity of Total ATPase,  $\text{Na}^{+}\text{-K}^{+}\text{-ATPase}$ ,  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$ , and ATP content, and the supernatant of muscle homogenate was used to measure the levels of glucose (Glu) and lactate (LA), as well as the activities of hexokinase (HK), pyruvate kinase (PK), and lactate dehydrogenase (LDH). All of the biochemical parameters were measured using commercial kits (Nanjing Jiancheng

Bioengineering Institute, Nanjing, China). Protein concentrations in the supernatants were determined using Bradford's procedure with bovine serum albumin as the standard (Bradford 1976).

**Statistical analysis.** All values were expressed as the mean  $\pm$  SD and analyzed by SPSS software for Windows 13.0. Analysis of variance (one-way ANOVA), followed by Tukey's HSD test when significant differences were found, was performed to determine the effect of TBT concentration on individual parameters. In addition, principal component analysis (PCA) to define the most important parameters usable as key factors for individual variations was conducted using STATISTICA 6.0.

## RESULTS

The parameters of energy metabolism in muscle tissue of common carp are summarized in Table 1. There was no significant change in Glu and LA levels in fish tissues in E1 group when compared with the control. However, with TBT concentrations increasing, Glu content was evaluated as significant ( $P < 0.05$ ) in E2 and E3 groups, but a significantly high La level was found only in E3 group. In muscle tissue, activities of HK and PK increased slightly in E1 group, and were significantly elevated in higher concentrations of TBT treatment groups (E2 and/or E3). While, compared with control, the activities of LDH were significantly inhibited in muscle of fish under higher TBT concentrations (E2 and E3 groups).

Indices of ATP enzymatic system determined in gill tissue of fish are shown in Table 2. The activities of Total ATPase and  $\text{Ca}^{2+}\text{-Mg}^{2+}\text{-ATPase}$  were increased in all TBT treated groups, and

Table 1. Parameters of energy metabolism in muscle of common carp after chronic exposure to tributyltin

Indices	Control	ACT	E1	E2	E3
Glu (mmol/l)	$30.12 \pm 4.11$	$33.75 \pm 3.52$	$38.92 \pm 5.05$	$43.87 \pm 4.77^*$	$48.26 \pm 5.34^*$
LA (mmol/g prot)	$1.09 \pm 0.16$	$1.03 \pm 0.13$	$1.16 \pm 0.21$	$1.29 \pm 0.14$	$1.37 \pm 0.11^*$
LDH (U/mg prot)	$2.12 \pm 0.28$	$2.20 \pm 0.32$	$1.97 \pm 0.21$	$1.84 \pm 0.12^*$	$1.59 \pm 0.13^*$
HK (U/mg prot)	$231.35 \pm 35.14$	$210.33 \pm 19.02$	$251.37 \pm 30.15$	$328.29 \pm 24.03^*$	$445.24 \pm 41.92^{**}$
PK (U/mg prot)	$91.73 \pm 10.25$	$89.53 \pm 9.31$	$95.74 \pm 8.26$	$101.39 \pm 12.18$	$141.22 \pm 13.10^*$

Glu = glucose, LA = lactic acid, LDH = lactic acid dehydrogenase, HK = hexokinase, PK = pyruvate kinase, ACT = acetone, prot = protein

groups: E1 = 75 ng/l, E2 = 0.75  $\mu\text{g/l}$ , E3 = 7.5  $\mu\text{g/l}$

data are means  $\pm$  SD,  $n = 6$

significant differences compared with control value  $^*P < 0.05$ ,  $^{**}P < 0.01$

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Table 2. Adenosine triphosphatase (ATPase) enzymatic system in gill of common carp after chronic exposure to tributyltin

Indices	Control	ACT	E1	E2	E3
ATP content ( $\mu\text{mol/g prot}$ )	242.72 $\pm$ 32.74	233.17 $\pm$ 19.89	212.75 $\pm$ 31.57	185.83 $\pm$ 16.09*	78.57 $\pm$ 10.87**
Total ATPase activity (U/mg prot)	3.42 $\pm$ 0.41	3.37 $\pm$ 0.32	4.02 $\pm$ 0.50	4.38 $\pm$ 0.37*	5.24 $\pm$ 0.53*
Na <sup>+</sup> -K <sup>+</sup> -ATPase activity (U/mg prot)	6.65 $\pm$ 1.04	6.33 $\pm$ 0.85	6.97 $\pm$ 1.12	6.29 $\pm$ 0.92	5.24 $\pm$ 0.74*
Ca <sup>2+</sup> -Mg <sup>2+</sup> -ATPase activity (U/mg prot)	3.73 $\pm$ 0.46	3.53 $\pm$ 0.29	4.74 $\pm$ 0.71	6.39 $\pm$ 0.94*	6.87 $\pm$ 0.53**

ACT = acetone, prot = protein

groups: E1 = 75 ng/l, E2 = 0.75  $\mu\text{g/l}$ , E3 = 7.5  $\mu\text{g/l}$ data are means  $\pm$  SD,  $n = 6$ significant differences compared with control value \* $P < 0.05$ , \*\* $P < 0.01$ 

were significantly induced in E2 and E3 groups. The activity of Na<sup>+</sup>-K<sup>+</sup>-ATPase was markedly inhibited in higher TBT treated groups, although it was slightly increasing in E1 group. Additionally, a statistically significant decrease in ATP content was noticed in E2 and E3 groups, as compared to the lowest concentration in TBT.

