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Accumulation and Depuration of Tributyltin Oxide and Its Effect on the Fertilization and Embryonic Development in the Pacific Oyster, *Crassostrea gigas*

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The profile of accumulation and depurtation of tributyltin oxide (TBTO) was investigated in various tissues of the Pacific oyster, *Crassostrea gigas.* The maximum concentration of accumulated TBTO in each tissue during exposure tended to be higher in the following order: gill > gonad≈ digestive diverticula > mantle > adductor muscle. Bioconcentration factors for TBTO in the oyster ranged from 2400 to 7800. Accumulated TBTO in the gill was readily eliminated up to the control level, while the gonad retained the highest level of TBTO. The influence of accumulated TBTO in the ovary and testis on fertilization and embryonic development was investigated. The rates of fertilization and development declined with increasing concentration of TBTO in the ovary, whereas no relationship between these rates and TBTO in the testis was observed. These results lead to the tentative conclusion that TBTO accumulated and retained in the eggs would interfere with embryonic development in the oyster.

Tributyltin compounds, including tributyltin oxide (TBTO), have been widely used as a marine antifouling paint for protection of hulls and other immersed surfaces and their toxicity to aquatic organisms has been well documented (Lapota *et al.* 1993; Widdows *et al.* 1993; Osada *et al.* 1993; Lawler *et al.* 1987; Ward *et al.* 1981). Acute and sublethal effects of organotin compounds on aquatic biota have been reported and their accumulation and depurtation have also been examined in a variety of fish and molluscs. Uptake and elimination of tributyltin oxide (TBTO) have been observed in the mussel *Mytilus edulis* (Laughlin *et a.l* 1986,1988a), the grayling *Thymallus thymallus* (Fent *et al* .1993), the rainbow trout *Oncorhynchus mykiss* (Martin *et al* .1989) and the dog-whelk *Nucella lapillus* (Bryan *et al* .1989). The accumulation of TBTO in *M. edulis* was different among a variety of tissues and TBTO burdens of gills and gonads were greater than that of the other organs. On the other hand, although a chronic effect of TBTO on gonadal development has been demonstrated in the dogwhelk *Nucella lapillus* (Spooner *et al* .1991), there have been few studies on the effects of accumulated TBTO in the gametes on the fertilization and development. Since *Crassostrea gigas* is one of the important species in aquaculture, it is very important to know whether accumulated TBTO in the gonad affects the reproduction and embryonic development of *C. gigas* or not.

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The purpose of the present study is to determine the characteristics of uptake and elimination of TBTO in various tissues of *C. gigas* and demonstrate how subsequent embryonic development is influenced by accumulated TBTO in the gonad.

MATERIALS AND METHODS

The cultured Pacific oysters, *Crassostrea aigus* (soft body weight; 21.3 ± 10.2 g; shell length, 6.3±0.9 cm; shell height, 10.1±1.0 cm.), were collected in Matsushita Bay, Miyagi Prefecture in May 1994. In this season, gonadal development was classified as being in the growing stage. They were acclimated in the aquarium at 20°C for about 2 weeks before beginning the experiment, which was conducted under the same temperature at three different concentrations of TBTO (0, 0.5 and 1.0 μgL^{-1}). During the experiment, eighty oysters were placed in a 120 liter aquarium and reared in running seawater which was prepared at the above concentrations of TBTO. Algal cultures of *Pavlova lutheri* were fed to the oysters at a density of 5×10^5 cells/mL every 3 days for three hours, during which constant flow in the aquaria was stopped. After exposure to TBTO for 42 days, the oysters were transferred to fresh seawater and reared for 14 more days.

Fifteen oysters were randomly collected on days 0, 28, 42, 49 and 56. The gill, mantle, digestive diverticula, gonad and adductor muscle were dissected from the oysters and frozen until use for measurement of TBTO by gas chromatography with a flame photometric detector (GC-FPD) .

