Effects of Sublethal Levels of Tributyltin Chloride in a New Toxicity Test Organism: The Chinese Rare Minnow (*Gobiocypris rarus*)

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Abstract. A newly developed toxicity test organism, the Chinese rare minnow (*Gobiocypris rarus*), which has similar merits to some common experimental fishes and could become a standardized test species for China, was used in a short-term study with emphasis on accumulation in fish muscles and cytological effects of sublethal concentrations of tri-n-butyltin chloride (TBT). The 1-year-old fish were exposed for 1 or 2 weeks to a concentration range of 50 to 5,000 ng TBT/L in static systems. Hepatosomatic Index (HSI) and Gonad Somatic Index (GSI) sensitively showed the adverse effects of TBT to the fish. Compared with the nominal TBT concentrations in water phase, 459–(5,000 ng TBT/L, 1 week) to 4,065–(50 ng TBT/L, 2 weeks)-fold higher concentrations of butyltin species were detected in the corresponding exposure fish muscle. Contaminations of TBT in muscle tissue increased with both the exposure levels and exposure time. The cellular pathological effects in the liver were studied. These included vacuoles with increasing number and size, swelling of mitochondria, abnormal nuclei, and decreases of rough endoplasmic reticulum cisternae. The results confirmed that the Chinese rare minnow (*G. rarus*) could be utilized as a useful species to evaluate water toxicity in the laboratory.

It is well known that tri-n-butyltin chloride (TBT) has been widely used as a biocide, especially as a component in anti-fouling paints for ships and for net cages in aquaculture (Piver 1973). Ablation and flaking off of these paints from ship hulls and factors like absorption by organic and inorganic particles with subsequent deposition in the sediment will contribute to environmental contamination by tributyltin and its derivatives. The potential for contamination would be especially high in areas of heavy boating and shipping traffic (Maguire *et al.* 1986). Bioconcentration factors ranging from 10^2 to 10^4 have been determined for various aquatic species, including fish, crustaceans, and algae (Evans and Laughlin 1984; Ward *et al.* 1981; Maguire *et al.* 1984). Levels that can be encountered in harbors and marinas, especially in sediment and the surface

microlayer (Maguire *et al.* 1986; Maguire and Tkacz 1987), have shown toxic effects in both short-term and long-term laboratory studies (Hall and Pinkney 1985; Cardwell and Sheldon 1986). Mortality has been reported in the salmon aquaculture industry after treatment of net pens with tributyltins (Short and Thrower 1986).

Laboratory studies on the long-term and short-term toxic action of tributyltin compounds have been conducted with a variety of organisms and in a number of target organs. Fish species are at the top position in the aquatic food chain and may directly affect the health of humans, as demonstrated by the Minimata episode in Japan (Kudo and Turner 1999). Many studies have focused on the toxicity effects of TBT on several kinds of experimental fish, such as guppy (*Poecilia reticulata*) (Wester and Canton 1987; Wester *et al.* 1990), medaka (*Oryzias latipes*) (Wester *et al.* 1990), fathead minnow (*Pimephales promelas*) (Alfred *et al.* 1988), and rainbow trout (*Oncorhynchus mykiss*) (Schwaiger *et al.* 1992).

A newly developed toxicity test organism, Chinese rare minnow, *Gobiocypris rarus*, has only been found in Sichuan Province in China until now, and it is usually common in small water systems such as paddyfields, ditches, and loblollies, especially in weedy ditches with small flows of water. It moves about frequently before and after rain. When a cold wave comes or water temperature drops, it always accumulates in hassock or stone gaps. The Chinese rare minnow is small in body size, which has a geographical range from 4.0 to 8.3 cm. It is easy to rear, stable to physical changes, and still quite sensitive to external chemicals. The period of its sexual development is usually shorter than 4 months, and it shows continuous batch spawning, about every 2–6 days. In laboratory, it can be raised in any type of fish jars without running water, aquatic grass, or any other equipment. It is a typical eurythermal fish and can live freely in the range of 0–36°C. Moreover, it can tolerate oxygen concentration low to 0.50 mg/L and carbon dioxide concentration as high as 70 mg/L (25°C), which benefits its transport and breeding. These merits were found to be similar to some of the aforementioned common experimental fishes used worldwide, which enable them to be an excellent kind of model fish (Wang and Cao 1997). To date, Chinese rare minnow was successfully utilized in several studies (Wang *et al.* 1993, 1994; Wang 1995). However, there have been no

reports concerned with its application in organotin toxicology research.

The present study dealt with the effects of TBT in the Chinese rare minnow after 1- and 2-week exposures to the toxicant. These responses are especially of interest as one of our projects is to investigate the usefulness of this small fish species in water toxicity studies. Hepatosomatic Index (HSI) and Gonad Somatic Index (GSI) were determined to evaluate the adverse effects of TBT to this kind of fish. The quantification of butyltin species' accumulation in fish muscle and the evaluation of cellular pathological effects under sublethal concentrations of TBT (50, 500, and 5,000 ng/L as compound) were the goals of this study.

