Bioaccumulation of tributyltin and its impact on spermatogenesis in mud crab Scylla serrata (Forskal)

Revathi PERANANDAM¹*, Iyapparaj PALANISAMY², Munuswamy NATESAN³, Arockia Vasanthi LOURDURAJ¹, Krishnan MUTHUKALINGAN¹

¹Department of Environmental Biotechnology, Bharathidasan University, Trichy, Tamil Nadu, India
²Center of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai, Tamil Nadu, India
³Department of Zoology, University of Madras, Guindy Campus, Chennai, Tamil Nadu, India

Abstract: The effects of tributyltin (TBT) on the spermatogenesis of mud crab Scylla serrata were studied. TBT-exposed crabs exhibited a decrease in the gonadosomatic Index and the hepatosomatic Index compared to the crabs in the control group. Bioaccumulation of the TBT levels also varied in different tissues. The treated crabs showed variation in the weight of the testes with respect to TBT concentrations. Histological results clearly indicated that the control crabs showed spermatophores and granular substances were seen filling up the lumen of the seminiferous tubules. The testes of the experimental crabs illustrated that disruption in the seminiferous tubule membrane architecture, arrangement of spermatophores, granular substance, vacuolation, and reduction in the number of epithelial cells of the membrane were apparent in the seminiferous tubules. Biochemical constituents such as protein and glycogen contents also decreased in experimental crabs. Thus, TBT had significantly retarded the reproductive activity and substantially reduced the biochemical constituents ultimately led to impairment of spermatogenesis in the mud crab S. serrata.

Key words: Scylla serrata, tributyltin, toxicity, spermatogenesis, biochemical variations

1. Introduction
Organotin compounds, such as tributyltin (TBT), have been employed as antifouling agents in paints for marine shipping and for a variety of other uses (1). The wide distribution, high hydrophobic condition, and persistence of organotin compounds have raised concerns about their bioaccumulation, biomagnification in the food webs, and adverse effects to human health and the environment (2). Bioaccumulation of TBT seems to be dependent on the lipid content of aquatic organisms and their environmental concentration (3). High concentrations of TBT have been detected in bivalves and fish collected from heavy organotin-contaminated sites (4). TBT was found to be very toxic to a number of marine invertebrates. It causes morphological abnormalities in mollusks and crustaceans (5,6). Although the mechanism of toxicity is unclear, triorganotins are thought to operate by inhibiting oxidative and photosynthetic phosphorylation processes essential to most organisms (7).

The wide range and fundamental toxicity has raised concern over the effects of triorganotins on nontarget organisms (8). In most of the studies, an aqueous solution of triorganotins was used to evaluate its effects on aquatic organisms. This procedure is perhaps most appropriate for small organisms at low trophic levels in which relatively large exposed membrane surfaces facilitate uptake of triorganotins directly from the solution (9). This mode of uptake would be particularly important for the TBT compound, which is lipophilic and thus likely to be accumulated through food chains (10).

The gonadosomatic index (GSI) is an indicator of sexual development and cycle in aquatic organisms. The hepatosomatic index (HSI) denotes the lipidic molecule storage capabilities of an organism. Both of these parameters are very sensitive towards feeding, reproductive performance, and environment. Hence, it is mandatory to analyze these parameters in toxicological studies. Jacobson et al. (11) have suggested that TBT probably reduces the reproductive fitness in the amphipod Monoporeia affinis. Previous studies have examined effects at gametic levels and whether exposure to organotin compounds affects the number of sperm produced (12).

Crustaceans are one of the bioindicators of TBT contamination (13). For instance, our previous study revealed the marked inhibition in organogenesis as well as embryonic development in Macrobrachium rosenbergii.
due to TBT toxicity (14). Brachydano rierio exhibited decreased growth and increased mortality at 10 mg/L zinc chloride (15). TBT metabolism has been mostly studied in mollusks (16); however, only limited research has been carried out on the impact of TBT in crustaceans, especially on brachyuran crabs. Scylla serrata is an economically important crab species due to its palatability, nutritive value, large size, high unit price, and great demand in the local and international markets (17). S. serrata is one of the targeted model organisms widely used to study the effects of contaminants like cadmium (18), naphthalene (19), and mercury (20). Therefore, the objective of the present study was to investigate the tissue-specific bioaccumulation rate of TBT and its consequences on spermatogenesis and associated biochemical parameters in mud crab S. serrata.