After forty ripe Pacific oysters were exposed to TBTO at the concentrations of 0, 3.0 and 6.0 μgL^{-1} for two weeks without feeding, the eggs and sperms were collected by mincing the ovary and testis. The gametes obtained from the oysters exposed to TBTO were crossed with the gametes from the control oysters reared without TBTO. The embryos were cultured at 25°C. The fertilization and development rates were calculated by counting the fertilized eggs at hour 2 and straight-hinge larvae at hour 24 post-fertilization. At the same time the accumulated TBTO in the gonad from which the gametes were collected was determined by GC-FPD below.

TBTO (96% pure) was from Aldrich Chemical Company (Milwaukee, USA). Grignard reagent, n-propyl magnesium bromide (2M in THF) and tri-n-butyltin chloride (TBTCI) were purchased from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). The latter was used as the standard. All other chemicals were extra grade from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). One gram of tissue was weighed and homogenized with lN HC1-methanol and ethylacetate. The homogenate was centrifuged at 10,000 rpm for 15 min at 4°C and distilled water, NaCl and ethylacetate/hexan (3:2) was added to the resulting supernatant. After vigorously shaking, the organic phase was collected and this extraction procedure was repeated again. The pooled organic phase was washed with distilled water and

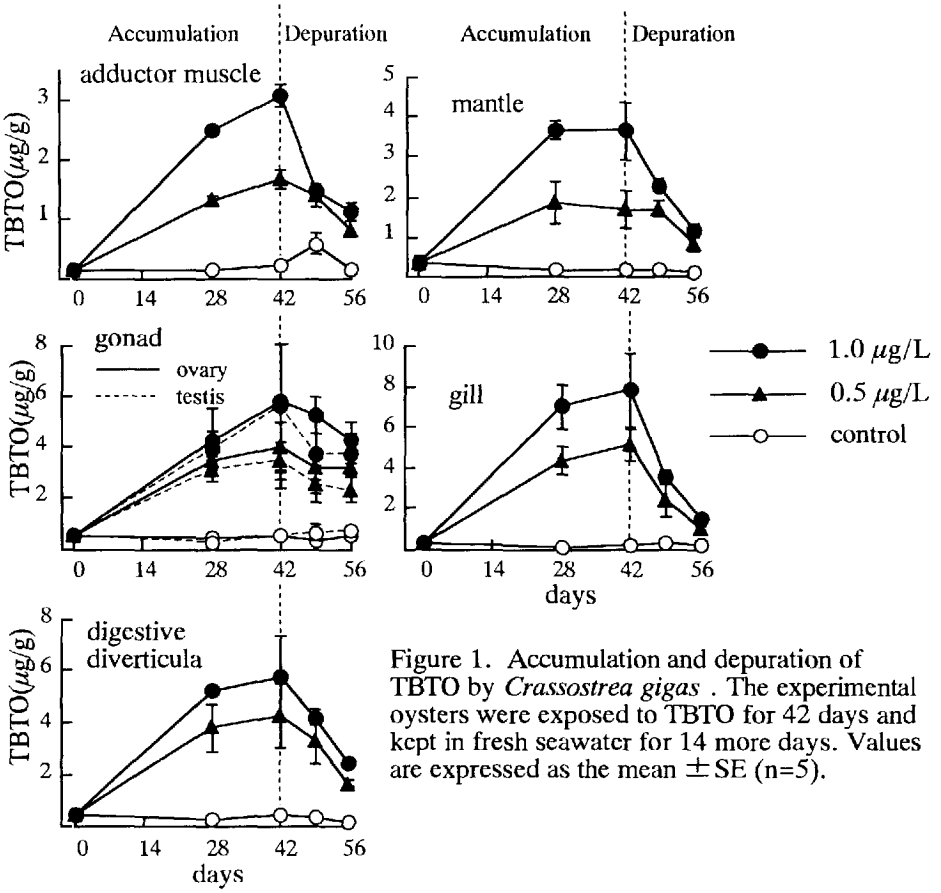

Figure 1. Accumulation and depuration of TBTO by *Crassostrea gigas* . The experimental oysters were exposed to TBTO for 42 days and kept in fresh seawater for 14 more days. Values are expressed as the mean ± SE (n=5).

