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# Effect of Tributyltin, Benzo[a]pyrene, and Their Mixture on the Hepatic Monooxygenase System in *Sebastiscus marmoratus*

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Polycyclic aromatic hydrocarbons (PAHs) are produced by any incomplete combustion of organic material and are therefore present worldwide due to anthropogenic activity. Many PAHs are known to act as potent carcinogens and/or mutagens and are therefore considered as important risk factors in epidemiological and epizootiological cancer. The cytochrome P450 system (or monooxygenase system) is universally distributed and plays a key role in the metabolism of xenobiotic compounds leading to their detoxification or bioactivation. The system is also important in the metabolism of endogenous substrates including steroids, arachidonic acid, prostaglandins and others. The monooxygenase system is a multi-component enzyme system containing several forms of cytochrome P450, cytochrome P450 reductase, cytochrome b5 and cytochrome b5 reductase. Numerous studies in the field and laboratory have demonstrated that CYP450 monooxygenase activity was affected by PAHs.

Organotin compounds, particularly tributyltin (TBT), are widely used as biocides in a variety of consumer and industrial products. Among them, antifouling paints are the most important contributors of organotin compounds to the aquatic environment, where they are known to cause deleterious effects to non-target organisms. The cytochrome P450 (CYP450) enzyme system also plays an important role in the metabolism of organotins (Padrós et al. 2003). In contrary to PAHs, TBT and triphenyltin (TPT) have been shown to markedly inhibit fish cytochrome P450 in vivo and in vitro (Fent and Stegeman 1991, 1993; Fent et al. 1998).

It was suggested that the monooxygenase system could be used as potential biomarkers to monitor marine pollution. TBT and benzo[a]pyrene (BaP) are widespread pollutants that occur simultaneously in the marine environment, and could have a combined effect on the monooxygenase system. Observations of Padrós et al. (2003) about the mutual metabolic interactions between TBT and BaP reinforce the need to further investigate the combined effects on biomarkers. However, limited information concerning combined effects of TBT and BaP on the monooxygenase system in fish is available. Accordingly, the present study was designed to investigate the combined effect of BaP and TBT on the monooxygenase system. The results will provide further evidence of the usefulness of the monooxygenase system to assess exposure/effects of both TBT and BaP.

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## MATERIALS AND METHODS

Tributyltin chloride (TBT) was obtained from Fluka AG, Switzerland, with a purity of greater than 97%. BaP (98% purity), NADPH, ethoxyresorufin, resorufin were obtained from Sigma Chemical Co, (St Louis, MO, USA). All other chemicals were of analytical grade and were obtained from commercial sources.

Cuvier (*Sebastiscus marmoratus*) weighing 25–50g, were captured from a coast in Xiamen, Fujian province, China. The fish at first were maintained in tanks containing 60 L of aerated sand–filtered seawater, with natural photoperiod for 7 days. Six fish per dose group were injected intraperitoneally with doses of 0.5, 1, 5 or 10 mg/kg body weight TBT, BaP or their mixture (at 1:1 concentration, for example, 10 mg/kg of the mixture was 5 mg/kg TBT plus 5 mg/kg BaP) in olive oil. Control fish received an equal volume of olive oil (injection volume in each case 1 ml/kg). The fish were fed with fresh clam *Meretrix meretrix* flesh for two hours before replacing the water. The clam was maintained in aerated sand–filtered seawater for 7-10d. This process was repeated every other day until the third day before sampling. The water temperature was maintained at 14±2℃ and salinity 22 −24. Seven days post-treatment, 5-6 fish per dose group were killed by a sharp blow on the head. The liver was frozen in liquid $N_2$ immediately after collection and stored at -80 ℃ until analyzed.

Homogenates of the liver were prepared in chilled buffered KCl (1.15% KCl buffered with 0.01mol/L tris-HCl, pH7.4) and centrifuged at 10000 g for 20 min at 4 ℃ to obtain post-mitochondrial supernatant, which was used as the source of enzyme. Ethoxyresorufin O-deethylase (EROD) activity was determined using a fluorometric assay according to the method of Peter (Peter et al. 1994). NADPH cytochrome c reductase activity was determined according to the procedure described by Livingstone and Farrar (1984). The activity was calculated using an extinction coefficient of 19.6/mM/cm (Shimakata et al. 1972). NADPH-cytochrome $b_5$ reductase activity was determined as described in Scott (1960). Protein concentrations in the supernatants were determined by the Bradford (1976) procedure using bovine serum albumin as the standard. All fluorometric assays were determined on a Hitachi F-4010 fluorescence spectrophotometer. The absorbance at UV and visible wavelengths was monitored on a Thermo GENESY$^{TM}$ 2 UV-Visible spectrophotometer.