According to the present results, the parameters of energy metabolism and ATP enzymatic system were altered in fish after long term exposure to TBT (Figure 1). Thus, 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 the present study, a data matrix was constructed with 9 analyzed biomarkers as independent variables and 30 sampled individuals as group variables. TBT concentration

and all of the parameters measured in the present study were distinguished on the ordination plots corresponding to the first (93.51%) and second (5.12%) principle components (Figure 2), which showed the correlations of all the biomarkers. Moreover, the individuals in the same area had the similar biochemical responses in fish brain in this study. Additionally, the observed correlations between the TBT concentrations and the parameters were confirmed and quantified by Spearman's test (Table 3).

## DISCUSSION

In fish there is an increase in energy demand as a result of stress situations (Santos et al. 2010), but most data are obtained from acute stress situations. Acute stress is often measured as a transient change

Table 3. Correlation coefficients among tributyltin (TBT) and biochemical parameters measured in tested fish according to Spearman's test

	TBT	ATP content	Total-ATPase	Na <sup>+</sup> -K <sup>+</sup> -ATPase	Ca <sup>2+</sup> -Mg <sup>2+</sup> -ATPase	Glu	LA	LDH	HK	PK
TBT	1.00									
ATP content	-0.97	1.00								
Total-ATPase	0.89	-0.97	1.00							
Na/K-ATPase	-0.95	0.94	-0.88	1.00						
Ca/Mg-ATPase	0.73	-0.86	0.93	-0.83	1.00					
Glu	0.81	-0.89	0.92	-0.73	0.83	1.00				
LA	0.82	-0.93	0.98	-0.86	0.98	0.93	1.00			
LDH	-0.90	0.98	-0.99	0.91	-0.93	-0.92	-0.98	1.00		
HK	0.94	-0.98	0.97	-0.97	0.92	0.91	0.96	-0.98	1.00	
PK	0.99	-0.98	0.93	-0.96	0.79	0.84	0.86	-0.94	0.96	1.00

Glu = glucose, LA = lactic acid, LDH = lactic acid dehydrogenase, HK = hexokinase, PK = pyruvate kinase, ATPase = adenosine triphosphatase



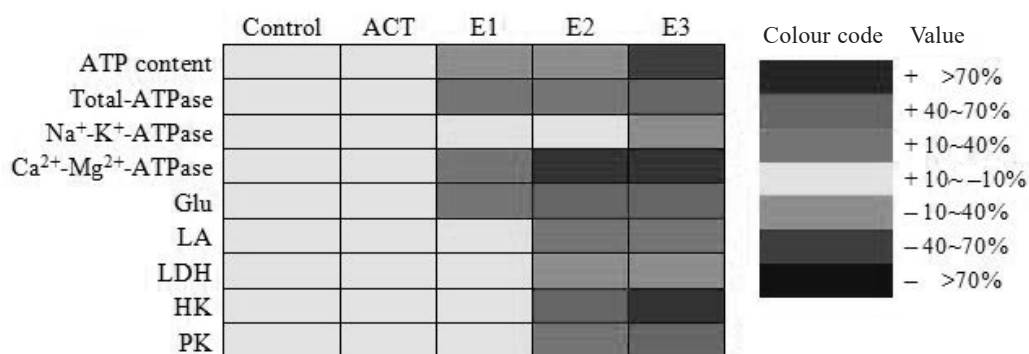


Figure 1. Heat map of changing levels of energy metabolism and adenosine triphosphatase (ATPase) enzymatic system parameters in juvenile common carp chronically exposed to tributyltin

Glu = glucose, LA = lactic acid, LDH = lactic acid dehydrogenase, HK = hexokinase, PK = pyruvate kinase, ACT = acetone groups: E1 = 75 ng/l, E2 = 0.75 µg/l, E3 = 7.5 µg/l

in metabolic activities in an attempt to counteract the stress event (Ruane et al. 2001; Xu et al. 2006). However assessing chronic stress in fish presents more challenges than assessing acute stress. Changes observed in glucose and lactate levels in muscle support an enhanced availability of fuels in TBT exposed fish (Tintos et al. 2007). The increase in glucose and lactate is in agreement with the general metabolic stress response observed in other studies after exposure to contaminants (Pacheco and Santos 2001; Teles et al. 2005), although there are no studies carried out with TBT. The capacity for using exogenous glucose appears to increase due to TBT treatment, based on increased HK activities in fish (Tintos et al. 2007). The increase in the activity of HK occurred with the increased levels

of glucose in muscle. Altogether, these metabolic changes suggest that TBT exposure induces an enhanced use of glucose in the muscle either from glycogen stores or from the blood stream (Carvalho Cdos and Fernandes 2008). Besides, the enhanced availability of glucose in TBT exposed fish appears to be used *in situ* through glycolysis, since increased PK activity was observed (Vijayan et al. 1997). In addition, a decrease was also noticed in LDH activity in fish of TBT treated groups suggesting a decreased importance of lactate oxidation in fish muscle, which can be related to the lower levels of lactate recorded in plasma simultaneously (Polakof et al. 2007a, b).