dehydrated with anhydrous sodium sulfate, followed by evaporation to reduce the volume to approximately 4 ml. The extracted TBTO was propylated by n-propyl magnesium bromide, passed through the Sep-pak Florisil column (Millipore Corporation, Millford, USA.), and analyzed by gas chromatography with flame photometric detection (Hitachi model 193). The detection limit for the method was 6.7 ng for TBTO and the recovery of TBTO from tissue was 87.4%.

RESULTS AND DISCUSSION

The burdens of TBTO on the tissues of the oysters exposed to either 0.5 or 1.0 μ gL^{-1} TBTO increased continuously, while in the control, marked fluctuations were not observed during the accumulation period (Fig. 1). In the 1.0 μgL^{-1} exposure, the TBTO concentrations of each tissue on day 42 was highest in gills (7.88 μg/g), followed in decreasing order (μg/g) by ovary (5.82), digestive diverticula (5.77), testis (5.61), mantle (3.65) and adductor muscle (3.08). This tendency was

Table 1. Bioconcentration factors (BCFs) for five tissues of oysters, *C. gigas*, exposed to two different TBTO concentrations.

Concentration ($\mu g\ L^{-1}$)	Tissue	Highest BCF	Steady-state BCF
0.617	Gill	7,850	—
	Gonad	5,230	—
	Digestive diverticula	6,162	—
	Mantle	2,474	2,161[a]
	Adductor muscle	2,458	—
1.125	Gill	6,742	—
	Gonad	4,618	—
	Digestive diverticula	4,713	—
	Mantle	2,971	2,937[a]
	Adductor muscle	2,606	—

(-), no steady state observed.

[a] calculated from the mean on day 42.

recognized also in the exposure at the lower concentration (0.5 μgL^{-1}). In general the TBTO contents of the mantle and adductor muscle tissues were much lower than those of other tissues. The TBTO levels in the rearing seawater prepared at 0.5 and 1.0 μgL^{-1} TBTO were determined and these effective concentrations were 0.617 and 1.125 μgL^{-1}, respectively, in this experiment. We attempted to estimate a bioconcentration factor (BCF) for each tissue in the oyster from the two different exposures (Table 1). An apparent steady state occurred only in the mantle during the period of exposure. The BCF was calculated as the ratio of the highest value in the tissue (taken on day 42) to the water concentration. The result showed that BCF for TBTO varied for the different tissues. In the 1.0 μgL^{-1} TBTO exposure, the BCF of adductor muscle showed the lowest value (2606) and the highest value (6742) was observed in the gill.

The TBTO elimination from all five tissues after the end of uptake on day 42 was shown in Fig. 1. In the 1.0 μgL^{-1} TBTO exposure group, TBTO concentrations of each tissue on day 56 was highest in ovaries (4.32 µg/g), followed in decreasing order (µg/g) by testis (3.69), digestive diverticula (2.42), gill (1.47), mantle (1.16) and adductor muscle (1.12). Such a similar decreasing tendency was observed in the 0.5 μgL^{-1} TBTO exposure group. Biological halflife was evaluated in each tissue to determine depurtation, according to Sato (1994). Accumulated TBTO in most tissues decreased by half within two weeks, whereas the gonad still kept a high level during the same period, indicating slower depurtation (Table 2).