Materials and Methods

Chemicals

TBT, 90%, was obtained from Acros Organics (NJ). Suitable amount of this compound was directly weighed into a brown volume vessel and dissolved in 25 ml acetone-water (1:1) to form a concentration level of 1 mg TBT/ml as the stock solution, wherein 25 μ l of concentrated HCl was added to ensure its stability. This stock solution was sealed and kept at 4°C in a refrigerator until used. Working standard solution (50 μ g/ml) was freshly prepared by diluting the stock solution with deionized water before use.

Test Materials

The test fish, Chinese rare minnow (*Gobiocypris rarus*) of 1-year-old were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan. The mean body length and mean body weight were 4.2 ± 0.2 cm and 1.5 ± 0.3 g, respectively. They were tamed for 1 week before being subjected to the experiment. These test fish were kept under standardized and disease and parasite controlled conditions and fed a commercial ration of eelworms twice a day. Water quality parameters were regularly checked for pH (6.9–7.9), dissolved oxygen concentration (5–7 mg/L), hardness as CaCO_3 (200 mg/L), and conductivity (650 $\mu\text{S}/\text{cm}$); the temperature was maintained at 24.2–27.1°C and the light/dark cycle was 12/12h.

Experimental Design

To observe cytological effects in the liver and evaluate the accumulation of tributyltin and its metabolites in the organisms, 20 test fish of both sexes per treatment were exposed to 50, 500, or 5,000 ng TBT/L over 1 or 2 weeks. Another 10 test fish were raised in clean water for 2 weeks as the control group.

In the previous reported study, TBT appeared to be stable in that the recoveries were 87% and higher during the 48-h cycle under the experimental conditions (Wester and Canton 1987). Regarding these high recoveries, the nominal values were used in this paper.

The exposure system was prepared by diluting appropriate quantities of standard solution into tap water that was preblown with air to dechlorinate for at least 24 h. The volume of each exposure system was adjusted so that the culture density was at 1 g fish/L. One-third of the exposure solution in each vessel was changed daily with newly prepared solution. This procedure helped maintain good water quality in the test vessels and provided relative more static test concentrations for

uninterrupted periods. All fish were kept under identical conditions for the duration of each test.

Daily observations were made for mortality, behavior, and general appearance. After 1 or 2 weeks of exposure, 10 fish of both sexes from each group were sampled, blotted dry with filter paper, and weighed. The liver and gonad were weighed immediately after the dissection for the calculation of the HSI and GSI, respectively. In addition, the liver of each fish was dissected and subjected to cytological analysis. Muscle tissue of the fish was kept frozen at -20°C until the chemical analysis for TBT.

Butyltin Species Analysis

Analyse for tri-, di-, and monobutyltin (TBT, DBT, and MBT) were carried out with 2-g (wet weight) homogenized muscle tissue samples from five fish in each test group. The technique applied here used *n*-hexane containing 0.01% tropolone extraction to concentrate the butyltins, which were then derivatized with Grignard reagent (*n*-propylmagnesium bromide) to form propylbutyltins. The samples were then cleaned up on florisil columns and reduced in volume under a gentle N_2 flow. The analysis was carried out by gas chromatography with a laboratory-made flame photometric detector using quartz surface-induced tin emission (Jiang *et al.* 1996; Jiang and Xu 1996). A sample blank using the control fish was processed to ensure against background contamination at the low concentration during these experiments. Butyltin concentrations were calculated by peak height response relative to that of the internal standard, tetrabutyltin. The reliability of this method was confirmed by the determination of a certified reference material CRM 477, which provided a precision of analysis less than 8.7% for five replicates. The recoveries of TBT, DBT, and MBT were 88.7%, 98.7%, and 107.3%, respectively. The minimum detectable concentrations were 6.9 ng/g for TBT, 5.2 ng/g for DBT, and 7.8 ng/g for MBT, respectively.

Histological Technique

The tissue specimen of the liver was fixed with 2.5% pentanediol (4°C, 1 month) and 1% osmic acid, then washed with a 0.1 M phosphoric acid buffer solution, dehydrated in a graded acetone series, and embedded in Epon 812 epoxy resin. Semi-thin sections of the specimen were obtained first with a LKB-V microtome for screening the liver position under an optical microscope. The ultra-thin sections of the specimens were double-stained in uranyl acetate and lead citrate, then observed and photographed on a JEM-100cx at the accelerating voltage of 100 kV.

Statistical Analysis

The significant differences of HSI and GSI among various groups were confirmed by variance analysis.