Results are reported as mean±SE (standard error). The data were analyzed by the t-test and $P<0.05$ was accepted as significant. Differences between treatments were determined by two-way ANOVA, with the factors being BaP and TBT.

## RESULTS AND DISCUSSION

Olive oil was used as the vehicle in the present study. Control groups received an equal volume of the olive oil. The results showed that the indicators were not affected by the vehicle control, except NADPH cytochrome c reductase activities were significantly induced by the vehicle control (Fig. 1). It was reported in previous studies that corn oil significantly reduced EROD activity in eel (*Anguilla*

*anguilla*) (Bonacci et al. 2003). Such effects may be related to an elevated omega-6 content in vegetable oil and to an increased susceptibility to peroxidative processes or to some modification of lipid metabolism in the exposed organism.

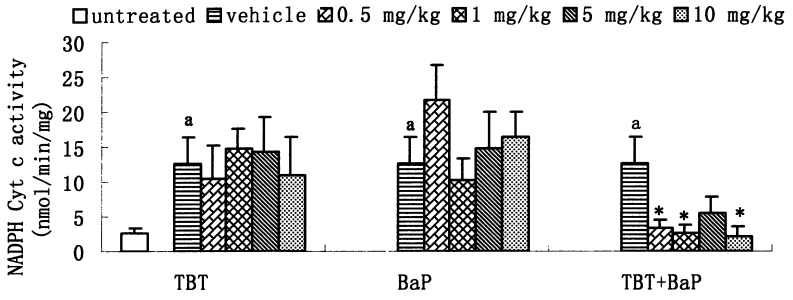
There was no effect on NADPH cytochrome c reductase activities of *Sebastiscus marmoratus* exposed to TBT or BaP alone for 7d. However, the NADPH cytochrome c reductase activities were significantly inhibited when the fish were exposed to TBT+BaP (Fig.1). There was a significant difference between TBT+BaP groups and TBT or BaP groups according to two-way ANOVA analysis.

Hepatic EROD activities in *Sebastiscus marmoratus* exposed to TBT were significantly induced (2.2-fold greater than the control) at the lowest dose (0.5 mg/kg), while such activities at the highest dose groups were significantly inhibited (Fig.1). The dose-response for fish exposed to BaP for 7 days showed that EROD activity significantly increased (1.4-fold over the control) at lower doses groups, but that at higher dose groups there were no significant differences between treated and control groups. Hepatic EROD activities in *Sebastiscus marmoratus* exposed to TBT+BaP were significantly induced (1.5-fold over the control) at the lowest dose (0.5 mg/kg). However, there was not a significant difference between TBT+BaP groups and TBT or BaP groups according to two-way ANOVA analysis.

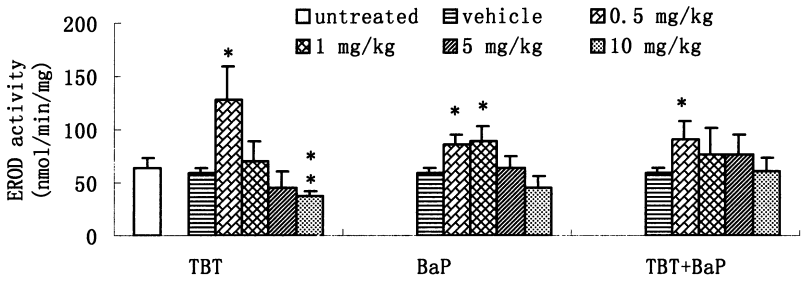
Hepatic NADH cytochrome b5 reductase activities in *Sebastiscus marmoratus* were induced after exposure to TBT or BaP for 7 days (Fig. 1). Cotreatment with TBT and BaP did not affect NADH cytochrome b5 reductase activity. There was not a significant difference between TBT+BaP groups and TBT or Bap groups according to two-way ANOVA analysis ($p=0.056$ and 0.627, respectively).

The results in a previous study showed that the level of BaP in surface sediment was 0.13-0.69 μg/g (Heit et al. 1981). Elevated levels of BaP and other PAHs have been detected in contaminated sediments. Specifically, Catallo and Gambrell (1987) reported a BaP concentration of 610 μ g/g in sediment taken from Bayou Bonfouca, LA. Many studies show sediment concentrations of TBT in the hundreds of ng/g to low μ g/g levels (Meador 1997). The lower doses of the two chemicals and their mixture (0.5-1mg/kg) used in the present study should be relevant to environmentally realistic concentrations. However, the higher doses of the chemicals (5-10 mg/kg) are higher than the commonly observed environmental exposure levels. Thus, they mimic highly contaminated field situations. In some cases high TBT concentrations (14.7 μ g/L) have been recorded (Hassan and Juma 1992). TBT is not readily biodegradable. Exposing *Cyprinidon variegates* to 1 ng/L TBT for 177 days led to a burden in the liver of 40800 ng/g (BCF=41000) (Ward et al. 1981). There could be an equal level between TBT and BaP in a realistic marine environment according to the previous reports. So in this study the concentration ratio of the TBT, BaP mixture was 1:1.