Higher burdens and slower depurtation of TBTO were observed in the gonads compared to the other tissues. When TBTO content in the eggs was compared with that in the original ovary, 86% of the TBTO was in the eggs (Table 3). Therefore, an attempt was made to assess the effect of accumulated TBTO in the eggs on

Table 2. Biological half life (days) of TBTO in different tissues of *C. gigas.*

treatment	gonad	digestive diverticula	adductor muscle	mantle	gill
0.5 μg/L	19.6	11.3	13.7	13.8	8.8
1.0 μg/L	17.1	12.1	11.0	10.3	9.6
Average	18.4	11.7	12.4	12.1	9.2

Table 3. TBTO distribution in the ovary in *C. gigas*.

	eggs	ovary
TBTO (ng/g)	479.8	555.6
percentage (%)	86	100

fertilization and embryonic development. The eggs and sperms obtained from the oyster exposed to TBTO for two weeks were separately crossed with the gamete of the control and the rates of fertilization and development of straight-hinge larvae were determined. Figure 2 showed TBTO concentrations of the ovaries and testes in three groups, the control, 3 and 6 μgL^{-1} TBTO exposure on day 14. The eggs from all three groups were inseminated with the sperms of a male control oyster. The fertilization rate after 2 hours and larval development rate after 24 hours are shown in Figure 3. Averaged fertilization rates for the control, 3 and 6 μgL^{-1} treatments were 97.1%, 41.8% and 31.4%, respectively. Development rate of straight-hinge larvae was 69.3% in the control, 26.0% in the 3 μgL^{-1}, and 22.7% in the 6 μgL^{-1} exposure. The relationship between these rates and TBTO content was investigated, resulting in a significant inverse relationship between the TBTO levels of ovaries and the rates of fertilization and development (ANCOVA, $P<0.05$). On the other hand, the sperms from all three groups were crossed with the eggs of a female control oyster and there was no significant relationship between the TBTO levels in the testes and the rates of fertilization and development (Fig. 3).

Pacific oysters rapidly accumulated TBTO in the various tissues throughout the experiment when exposed to two different TBTO concentrations, however, TBTO content reached a plateau only in the mantle. Laughlin *et al.* (1988a) exposed mussels, *Mytilus edulis,* to 23, 45, 63, 141 and 670 ngL^{-1} TBT for 20-52 days and reported that BCF for TBT varied for different tissues. Muscle had the lowest BCF, 1500, with increasing BCF in other tissues, up to 7300 in the gill which accumulated the highest burdens. These values agreed with the results obtained in our experiment.

The differences of TBT burdens in the various tissues are suggested to be mainly

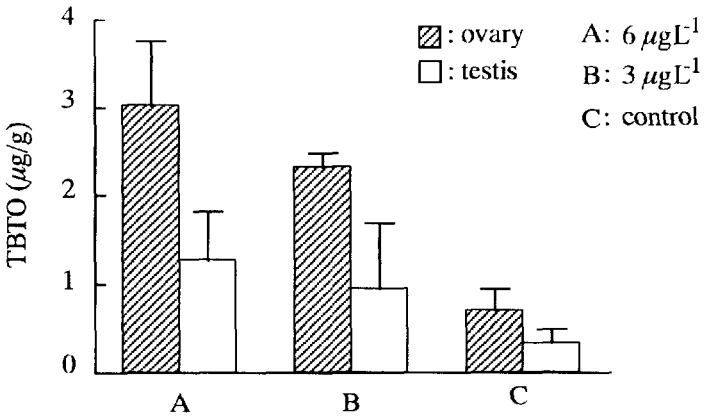

Figure 2. Concentration of TBTO in the gonad of *C.gigas*. Values are expressed as the mean ± SE (n=5).

based on physiological and constitutional differences, presumably lipids, which provide a hydrophobic refuge for accumulated organic compounds. A ranking of TBT burdens in different tissues would correlate well with relative lipid content because mantle and muscle tissues, containing low lipid levels compared to the others, had low TBT burdens. In addition, mucopolysaccharides occurring on bivalve gills may be the chemical analog of bacterial biofilm which could bind or conjugate with the tin atom, and may act similarly to influence uptake of TBT by bivalves (Laughlin *et al* .1988b). In our experiment, the seawater used was filtered by absorbent wool and activated charcoal before use. Any contamination of microalgae, even if present in the aquarium, would be extremely limited for so many oysters. Therefore, it is assumed that the route of TBTO uptake for the oysters was directly from water rather than food.

