

# Effects of tributyltin exposure in the embryonic stage on sex ratio and survival rate in the caprellid amphipod *Caprella danilevskii*

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**ABSTRACT:** In order to examine the biological effects of tributyltin (TBT) exposure, the caprellid amphipod *Caprella danilevskii* was exposed to 5 levels (0, 10, 100, 1000 and 10 000 ng l<sup>-1</sup>) of TBT during the embryonic stage (5 d). The male and female ratios changed dramatically in the hatched juvenile with increases in TBT concentrations; i.e. the proportion of females was found to increase to 55.6% at 10 ng l<sup>-1</sup>, 85.7% at 100 ng l<sup>-1</sup> and 81.8% at 1000 ng l<sup>-1</sup>. All specimens died in 10 000 ng TBTCI l<sup>-1</sup> within 5 d after spawning due to the acute toxic concentration for the species. No significant differences were observed to occur in the sex ratio in response to the exposure after hatching (50 d) in a previous study. Sex disturbance might therefore be induced during the embryonic stage in the caprellid. Reproductive inhibitions such as brood loss and oogenesis inhibition occurred even at 10 to 100 ng TBTCI l<sup>-1</sup> exposures in the short term in both parental females and their offspring females. The survival rate decreased drastically as the TBT concentrations increased, with the decrease being observed at TBT concentrations as low as 10 ng l<sup>-1</sup> (69%) in the offspring. In parental females, the survival rate also decreased at more than 100 ng TBTCI l<sup>-1</sup>, despite movement after 5 d into the no-TBT-added seawater. Therefore, our data suggest that nanogram concentrations of TBT similar to those encountered in coastal waters can directly affect sex ratio, reproduction and survival in the caprellid, and this phenomenon occurs at environmentally realistic concentrations in the coastal ecosystem.

**KEY WORDS:** Tributyltin · Caprellid · Sex ratio · Survival rate · Reproduction

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## INTRODUCTION

In organotin compounds, it has recently become clear that antifouling chemicals with biocide properties such as those of tributyltin (TBT) exert adverse effects on environmental components of the marine ecosystem. TBT accumulation in the marine ecosystem along the food chain is different from that of other chemicals, i.e. no significant biomagnification is observed in the higher levels of the food chain (Takahashi et al. 1999). However, high concentrations have been found in lower trophic organisms such as caprellids because of

their lower metabolic capacity to degrade TBT, causing them to accumulate butyltins (BTs) at elevated concentrations (Takahashi et al. 1999, Ohji et al. 2002).

Caprellids are an important trophic link as one of the dominant secondary producers between unicellular algae and fishes in coastal water ecosystem. Furthermore, these organisms are important prey resources for small fishes in coastal ecosystem (Fuse 1962, Caine 1989, Holbrook & Schmitt 1992). Recently, the use of caprellids in monitoring temporal and spatial changes in baseline concentrations of BTs has been proposed (Takeuchi et al. 2001, Ohji et al. 2002). However, little information is available regarding the biological effects in relation to sex ratio, survival rate, growth rate and reproduction as a function of BTs exposure. Such

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verification is likely a prerequisite to understanding the biological impacts of chemical toxicants as well as the interpretation of the BTs accumulation process in the coastal ecosystem.

The objectives of the present study were to examine the biological effects of TBT exposure during the embryonic stage of the caprellid amphipod *Caprella danilevskii* Czerniavski. The results form the basis of discussions on the fluctuation of abundance of this species in the coastal ecosystem as well as the biological impact of TBT on it.

## MATERIALS AND METHODS

**Specimens, seawater and TBT solution.** *Caprella danilevskii* was collected from the rocky shore in Uchiura Bay, central Japan. Premature females and mature males were sorted and provided for the experiments.

The seawater used for the present experiments was collected from a depth of 10 m outside Otsuchi Bay, NE Japan, where TBT concentrations at 0.5 and 10 m depths were confirmed to be less than the detection limit (Takahashi et al. 1999, Ohji et al. 2002).

A TBT-seawater solution and the control seawater were made following Ohji et al. (2002). In the present study, 5 test concentrations of TBTCI (0, 10, 100, 1000 and 10 000 ng l<sup>-1</sup>) were prepared using dilute solution. These solutions were made every week.