2. Materials and methods

2.1. Collection and maintenance of crabs
Male individuals of S. serrata were collected from Aqua Nova hatchery, Kanathur, near Chennai, southern India. The crabs were transported to the laboratory in a plastic container with battery aerator and maintained in an aquarium with continuous aeration. As per the methods of Ruscoe et al. (21), optimum water temperature (30 ± 2 °C) and salinity of 20 ppt were maintained in the culture tanks. Crabs were acclimatized in the laboratory for 3 weeks before the start of experiment. During this period, the crabs were fed ad libitum with Donax sp. and the water was changed daily using saline water from the hatchery.

2.2. Reagents preparation

2.2.1 Stock solution
TBT (95% pure) was purchased from HiMedia (HiMedia, Maharashtra, India). TBT stock solution of 50 mM concentration was prepared in 2% ethanol (14).

2.2.2 Working solution
TBT (1.627 mL) was dissolved in 100 mL of 2% ethanol. Sublethal concentrations such as 10 nL, 100 nL, and 1000 nL of TBT were prepared and stored at 4 °C until use.

2.3. Experimental procedure
Five-month-old male crabs were divided into 5 groups, each consisting of 10 healthy adult individuals weighing 32 ± 2 g. One group served as normal specimens, which did not receive any chemical treatment, and was marked as control I. The next group served as control II and received ethanol (2%) treatment. The other 3 groups were exposed to TBT at 10 nL/L, 100 nL/L, and 1000 nL/L concentrations. The experiments were conducted for a period of 50 days in a static microcosm with brackish water. Three replicate tanks were maintained for each treatment.

2.4. Assessment of GSI and HSI
At the end of the experiment, the body weight of crabs was recorded. The gonad tissues were then dissected out and the wet weight was recorded. The GSI and HSI were calculated using the following formulas:

\[
\text{GSI} = \frac{\text{Gonad weight}}{\text{Body weight}} \times 100
\]

\[
\text{HSI} = \frac{\text{Hepatopancreas weight}}{\text{Body weight}} \times 100
\]

2.5. TBT analysis

Hepatopancreas and testis (1.5 g wet weight) tissues were homogenized individually with concentrated hydrochloric acid in a Soveril centrifuge tube. Hexane (10 mL) was added and the extract was shaken for 30 min and centrifuged at 2500 \(\times\) g for 15 min. To 4 mL of supernatant, 8 mL of 3% sodium hydroxide was added. The supernatant was shaken for 10 min and centrifuged, and an aliquot of hexane extract was analyzed using an atomic absorption spectrophotometer (22).

2.6. Histology

The testes of the male crabs in both the control and treated groups were dissected and fixed in Bouin's fixative for histological studies. The tissues were dehydrated through a graded alcohol series and embedded in paraffin wax. Sections of 6–8 µm in thickness were taken and stained with hematoxylin and eosin. The stained sections were mounted using DPX and photomicrographs were taken using a Leica 2500 microscope (Germany) (23).

2.7. Biochemical analysis

2.7.1. Protein content
After 50 days of treatment with TBT, crabs were dissected and test tissue samples were collected and used for protein estimation. The samples such as hemolymph (100 µL), testis, and hepatopancreas (100 mg wet weight) were taken and homogenized individually with 10% trichloroacetic acid and centrifuged at 9000 \(\times\) g for 10 min at 4 °C. The precipitate was then dissolved in 1.0 N NaOH and used to measure the protein content. For each sample, the soluble protein concentration was determined spectrophotometrically at a wavelength of 595 nm by the Coomassie Brilliant Blue G-250 method as described previously (24). Bovine serum albumin was used as a standard.

2.7.2. Glycogen content

Glycogen contents in the hepatopancreas and testis samples were quantified following the standard method (25). The hepatopancreas and testes (100 mg) were taken individually, homogenized with 5 mL of 30% KOH, and kept in a boiling water bath for 15 min, to which 2 mL of 96% ethyl alcohol was added. This mixture was kept overnight in a refrigerator. After 24 h, this mixture was centrifuged at 3000 \(\times\) g for 15 min. The glycogen pellet settled to the bottom. Two milliliters of distilled water was added and the mixture was placed in a boiling water bath at 70 °C.
for 5 min. From this solution, an aliquot of 100 µL was mixed with 900 µL of distilled water and 5 mL of anthrone reagent. Again it was kept in a boiling water bath for 10 min and the absorbance was measured at 520 nm using a UV spectrophotometer (Shimadzu, Japan).

