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Spermiotoxicity and embryotoxicity of triphenyltin in the sea urchin *Paracentrotus lividus* Lmk†

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The most important sources of pollution by triphenyltin (TPT) in marine coastal ecosystems are its employment as a fungicide in agriculture and, in association with tributyltin, as a biocide in antifouling paints. In this study, spermiotoxicity and embryotoxicity (from post-fertilization to pluteus stage) experiments were carried out to clarify better the ecotoxicological effects of TPT during the development of the sea urchin *Paracentrotus lividus*. Sperm exposed to triphenyltin acetate (TPTA) for 60 min showed a significantly reduced capability to fertilize eggs even at the lowest TPTA concentration of 0.1 $\mu g\ l^{-1}$. In proportion to increasing TPTA concentrations, the percentage of fertilized eggs decreased, falling to 45% at 10 $\mu g\ l^{-1}$, the maximum TPTA concentration tested. In embryotoxicity experiments at 48 h post-fertilization, the length of the pluteus somatic rods was significantly reduced ($P < 0.001$) at 1.5 $\mu g\ l^{-1}$ and above. Progressive increases in skeletal anomalies were also detected, which were highly significant ($P < 0.001$) at 2 $\mu g\ l^{-1}$. Embryonic development was greatly slowed at the highest TPT concentrations: embryos never reached the pluteus stage at 5 $\mu g\ l^{-1}$, and development was blocked at the gastrula stage at 10 $\mu g\ l^{-1}$. As observed in previous experiments using butyltin compounds, embryotoxic effects on both skeletal deposition and blocked development are presumed to be due to interference of TPT with intracellular calcium homeostasis. Sea urchin gametes are more sensitive to TPT than embryos, this condition emphasising the environmental risk due to TPT contamination.

KEYWORDS: triphenyltin; sea urchin; *Paracentrotus lividus*; spermiotoxicity; embryotoxicity

The biocidal use of organotin compounds includes formulations of insecticides, fungicides, bactericides, wood preservatives and antifouling agents in the form of triorganotins, in particular tributyltin (TBT) and triphenyltin (TPT). It was estimated that the annual world production of organotins reached 50 000 tonnes in 1992; despite legislative restrictions about the use of paints containing organotins, the consumption of and the contamination by triorganotin biocides are still causes of concern for aquatic life.[1–3]

TPT enters freshwater and marine ecosystems after use in antifouling paints as a co-toxicant of TBT on vessels, nets, buoys, and all materials remaining underwater for long periods.[4,5] Another source of contamination is leaching from soil, because of its use in agriculture as a non-systemic fungicide and a rodent and insect repellent.[6,7]

Few data are available from the literature concerning the contamination of the marine environment by TPT. Along Mediterranean coasts a maximum TPT concentration of about 0.1 $\mu g\ l^{-1}$ was reported in water samples from a marina near Barcelona.[8] More recent data indicated concentrations ranging from <1 to 28.6 $ng\ l^{-1}$ along the Côte d'Azur, France.[2] TPT concentrations of up to 3800 $ng\ g^{-1}$ dry weight were found in sediment samples from Baltic Sea marinas, showing the considerable input and persistence of the pollutant and long-term contamination of marine sediments.[9] Marine organisms are subjected to TPT exposure and can accumulate it from sediments, water and food; some studies have demonstrated its deleterious effects in non-target species. TPT has been detected in various tissues and organs of freshwater and marine fish;[10–12] concentrations in

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muscle have been found to range from <0.001 to 0.130 mg kg^{-1} wet weight, with higher values in fish collected in marine coastal areas near Osaka (Japan) with respect to freshwater specimens.[12] In a lake foodweb in the Netherlands, Stäb *et al.*[11] reported an accumulation potential that was higher for TPT than for TBT: high levels of biodegradation products of TBT, but not of TPT, were found in the liver of fish and birds, indicating the latter as a more persistent and less easily metabolized compound.