**Embryonic exposure experiments.** After reaching the mature stage, these parental females were allowed to copulate with males, and spawning was stimulated (first mature stage in parent). After spawning in the brood pouch, ovigerous mature females were transferred to Petri dishes containing each concentration of TBTCI, respectively. One ovigerous mature female was allocated per dish, and a total of 11 females were used for the exposure experiment (55 females in 5-concentration exposure experiments). The conditions of ovigerous parental females and egg number in the brood pouch were observed each day. The rearing method was otherwise the same as that described by M. Ohji et al. (unpubl.).

Specimens were exposed to 5 concentrations of TBTCI for 5 d, which corresponded to the period of embryonic development. After being released from the brood pouch, the juveniles were transferred into the filtered seawater containing neither TBTCI nor acetone. Two juveniles were allocated per dish, and a total of 11 to 25 specimens were used for the exposure experiment (68 juveniles in 5 exposure experiments).

Furthermore, parental females were transferred to the filtered seawater. After molting, these females were recopulated. After spawning (second mature

stage in parent), the eggs were counted at each concentration of TBTCI to examine the effects of TBTCI on the oogenesis stage.

After the TBTCI-exposed female juveniles reached maturity, they were allowed to copulate with males, and spawned (first generation of offspring). The eggs were counted each day. After juveniles were released, these juveniles continued to rear until instar II, and the sex was determined (second generation of offspring). The specimens that survived over 50 d or died during the experimental period were fixed with 10% formalin.

**Statistical analysis.** Comparisons of life span between the control condition (0 ng TBTCI l<sup>-1</sup>) and each concentration (0, 10, 100, 1000 and 10 000 ng l<sup>-1</sup>) of TBTCI were carried out by the log-rank test. A comparison of sex ratio between the control and each concentration of TBTCI was carried out by the chi-squared test. Differences in both reproduction and growth between the control and each concentration of TBTCI were tested by the Mann-Whitney *U*-test. Comparisons between the number of eggs spawned and number of juveniles hatched, and between the number of eggs spawned in the first mature stage and the number of eggs spawned in the second mature stage in the parental female were carried out by Wilcoxon's signed-rank test. All statistical analyses were carried out by StatView 5.0 (SAS Institute).

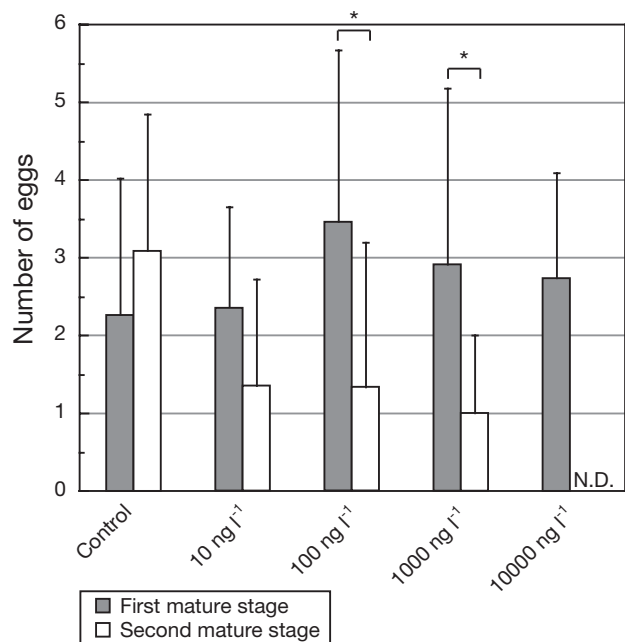


Fig. 1. Comparison of the number of eggs released into the brood pouch between the first and second mature stages in the parental female. N.D.: no data because of death of all specimens. Wilcoxon's signed-rank test, \**p* < 0.05

**RESULTS**

**Condition of parental females**

Eleven ovigerous females were allocated to each concentration compartment of TBTCI (0, 10, 100, 1000 and 10 000 ng l<sup>-1</sup>). The average (± standard deviation [SD]) number of eggs of the females ranged from 2.3 ± 1.7 to 3.5 ± 2.2 in the brood pouch (Fig. 1). No significant differences were found in the number of eggs spawned between the control and the other 4 concentrations of TBTCI (Mann-Whitney *U*-test, *p* > 0.1). Some deaths of ovigerous females exposed for 5 d were observed at more than 100 ng TBTCI l<sup>-1</sup>, and all specimens died at 10 000 ng TBTCI l<sup>-1</sup> due to the acute toxic concentration for the species (Ohji et al. 2002) (Fig. 2). Brood loss of the females also occurred at concentrations higher than 10 ng TBTCI l<sup>-1</sup>, ranging from 3 to 6 specimens, while no brood loss was observed in the control (0 ng TBTCI l<sup>-1</sup>).