Results and Discussion

Fish Behavior

Compared with the control group, the fish exposed to 5,000 ng TBT/L showed hyperactivity within the first 2 h after exposure. There was generalized agitation and accelerated ventilation with rapid mouth and opercular movements. Individual fish

Table 1. Hepatosomatic Index (HSI) and the Gonad Somatic Index (GSI)^a

Exposure (ng TBT/L)		1 Week				2 Weeks		
		Control	50	500	5,000	50	500	5,000
HSI	Male	6.5 ± 0.2	5.7 ± 0.2	5.3 ± 0.1	4.6 ± 0.1	4.9 ± 0.3	3.2 ± 0.2	2.8 ± 0.3
	Female	6.8 ± 0.2	6.4 ± 0.3	4.3 ± 0.5	3.7 ± 0.3	4.2 ± 0.1	2.9 ± 0.2	1.9 ± 0.1
GSI	Male	1.9 ± 0.1	2.8 ± 0.3	3.3 ± 0.2	3.6 ± 0.1	2.2 ± 0.1	4.0 ± 0.3	4.6 ± 0.3
	Female	24.2 ± 1.2	23.8 ± 1.0	22.9 ± 0.8	22.6 ± 0.5	21.1 ± 1.1	19.1 ± 0.8	9.0 ± 0.6

^a Five fish were sampled in each group (mean ± SD). Significantly different from control and exposure groups from each other, $p < 0.05$.

Table 2. Concentrations of butyltins in test fish muscles (ng Sn/g)^a

Exposure Time		Exposure Levels (ng TBT/L)	BuSn ³⁺	Bu ₂ Sn ²⁺	Bu ₃ Sn ⁺
1 week		Control	ND ^b	ND	ND
		50	9.64 ± 0.83	8.21 ± 0.61	45.10 ± 2.82
		500	16.26 ± 0.97	27.31 ± 1.37	158.84 ± 11.23
		5000	33.92 ± 2.55	124.24 ± 9.34	686.09 ± 20.68
2 weeks		50	10.80 ± 0.85	12.32 ± 1.03	51.07 ± 3.75
		500	16.17 ± 1.20	47.76 ± 3.15	306.35 ± 16.65
		5000	49.93 ± 3.34	223.61 ± 17.45	2470.33 ± 114.92

^a Mean ± SD (n = 5 measurements/sample).

^b Not detected.

swam fleetly from one side to another, up and down. Then the fish progressively showed signs of tiredness and rested at the bottom of aquarium after a period of weakness. After 24 h, all fish had acclimatized themselves to the new condition. The exceptional behavior patterns of the other two exposure groups were much less obvious. Throughout the exposure, no fish mortality, general appearance, abnormal behavior, or difference in food intake was observed in all experimental groups.

HSI and GSI

The hepatosomatic index [$100 \times$ liver weight (g)/fish body weight (g)] and the gonad somatic index [$100 \times$ gonad weight (g)/fish body weight (g)] of each test system after 1 or 2 weeks of exposure to TBT are presented in Table 1. With the increasing exposure concentration of TBT and the exposure time, a significant decrease of HSI was determined in both male and female test groups compared with the control group. Accordingly, it might be concluded that the extent of damage to the liver that served as an antidotal organ, was related to the suspect TBT exposure concentrations. The decrease in HSI in exposed fish was expected based on the common finding of hepatocellular trauma.

For the GSI, an obvious difference existed between the male and the female test fish. For male test fish, the GSI slowly increased with the exposure levels of TBT and the exposure time, whereas for the female fish, the trend was reversed. The GSI of the male fish exposed to 5,000 ng TBT/L for 2 weeks were nearly 2.5 times higher than in the control fish. In contrast, the GSI of the female fish exposed to TBT was only about 40% of that of the control group. The androgenic effects of organotin compounds (Bryan *et al.* 1988; Smith 1981) may explain these trends in fish exposed to TBT in this study. A

previous paper (Oehlmann *et al.* 1996) reported that TBT interfered with steroid biosynthesis at the level of estrogen synthesis by inhibition of the androgens into estrogen, which resulted in the high level of the androgens androstenedione and testosterone in the TBT-exposed biota. It might be understandable that the male gonad was probably strengthened and the female one was probably weakened as the result of the imbalance of the sex hormone described above.

Chemicals Analysis

The exposure of rare minnow to several TBT concentrations resulted in the higher bioaccumulation of tributyltin and its dealkylated metabolites dibutyltin and monobutyltin in the whole fish muscle homogenate (Table 2). Concentrations of butyltin species in the muscle tissue of exposed fish were 459- (5,000 ng TBT/L, 1 week) to 4,065- (50 ng TBT/L, 2 weeks) fold higher than the nominal TBT concentrations in the exposure water. Similar results were reported by Schwaiger *et al.* (1992). Total body concentrations of butyltin species increased with both the TBT-concentration of the test solutions and the duration of exposure. The bioconcentration factors of total butyltin species for test fish muscle decreased with the increasing exposure levels, however, they all increased with the exposure time at each exposure level (Table 2).