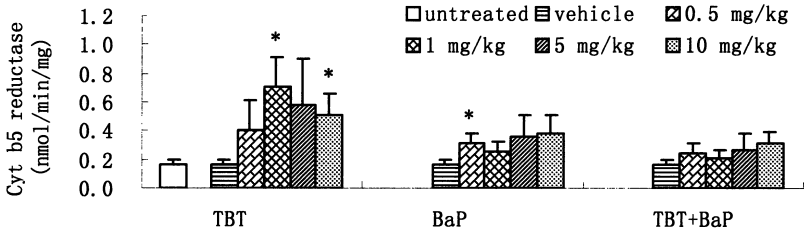
Cytochrome P450 monooxygenase activities are probably the biomarkers investigated most extensively in fish. Previous studies have shown that TBT or TPT can strongly act in vitro or in vivo on hepatic microsomal cytochrome P450 in a marine bivalve and fish leading to destruction of native enzyme and inhibition of




The activity of hepatic NADPH cytochrome c reductases




The activity of hepatic EROD




The activity of hepatic NADH cytochrome b5 reductases

**Figure 1.** The activity of hepatic NADPH cytochrome c reductases, EROD, NADH cytochrome b5 reductases in *Sebastiscus marmoratus* exposed to TBT, BaP and their mixture at 1:1 concentration ratio for 7 days. n=5-6 *, $P<0.05$ vs the vehicle group; a, $p<0.05$ vs the untreated group

enzyme activity (Fent and Stegeman 1991, 1993; Fent et al. 1998; Morcillo and Porte 1997). EROD activity tended to be decreased in TPT-treated (1.9–19.3 mg/kg) scup (*Stenotomus chrysops*), while NAD(P)H cytochrome c reductase activities were significantly inhibited (Fent et al. 1998). In scup, doses of 3.3, 8.1 and 16.3 mg/kg of TBT led to conversion of P450 to its degraded form cytochrome P420 in

vivo and loss of EROD activity (Fent et al. 1998). However, it was reported in juvenile Arctic charr (*Salvelinus alpinus*) that repeated exposure to a low dose of TBT (0.3 mg/kg) produced a modest, but significant induction of hepatic P4501A activity (Padrós et al. 2003). Similar results have been observed in another in vivo study with fish repeatedly injected with 1.7, 17, or 170 μg/kg of TBT for 16 days (Rice and Roszell 1998). The present results show that the EROD activity was induced at a low TBT dose, but inhibited by high dose TBT. We suggest that there is a threshold concentration of TBT to inhibit EROD activity. However, in the present study, treatment with 10 mg/kg TBT resulted in a significant induction of hepatic NADH cytochrome b5 reductase activity. This indicates that NADH cytochrome b5 reductase would be more tolerant to exposure of TBT than EROD.

There are many reports about phase Ⅰ biotransformation enzymes induced by PAHs. However, the conclusions in some studies are not similar. In a few case, it was observed that EROD activity was inhibited. Di Giulio et al. (1993) reported that, in channel catfish (*Ictalurus punctatus*) exposed in the laboratory to sediments highly contaminated with PAHs, EROD activities gradually declined during the course of the experiment. In the present work, the EROD activity was not induced at higher doses ($\geqslant$5 mg/kg) of BaP, which is probably due to competitive inhibition of the enzyme activity.

At environmentally relevant doses, organotins such as TBT intensified the PCB-induced CYP1A induction in channel catfish, while a decrease of induction was observed at higher TBT doses (Rice and Roszell 1998). Padrós et al. (2003) observed in juvenile Arctic charr that after two ip injections (BaP 3 mg/kg, TBT 0.3 mg/kg, or both in combination), cotreatment with BaP and TBT significantly antagonized the EROD increase by 43% relative to BaP alone. In the present study cotreatment with BaP and TBT did not change the general effect of BaP or TBT alone on EROD activity according to two-way ANOVA analysis, while cotreatment with BaP and TBT resulted in significant inhibition of NADPH cytochrome c reductase activity, which was not affected by TBT or BaP alone. This result showed usefulness of EROD activity as a biomarker to assess exposure/effects of both TBT and BaP, which should be further confirmed by chronic exposure at environmental levels.

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