2.7.3. Glucose content
Glucose content in the hemolymph was estimated using the standard procedure (26). To determine glucose content in hemolymph, 3 mL of glucose monoreagent was added to 30 µL of hemolymph and gently mixed. The assay mixture was incubated at 37 °C for 10 min and the absorbance was measured at 505 nm using a UV spectrophotometer (Shimadzu, Japan).

2.8. Statistical analysis
Data are presented as the mean ± SD of 5 individuals. Using SPSS 7.5, ANOVA was performed to determine statistical significance between the mean values of groups at P < 0.05.

3. Results and discussion
In mud crabs, the testis is situated in the midthoracic region. Control crabs showed fully mature elongated testes. The crabs treated with TBT showed variation in the weight of the testes with respect to TBT concentrations (Figure 1). In control crabs, the GSI value was 1.53 ± 0.16% and the HSI was 6.65 ± 0.36%. At concentrations of 10 nL/L and 100 nL/L, the GSI and HSI values were reduced to 1.35 ± 0.18% and 0.98 ± 0.10% and to 6.19 ± 0.28% and 5.27 ± 0.22%, respectively. Both GSI and HSI values remarkably reduced to 0.28 ± 0.09% and 4.03 ± 0.20%, respectively, at 1000 nL/L (Figure 2). The changes in the GSI and HSI values between the control and treated groups are statistically significant (P < 0.05). The present results obviously suggest that TBT possesses adverse effects on male reproductive systems of crabs S. serrata as evidenced by reduced testis weight and GSI and HSI values. A decrease of 5.46-fold in the GSI and 1.65-fold in the HSI was recorded at higher concentrations of TBT. Similar observations on the decrease of GSI in male goby (Chasmichthys dolichognathus) have been reported (27).

Omura et al. (28) reported that daily doses of 10 mg/kg of TBT affected both testicular development and sperm counts. Jegou (29) found that dysfunction may lead to a reduction in sperm quality and possible infertility. Likewise, Fent and Hunn (30) suggested that TBT can also affect sperm count and male reproductive systems in aquatic organisms. Similarly, TBT at environmentally realistic concentrations had an adverse effect on gametogenesis in cuvier Sebastiscus marmoratus (31). Thus, it is obvious that TBT has reproductive toxicity in crab S. serrata.

It appears that TBT induced toxicity and negatively affects reproductive physiology in aquatic organisms including S. serrata. In the present study, the lowest concentration of TBT (10 nL/L) showed very low accumulation of 0.012 ± 0.0001 µg/g in the hepatopancreas and was below the detection limit in the testes of S. serrata. Crabs treated with 100 nL/L TBT had moderately increased bioaccumulation of TBT in the hepatopancreas (0.079 ± 0.021 µg/g), and nondetectable levels were observed in

Figure 1. Anatomy of Scylla serrata crabs showing effects of TBT on testis development. A- control crab showing fully mature testis (T). B and C- Reduction in size of testis in 10 and 100 nL/L TBT-treated crabs, respectively. D- Rudimentary testis in 1000 nL/L TBT-treated crab. Bar: 5 mm.
the testes. The maximum level of TBT accumulation was recorded in the hepatopancreas (0.150 ± 0.038 µg/g) and testes (0.013 ± 0.002 µg/g) of 1000 nL/L TBT-treated crabs (Table). The bioaccumulation of TBT in the hepatopancreas and testes varied significantly between experimental and control groups (P < 0.05). TBT was unequally distributed in the tested organs such as the hepatopancreas and testes of treated crabs. The testes had comparatively lesser accumulation of TBT than the hepatopancreas. This could suggest that the hepatopancreas is the location for TBT degradation because of the presence of respective enzymes like mixed-function oxygenase and conjugating enzymes (glutathione-S-transferase), cytochrome P450, and other components such as cytochrome b (5), NADPH-cytochrome c reductase, and NADH-cytochrome c-reductase. In accordance, Lee (32) reported that TBT distribution exists in crabs, where it was mainly recovered in F-cells of the hepatopancreas. Another report explained that the pollutants are majorly recovered in the hepatopancreas because of its proximity to the ingestion site and its high lipid content. TBT is a lipophilic compound (33), whereas its metabolites bear higher ionic charges and can more readily circulate in hemolymph, from where they could reach the gonads. Nevertheless, here the crab gonad seems to be kept almost free of TBT accumulation at lower concentrations like 10 nL/L and 100 nL/L TBT.