The average number of eggs spawned in the brood pouch in the second mature stage ranged from 1.0 ± 1.0 to 3.1 ± 1.8 (Fig. 1). Significant differences were found in the number of eggs between the control and 3 concentrations (10, 100 and 1000 ng l<sup>-1</sup>) of TBTCI (Mann-Whitney *U*-test, *p* < 0.05 to *p* < 0.01). Furthermore, significant differences in the number of eggs were found between the first and second mature stages at 100 ng TBTCI l<sup>-1</sup> and 1000 ng TBTCI l<sup>-1</sup> (Wilcoxon's signed-rank test, *p* < 0.05) (Fig. 1).

**Survival rate in the first generation of offspring**

The survival rate (estimated from the amount of brood loss, the number of eggs in the brood pouch in

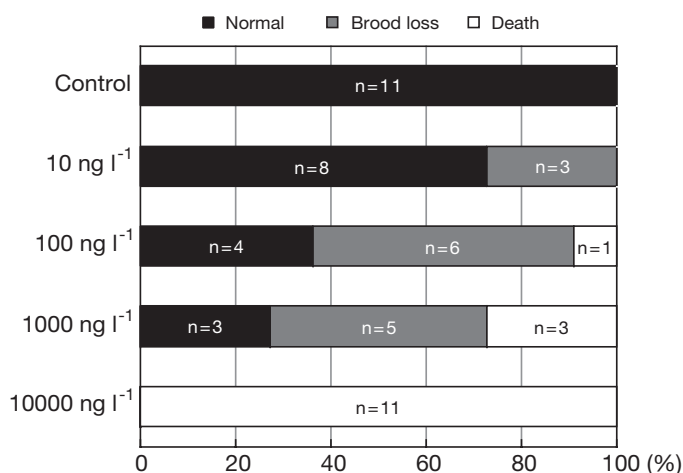


Fig. 2. Condition of the parental female in the first mature stage after 5 d exposure to tributyltin chloride (TBTCI)

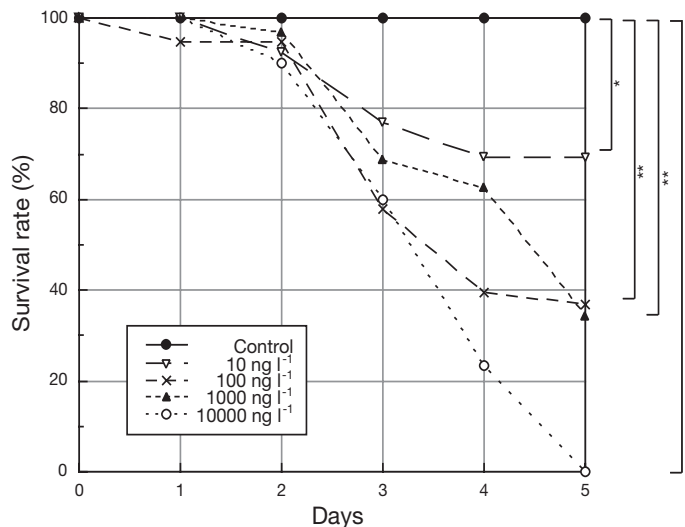


Fig. 3. Changes in the survival rate in embryos exposed to TBTCI during the embryonic stage (5 d). Log-rank test, \**p* < 0.05, \*\**p* < 0.0001

dead specimens and the total number of eggs) during the TBTCI exposure period decreased as the TBTCI concentrations increased, i.e. 69.2% at 10 ng l<sup>-1</sup>, 36.8% at 100 ng l<sup>-1</sup>, 34.4% at 1000 ng l<sup>-1</sup> and 0% at 10 000 ng l<sup>-1</sup> (Fig. 3). Significant differences were found in the survival rates between the control and the other 4 concentrations (log-rank test, *p* < 0.05 to *p* < 0.0001).