The prominent effects on the ATP enzymatic system reveal influences on the stability and per-

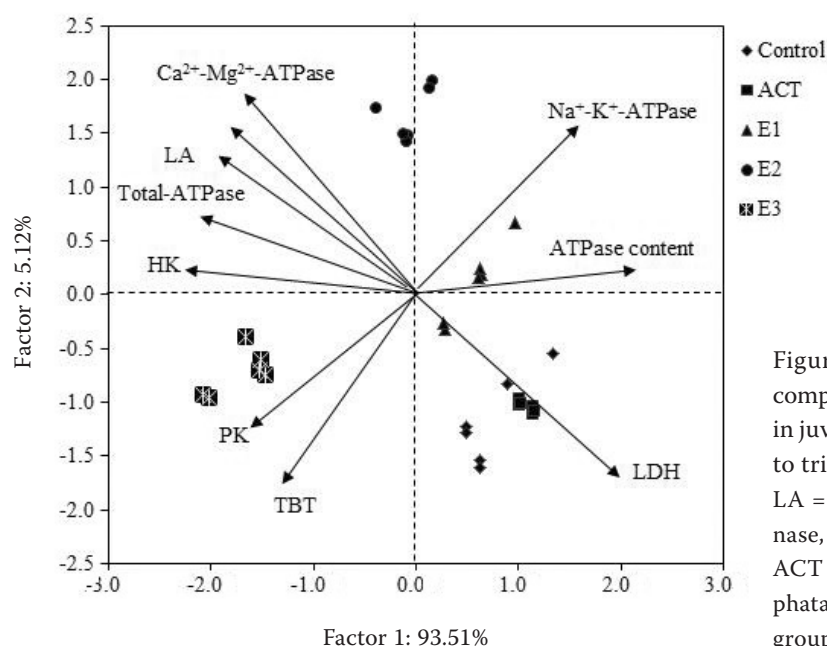


Figure 2. Ordination diagram of principal component analysis of biochemical parameters in juvenile common carp chronically exposed to tributyltin (TBT)

LA = lactic acid, LDH = lactic acid dehydrogenase, HK = hexokinase, PK = pyruvate kinase, ACT = acetone, ATPase = adenosine triphosphatase

groups: E1 = 75 ng/l, E2 = 0.75 µg/l, E3 = 7.5 µg/l

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meability of the cellular membranes leading to the loss of osmoregulation (Cotou et al. 2001). In the present study, fish had the ability of active ion transport and ion regulation, which was confirmed by the increasing ATPase activity. The simultaneous decrease of the ATP content levels is another confirmation that excessive energy was consumed in fish under TBT-induced stress. The inhibited  $\text{Na}^+$ - $\text{K}^+$ -ATPase activity reveals disturbances of the cellular membranes function, such as loss of ion regulation, which may lead to osmoregulatory failure (Augenfeld 1969). The  $\text{Na}^+$ - $\text{K}^+$ -ATPase is generally accepted as a membrane pump related with ion transport of  $\text{Na}^+/\text{K}^+$  across the cell membrane and osmoregulation (Li et al. 2009, 2011c).  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -ATPase is always associated with both the oxidative phosphorylation and  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ion transport across the cell membrane (Isaia and Masoni 1976; Cotou et al. 2001). The increased activity of  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -ATPase suggests that the  $\text{Ca}^{2+}/\text{Mg}^{2+}$  ion transport rates are enhanced implying alterations in cellular membrane stability and permeability. Moreover, the enhancement of  $\text{Ca}^{2+}/\text{Mg}^{2+}$  activity may represent a rise in energy expenditure and metabolism of the organism because of the increased rate of ATP hydrolysis during oxidative phosphorylation and therefore respiration (Ulrich 1963). In line with previous studies (Cotou et al. 2001; Carageorgiou et al. 2004), the increased  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -ATPase activity in our study may represent a compensatory physiological mechanism operating to maintain haemolymph ion concentrations and osmoregulatory function in fish exposed to TBT.

## CONCLUSION

In summary, the present study has demonstrated that TBT altered the parameters of energy metabolism and ATPase enzymatic system in juvenile common carp. The regulated energy metabolism proceeding indicated the energy demands for detoxification and repair processes in fish exposed to TBT. Based on the results, the inhibition of  $\text{Na}^+$ - $\text{K}^+$ -ATPase and the stimulation of  $\text{Ca}^{2+}$ - $\text{Mg}^{2+}$ -ATPase activities were observed in the present study, which could be used to indicate toxic stress of TBT. Thus, although the present findings may represent some new aspects of TBT toxicity, more detailed studies need to be done to fully understand the molecular mechanisms in future.

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