Testicular inflammation was documented as one of the common responses in aquatic animals exposed to environmental toxicants (34–36). Histological observations of control testes showed the normal architecture of seminiferous tubules and lumen, along with granular substances and the spermatophores. However, in TBT (10 nL/L)-treated crabs, retarded testicular development resulting in the reduction of the seminiferous tubule and disruption in the tubule membrane architecture were noticed. Vacuole formation and reduction in the number of epithelial cells of the seminiferous tubule membrane were also recorded. Interestingly, 100 nL/L TBT-treated crabs exhibited disruption in the tubular architecture, arrangement of epithelial cells, reduction in number of spermatophores, and aggregation of the granular substances. At 1000 nL/L of TBT exposure, severe disruption in the arrangement of spermatophores and epithelial cells and the reduction in seminiferous tubule membrane thickness were reported. (Figures 3A–3H). Our results clearly indicate that the abnormal cellular architecture of the testis was noticed in S. serrata on treatment with TBT. Reduction in the seminiferous tubule membrane thickness, granular substance, and disruption of the spermatophores were also noticed. All these effects led to reduction in the number of spermatophores, indicating an adverse effect on spermatogenesis. Overall, the results show a dose-dependent toxicity of TBT on the reproductive performance of S. serrata. Mercier et al. (37) also suggested that the histological result is the probable loss of reserves reflected by the thinning of gonad epithelia, as well as the reduced ability to sustain gonad development.

**Table.** Bioaccumulation of TBT (µg/g) in different tissues of male crab Scylla serrata.

<table>
<thead>
<tr>
<th>Experimental groups treated with TBT (nL/L)</th>
<th>Hepatopancreas</th>
<th>Testis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Control-ethanol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>10</td>
<td>0.012 ± 0.0001</td>
<td>ND</td>
</tr>
<tr>
<td>100</td>
<td>0.079 ± 0.021*</td>
<td>ND</td>
</tr>
<tr>
<td>1000</td>
<td>0.150 ± 0.038*</td>
<td>0.013 ± 0.002*</td>
</tr>
</tbody>
</table>

ND = not detected. *: F-test significant at P < 0.05. Values are means ± SDs of 5 observations.
**Figure 3.** A and B- Cross-section of testes of control crabs showing normal architecture of seminiferous tubules (ST), lumen (L) along with granular substances (GS), and spermatophores (SP). C and D- TBT-treated crabs showing decrease in the size of the seminiferous tubules, aggregation of granular substances (AGS), and vacuoles (Va) in the seminiferous tubules at 10 nL/L TBT exposure. E and F- At 100 nL/L exposure, note disruption in the tubule architecture (arrows, E) reduction in the number of spermatophores, and aggregation of granular substance (arrows, F). G and H- At 1000 nL/L, note the disruption in the arrangement of spermatophores (arrow, H), decrease in granular substances (DGS), and reduction in membrane thickness (arrows, G) of the seminiferous tubules. Bar: 50 µm.
in TBT-contaminated starfish. Supportively, Kinnberg et al. (38) also documented concentration-dependent effects of nonylphenol on the testicular structure of the fish Xiphophorus maculates. Zutshi and Murthy (39) observed appreciable reduction in size, with spermatids and sperms in degenerating condition, and necrosis of interstitial cells after fenthion treatment in the fish Glossogobius giuris. They also reported extensive cytotoxic damage in the testes of G. giuris after fenthion exposure. Hence, the cellular level damages recorded in the testes of S. serrata were due to the toxicity of TBT.

Furthermore, biochemical constituents were also outstandingly varied in the TBT-treated and control groups. In control crabs, the protein content of hemolymph, hepatopancreas, and testis amounted to 55 ± 2.03 mg/mL, 11.22 ± 2.5 mg/g, and 9.22 ± 1.43 mg/g, respectively. However, in TBT (10 nL/L)-treated crabs, it decreased to 46 ± 3.8 mg/mL, 10.95 ± 1.5 mg/g, and 8.68 ± 1.29 mg/g, respectively, after 50 days of exposure. The maximum reduction in protein content of the hemolymph (28 ± 2.9 mg/mL), hepatopancreas (7.22 ± 1.4 mg/g), and testis (2.44 ± 0.07 mg/g) was noticed in 1000 nL/L TBT-exposed crabs (Figure 4). Overall, the protein content in tested tissues varied significantly in the TBT-treated groups compared to the control groups (P < 0.05). Furthermore, in the present study, major biochemical components like total protein content had significantly decreased in crabs, especially at higher concentrations of TBT. A decrease of protein content of 1.96-fold in hemolymph, 1.55-fold in the hepatopancreas, and 3.77-fold in the testis were recorded at a higher concentration of TBT (1000 nL/L). In this way, Reddy and Bhagyalakshmi (40) also reported that cadmium induced biochemical changes in S. serrata. Similar observations have been reported in the trophic estuarine clam by exposure to tributyltin oxide (TBTO) (41). Significant reduction in protein content was observed in Mystus guilio due to toxic stress (42). The loss of protein content in the hemolymph, hepatopancreas, and gonads is due to excessive proteolysis to overcome the metabolic stress (43). The observed reduction in protein content of experimental S. serrata was because of the toxic stress of TBT.