The number of juveniles hatched decreased as the TBTCI concentrations increased, i.e. 2.3 ± 1.7 in the control, 1.6 ± 1.6 at 10 ng l<sup>-1</sup>, 1.3 ± 1.9 at 100 ng l<sup>-1</sup>, 1.0 ± 1.3 at 1000 ng l<sup>-1</sup> and 0 at 10 000 ng l<sup>-1</sup> (Fig. 4). Significant differences were found between the control and 1000 ng TBTCI l<sup>-1</sup> and between the control and 10 000 ng TBTCI l<sup>-1</sup> (Mann-Whitney *U*-test, *p* < 0.05 to *p* < 0.0001). Furthermore, significant differences were found between the number of eggs spawned in the brood pouch and the number of juveniles hatched at 100 ng TBTCI l<sup>-1</sup>, 1000 ng TBTCI l<sup>-1</sup> and 10 000 ng TBTCI l<sup>-1</sup> (Wilcoxon's signed-rank test, *p* < 0.05 to *p* < 0.01) (Fig. 4).

At all concentrations, the survival rate in offspring continued to decrease despite the movement of hatched juveniles into seawater that did not contain either TBTCI or acetone (Fig. 5). Significant differences were found in the survival rate between the control and the other 4 concentrations (log-rank test, *p* < 0.0001). The survival rate of females at maturity decreased to 38.5% at 10 ng TBTCI l<sup>-1</sup>, 21.1% at 100 ng TBTCI l<sup>-1</sup>, 15.6% at 1000 ng TBTCI l<sup>-1</sup> and 0% at 10 000 ng TBTCI l<sup>-1</sup>, although the survival rate in the control was 100% (Fig. 5). The drastic change in survival rate was observed twice, at 10 to 15 d and during 35 to 45 d after spawning (Fig. 5).

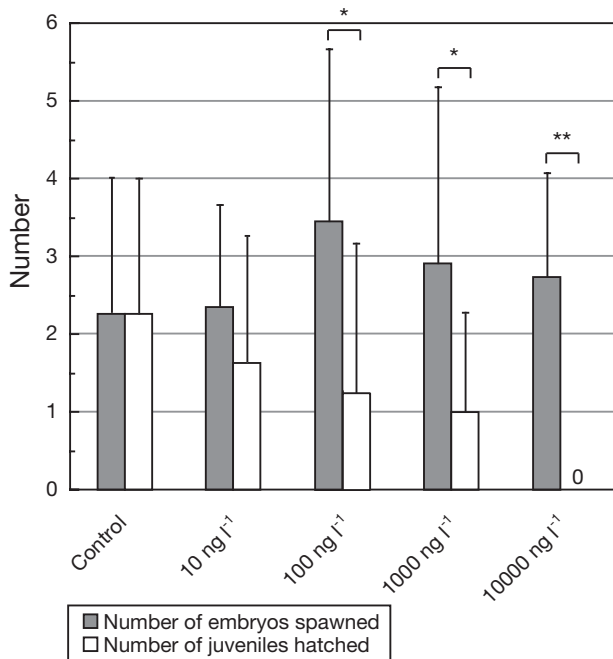


Fig. 4. Comparison between the number of eggs released to the brood pouch and the number of juveniles hatched. Wilcoxon's signed-rank test, \*p < 0.05, \*\*p < 0.01

**Sex ratio in the first generation of offspring**

The male and female ratios were 64 and 36%, respectively in the control (Fig. 6), corresponding to previous field observations (Takeuchi & Hirano 1991). However, as the TBTC1 concentration increased, the

proportion of females increased, i.e. 55.6% at 10 ng l<sup>-1</sup> (44.4% in males), 85.7% at 100 ng l<sup>-1</sup> (14.3% in males) and 81.8% at 1000 ng l<sup>-1</sup> (18.2% in males) (Fig. 6). Significant differences occurred in the sex ratio between the control and 100 ng TBTC1 l<sup>-1</sup> and between the control and 1000 ng TBTC1 l<sup>-1</sup> (chi-squared test, p < 0.01).

**Growth and reproduction in the first generation of offspring**

In the present study, no significant differences were found in the body length of each instar and in the time taken for each instar from hatch between the control and each concentration of TBTC1 in either males or females (Mann-Whitney U-test, p > 0.05). These results suggest that growth and molting are not inhibited after hatching in response to exposure to TBTC1 in the embryonic period.