When the fish were newly subjected to the exposure solution, the diffusion rates of butyltins from water to fish were similar at all exposure levels. As time passed, the diffusion rates slowed down more rapidly at lower exposure levels than at higher ones with the increase of butyltin concentrations in fish muscles. Although the absolute amounts of TBT accumulated in the fish muscle at higher exposure levels were much higher than those at lower ones, it is still difficult to conclude that the

Fig. 1. Transmission electron microscopy micrograph of hepatocytes of the control fish. a. Many layers of RER cisternae abundant with the ribosomes were regularly arranged; lots of mitochondria with clear regular structures were well distributed in the cytoplasm, $\times 490,000$. b. Only a few vacuoles with small size could be found in the control, $\times 49,000$. c. Large nucleus with distinct nucleolus clearly located in the center of hepatocytes; electron-dense heterochromatin was situated around nuclear membrane, $\times 97,000$

accumulation ratios increased with the exposure levels compared to the respective exposure levels. Indeed, the muscle tissue exhibited a negative dosed-related accumulation ratio of total butyltins here (Table 2).

Fig. 2. Transmission electron microscopy micrograph of hepatocytes from fish exposed to 5,000 ng TBT/L for 2 weeks. a. Many mitochondria were swelling and distorted; RER cisternae could rarely be found, $\times 120,000$. b. Many vacuoles with increasing number and size were full of the hepatocyte, $\times 33,000$. c. The nuclei became rather abnormal, $\times 170,000$

Ultrastructural Findings

No apparent changes were noted in the organs and tissues *in situ* in the total-body sections. Pathological changes were observed in cellular structure in the liver of the fish exposed to 5,000 ng TBT/L for 2 weeks. The incidence of the lesions is described in detail below.

As the ultrastructure of the control fish liver (Figure 1)

showed that large nucleolus with distinct nucleolus were clearly located in the center of hepatocytes, and electron-dense heterochromatin was situated around nuclear membrane; many layers of rough endoplasmic reticulum (RER) cisternae abundant with the ribosomes were regularly arranged. Many mitochondria with clear regular structures were distributed throughout the cytoplasm. Only a few vacuoles with small size could be found in the control. Pronounced changes in hepatocytes of TBT-exposed fish could be clearly observed (Figure 2). The most common phenomenon was that many vacuoles with increasing number and size were full of hepatocytes. Many mitochondria were swollen and distorted and the nucleus became rather abnormal. RER cisternae could rarely be found.

Among the cytoplasmic organelles, the nucleus was the controlling center of the cell. It offered the place where the transcript and translation of DNA and RNA took place. In our study, the alterations in the morphology of the cell nucleus might be a direct response of fish liver to the high concentration of TBT in water. As the result of the abnormal cell nucleus, the normal function of the cell (such as protein synthesis) might be seriously affected. The mitochondria were sensitive indicators of exposure to TBT. Swollen mitochondria and deformation of cristae were found in the exposed fish in our study. This phenomenon might be a common response in fish hepatocyte to various chemical stressors. The increase in vacuolation of the liver indicated a higher hepatocellular lipid, water, and/or glycogen content, which was in agreement with the results of other reports (Wester and Canton 1987; Wester *et al.* 1990). A trend involving a decrease of RER was also induced in hepatocytes after fish were exposed to contaminated water. Some reports described the reduction of RER and an increase of heterogeneity (Braunbeck 1992; Braunbeck *et al.* 1990) and vesiculation after polychlorinated biphenyl or polyaromatic hydrocarbon exposure in fish.

In our observations, butyltin compounds could also induce the similar alteration of RER. Because abundant RER in control fish liver was arranged in a highly ordered pericentral stacks in the hepatocytes, the rare existence of RER was conspicuously recognized in exposed fish. It was concluded that butyltin compounds appeared directly related to the serious cytological alterations in *G. rarus* hepatocytes.

Conclusion

The present study clearly demonstrated that TBT concentrations reached or exceeded in natural aquatic systems (Jiang *et al.* 2001), resulted in accumulation in the muscle tissue of *G. rarus*. Both the HSI and GSI were sensitive indexes to evaluate the adverse effects of environmental hormonal pollutants in *G. rarus*. The pathologic alterations in fish exposed to TBT included vacuoles with increasing number and size, swollen mitochondria, abnormal nuclei, and decreases in RER cisternae. These responses clearly indicated that the cytological investigation was a valuable tool in assessing toxicological responses in fish. All the findings obtained above demonstrated that the Chinese rare minnow (*G. rarus*) could be applied as a useful approach to evaluate water toxicity in aquatic habitats in China and other suitable areas of Asia.

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