The glycogen contents in the hepatopancreas and testes were recorded as 9.27 ± 0.05 mg/g and 0.096 ± 0.0034 mg/g, respectively, in control crabs. They decreased to 8.53 ± 0.07 mg/g in the hepatopancreas and 0.085 ± 0.007 mg/g in the testes of crabs treated with 10 nL/L of TBT. At 100 nL/L of TBT exposure, the glycogen contents of the hepatopancreas and testes were 7.64 ± 0.08 mg/g and 0.044 ± 0.0025 mg/g, respectively. They further reduced in the hepatopancreas (7.05 ± 0.03 mg/g) and testes (0.021 ± 0.0033 mg/g) in 1000 nL/L TBT-exposed crabs (Figure 5). The glycogen content of the hepatopancreas and testes varied significantly in the experimental groups compared to the controls (P < 0.05). The glucose content of hemolymph was 25.03 ± 0.89 mg/dL in the control crabs, whereas the glucose contents of crabs treated with 10 nL/L, 100 nL/L, and 1000 nL/L TBT were 26.01 ± 0.63 mg/dL, 28.54 ± 0.48 mg/dL, and 30.94 ± 0.34 mg/dL, respectively. Gradual increase in glucose content was obtained with subsequent increase in TBT concentrations (Figure 5). The changes in the hemolymph glucose content of the experimental groups differed significantly (P < 0.05) compared to both of the control groups. In the present study, the glycogen content decreased in TBT-treated crabs. A 1.31-fold decrease of glycogen content in the hepatopancreas and 4.57-fold in testes was recorded at 1000 nL/L TBT. On the other hand, an increase of 0.80-fold glucose content was observed in hemolymph at 1000 nL/L TBT. Similarly, Shah et al. (44) reported changes in glycogen content in an estuarine edible clam, Anadara rhombea, exposed to TBTO. Depletion of glycogen content indicates its rapid utilization to meet the energy demands during stress conditions created by tributyltin chloride (TBTCI). The decrease in glycogen content may be due to the enhanced breakdown of glycogen to glucose through glycogenolysis under the toxic stress of TBT (45). In crustaceans, elevation of hemolymph glucose was also observed in vivo when they were subjected to stressful pollutants, such as exposure to cadmium in the red crayfish, Procambarus clarkia (46), and in the fiddler crab, Uca pugilator (47). Sarojini et al. (48) reported declined glycogen content in Barytelphusa guerini exposed to zinc sulfate. Mane and Kulkarni (49) suggested that TBT had significantly decreased the glycogen content in bivalves. Suresh (50) and Mali (51) indicated depletion of glycogen content in Lamelidens marginalis, Barytelphusa guerini, and Uca annulipes exposed to cadmium, mercury, and copper sulfate. Therefore, it appears that a decrease in the

Figure 4. Total protein content in different tissues of S. serrata treated with TBT. The values in a bar represent mean ± SD. *: F-test significant at P < 0.05.
glycogen content in different tissues of *S. serrata* was due to enhanced glycogenolysis to combat TBT-induced stress.

In conclusion, the results of our study indicate that exposure to TBT adversely affects the male reproductive performance, as well as spermatogenesis, in the mud crab *S. serrata*.

**Acknowledgments**

Financial assistance from the University Grants Commission Dr DS Kothari Postdoctoral Fellowship to Dr P Revathi is gratefully acknowledged. Thanks are due to Mr GS Samarasam for providing the hatchery facility for the experiment.

**References**


50. Suresh V. A study on the effects of heavy metals toxicity on a brackish water crab *Uca (celuca) Lacteal annulipes* (Crane 1975) of Pulicat Lake Tamil Nadu. PhD, University of Madras, Tamil Nadu, India, 2001.