In the first mature stage in offspring, oogenesis inhibition and brood loss were observed at 100 ng TBTC1 l<sup>-1</sup> and 1000 ng TBTC1 l<sup>-1</sup>. Three of 6 mature females exhibited apparent oogenesis inhibition at 100 ng TBTC1 l<sup>-1</sup> and 3 of 5 at 1000 ng TBTC1 l<sup>-1</sup>. Brood loss was apparent in 1 of 6 mature females at 100 ng TBTC1 l<sup>-1</sup> and in 2 of 5 at 1000 ng TBTC1 l<sup>-1</sup>. These abnormal ratios during the mature stage increased as the TBTC1 concentrations increased, i.e. 0% at the control and at 10 ng TBTC1 l<sup>-1</sup>, 66.7% at 100 ng TBTC1 l<sup>-1</sup> and 100% at 1000 ng TBTC1 l<sup>-1</sup>.

**Sex ratio in the second generation of offspring**

The ratios of males and females in the control and at 10, 100 and 1000 ng TBTC1 l<sup>-1</sup> were 71.4 and 28.6%, 71.4 and 28.6%, 77.8 and 22.2%, and 66.7 and 33.3%,

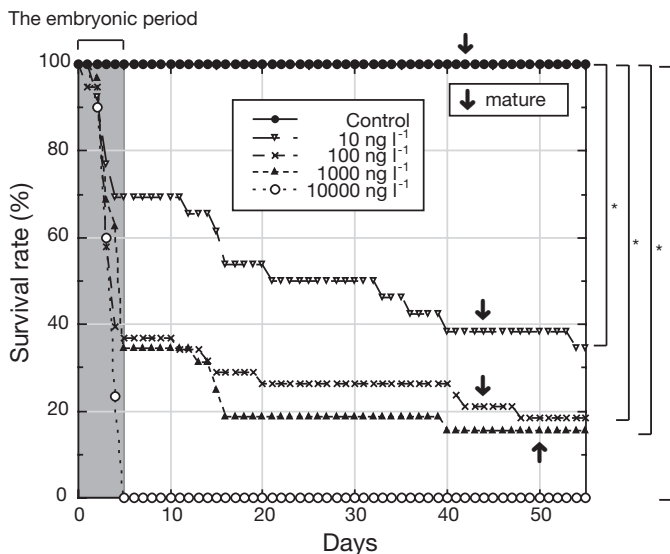


Fig. 5. Changes in the survival rate during spawning and mortality in offspring exposed to TBT during the embryonic stage and thereafter reared in seawater with no TBTC1 added. Log-rank test, \*p < 0.0001

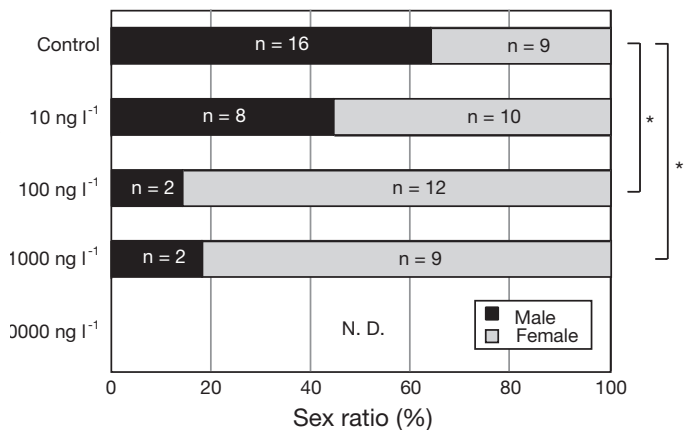


Fig. 6. Sex ratio in offspring of the first generation exposed to TBTC1 during the embryonic stage. Chi-squared test, \*p < 0.05

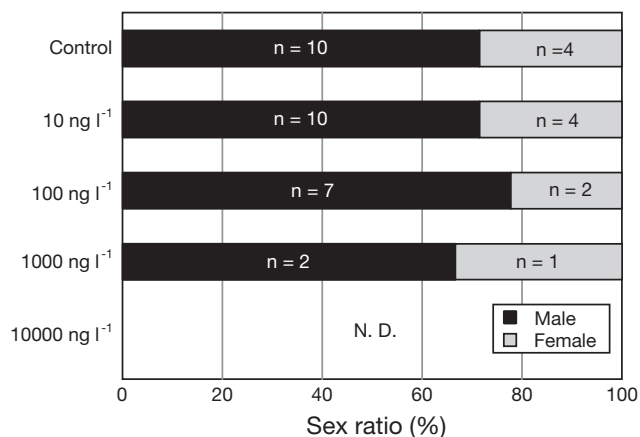


Fig. 7. Sex ratio in offspring of the second generation exposed to TBTCI during the embryonic stage

respectively (Fig. 7). No significant differences in the sex ratio between control and other concentrations of TBTCI were observed (chi-squared test,  $p > 0.5$ ). These results suggest that TBTCI exposure in the embryonic period does not affect the sex ratio in the second generation.