After the oysters were transferred into fresh seawater, the major distinction of depurtation among the tissues was that the highest TBTO burden (gill) rapidly declined to reach the lowest level, compared to the other tissues, on day 14. TBTO depuration in the gonads was the slowest, with a biological halflife of 18.4 days. The rapid TBT decrease in gill was presumably due to active exchange of seawater there, while the slow TBTO elimination in the gonad may be caused either by high lipid content, which could have a strong affinity with organotin, or by the limited ability of the oyster to degrade TBT via mixed function oxygenates (Lee 1981, 1985). The rapid uptake and slow elimination of TBTO in gonads indicate that TBTO has a considerable potential for bioaccumulation in the gonad of the oyster, both in the experimental condition and in the natural environment. We showed that 86% of TBTO in the ovary was in the eggs. This result led us to investigate the effect of accumulated TBTO in the eggs on embryonic development. Eggs and sperms contaminated with TBTO were crossed with normal ones reared in filtered seawater (TBTO< 5 ngL^{-1}). Insemination of the eggs contaminated with

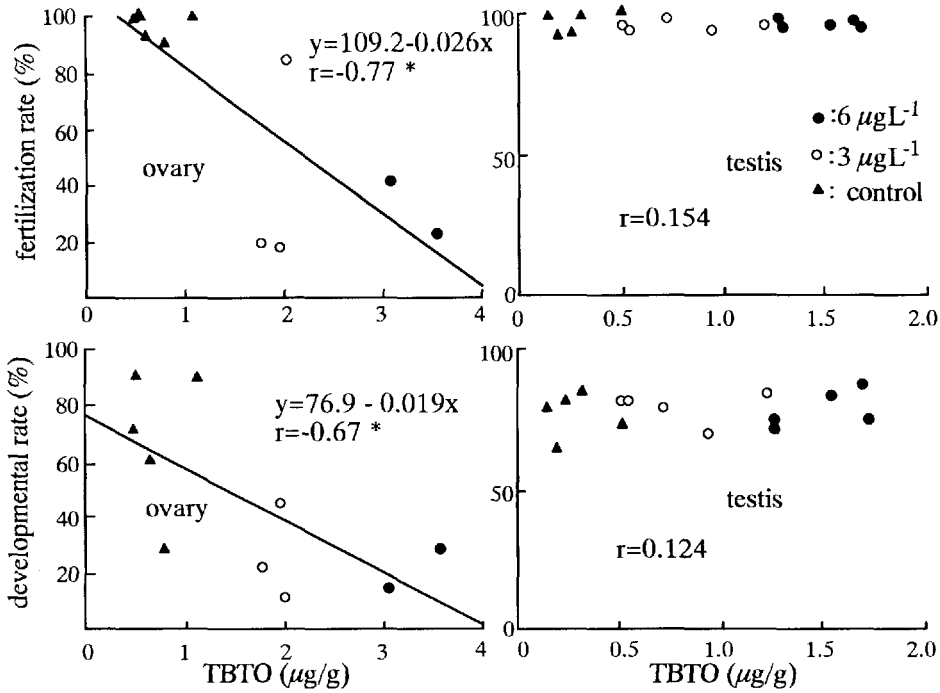

Figure 3. Relationships between the TBTO concentration in the ovaries and testes and the rates of fertilization and development of straight-hinge larvae in *C.gigas.*
* ANCOVA, $P<0.05$.

TBTO resulted in decreased fertilization and development rates. A similar result was not obtained using the sperms from the testis contaminated with TBTO. Furthermore, during development, TBTO accumulated eggs led to many malformed larvae and a delay in development to straight-hinge larvae, although the mortality was as low as the control. It seems that TBTO may be incorporated in the yolk lipid during the process of yolk formation and this inhibited embryonic development. Nevertheless, further study is necessary to elucidate the toxic function of TBTO in eggs during the developmental process.

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