## DISCUSSION

The present study first demonstrates that the sex ratio in the crustacean changes dramatically even with short exposure to TBT in the embryonic period (5 d). With increases in TBT concentrations, the proportion of females was found to increase to 55.6% at 10 ng l<sup>-1</sup>, 85.7% at 100 ng l<sup>-1</sup> and 81.8% at 1000 ng l<sup>-1</sup>. However, no significant difference was observed in the sex ratio in response to long-term exposure to TBT at these levels after hatching (50 d) in a previous study (M. Ohji et al. unpubl.). These findings suggest that sex disturbance might be induced during the embryonic stage in the caprellid.

Though the sex ratio in the present study was changed in response to exposure to TBT, the number of females was almost constant (9 to 12) regardless of increases in TBT concentrations (Fig. 6). Accordingly, males seem to have a higher sensitivity to TBT than females. However, the survival rate in response to exposure to TBT has been found to be similar regardless of sex in the juvenile stage (M. Ohji unpubl.). TBT acts as a competitive inhibitor of cytochrome P450-mediated aromatase, resulting in an increase in androgens (Spooner et al. 1991, Bettin et al. 1996) and inhibition of androgen elimination (Bettin et al. 1996) in gastropods. These *in vivo* increases in androgens may result in an androgenization of organisms. Jobling et al. (1996) have shown that some of the breakdown

products of alkylphenol polyethoxylate surfactants induce vitellogenesis in male fish as an estrogen receptor-mediated effect. Therefore, receptor-mediated disruption by TBT might occur during the embryonic stage in caprellids. Nevertheless, identification of androgen receptors has not yet been reported in crustaceans. Further experiments are needed to clarify the action of TBT as a hormone disrupter in the endocrine system of caprellids.

Conspicuous reproductive inhibitions such as brood loss and oogenesis inhibition occurred in both parental ovigerous females and ovigerous females of offspring in the first generation, even at nanogram per liter levels of TBT exposure (corresponding to present TBT levels in the coastal environment) during the embryonic stage, although such inhibitions were not apparent in the control in *Caprella danilevskii*. A similar phenomenon of impairment of egg production has been reported in the copepod *Acartia tonsa* (Johansen & Møhlenberg 1987) and in the sea urchin *Paracentrotus lividus* (Girard et al. 1997, 2000) in response to TBT exposure. The cytotoxicity of TBT often results in an arrest of cellular dynamics, leading to apoptosis (Stridh et al. 1999) or a blocking of cell division (Girard et al. 1997) primarily occurring through an alteration of macromolecular syntheses (Snoeijs et al. 1988, Girard et al. 1997) or membrane-mediated processes controlling cell signaling. These processes consist primarily of a disruption of calcium homeostasis (Chow et al. 1992, Matsuoka & Igisu 1996) or calcium signaling (Corsini et al. 1997, Girard et al. 1997). Girard et al. (1997, 2000) have found that TBT inhibits sea urchin egg cleavage by altering many of the cellular events related to cell division. Furthermore, Girard et al. (2000) have suggested that the inhibition occurs in response to a few hours of TBT exposure and is sufficient to damage the organism during its embryonic life. A similar inhibition related to egg cleavage might occur in the caprellid, resulting in brood loss and oogenesis inhibition in the species. In the present study, impaired reproductive success also occurred in the short-term exposure to TBT during the embryonic stage (5 d). Therefore, our data suggest that nanogram concentrations of TBT similar to those encountered in coastal waters can directly affect reproduction in the caprellid and that this phenomenon is an environmentally realistic scenario in the coastal ecosystem.

The survival rate decreased drastically as TBT concentrations increased in the present study, with the decrease being found even at 10 ng l<sup>-1</sup> (69%) despite the short exposure period corresponding to the embryonic period in offspring of the first generation. In parental females, the survival rate also decreased at TBT concentrations more than 100 ng l<sup>-1</sup> despite movement of females into the no-TBT-added seawater after

the 5 d exposure. These results suggest that TBT exposure even at present levels in ambient water and even for a short period might influence the population in the coastal environment.

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