Like TBT, TPT is considered as an endocrine disruptor because it induces 'imposex' (imposition of male sexual organs on female) in various species of gastropod. A positive correlation was found between TPT concentrations in tissues, ranging from 3 to 2460 ng g^{-1}, and the degree of imposex in females of *Thais bronni*,[13] *Thais clavigera*[13–15] and *Bolinus brandaris*,[16] although it did not induce imposex in *Nucella lapillus*.[17]

The effects of TPT have also been studied in the early life stages of fish and molluscs. An LC_{50} (96 h) value for TPTH of 7.1 μg l^{-1} was reported in fathead minnow larvae (*Pimephales promelas*).[18] Moreover, TPT was rapidly accumulated and apparently not metabolized in larvae of the European minnow (*Phoxinus phoxinus*).[19] Larval mortality in this species increased at TPT concentrations ≥3.9 μg l^{-1}, and complete mortality occurred after 7 days and 9 days at 15.9 μg l^{-1} and 5.1 μg l^{-1} respectively.[7] In 24 h and 48 h acute toxicity tests on the larvae of the rock shell *T. clavigera*, LC_{50} values for TPT were 8.6 μg l^{-1} and 4.6 μg l^{-1} respectively.[20]

In several studies, sea urchin gametes and embryos have been recognized as useful tools for evaluating the toxicity of xenobiotics, such as heavy metals and pesticides,[21,22] as well as for monitoring marine coastal environments subjected to various sources of pollution.[23–25]

In the present work, the toxic effects of TPT were evaluated on the sperm activity and embryonic development of the sea urchin *Paracentrotus lividus*. It is well known that embryos and larvae are less tolerant to pollutants than adults of the same species and that they represent critical stages in the life history.[26,27] Therefore, assessing the toxicity of environmental contaminants that affect reproductive success is essential, in order to highlight their potentially detrimental effects on marine and freshwater ecosystems.

MATERIALS AND METHODS

Sea urchins were collected in the littoral zone outside the Lagoon of Venice, kept in the laboratory in sea water, changed every 2 days, maintained at a temperature of 16 ± 1 °C and a salinity of 35 ± 1‰, and fed with *Ulva laetevirens*. Specimens were used after an acclimatization period of about 1 week.

As the high degree of interindividual variability in sensitivity to environmental conditions is well known in *P. lividus*, each experiment was performed with gametes from a single male and a single female according to Bougis.[28] Gametes were obtained by 0.5M KCl injection into the coelomic cavity through the peristomal membrane.[29] TPT, purchased from Sigma as acetate, was dissolved in 95% ethanol (stock solution). The spermio- and embryo-toxicity of this organotin compound were assessed in six and four experiments respectively, at concentrations ranging from 0.1 to 10 μg l^{-1}. Experimental solutions were prepared by diluting the stock solution into artificial sea water (ASW; 35‰ salinity). ASW was mainly used to avoid the interference of unknown pollutants, which may be present in natural sea water, on the experimental results.[30] The maximum ethanol concentration (2.5 μl l^{-1}) tested in solvent controls had no effect on either the fertilization or embryonic growth of sea urchin eggs and embryos.

Spermiotoxicity experiments

All bioassays were performed using a constant sperm:egg ratio (1250:1) in order to standardized experimental conditions, according to the recommendations of Dinnel *et al.*[31] Some drops of sperm, collected dry directly from gonopores, were resuspended in 5 ml ASW; aliquots of this suspension were diluted 1:10 and fixed with 10% neutralized formalin, and sperm density was then determined using a Neubauer haemacytometer.

Five replicates per TPT concentration were set up: sperm was exposed to TPT for 60 min in glass test tubes containing 10 ml of experimental solution and kept in an incubator at a constant temperature of 15 °C.[24,31] Eggs (200 ml^{-1}) were then added and fertilization allowed to proceed; after 20 min, samples were fixed with 10% neutralized formalin.

Fertilization success was determined by assessing the presence or absence of the fertilization membrane in subsamples of 200 eggs per replicate.

Embryotoxicity experiments

Gametes were mixed in filtered (0.45 μm) natural sea water; after fertilization, eggs were washed and distributed in glass beakers containing 100 ml of TPT solution to obtain a suspension of approximately 50 eggs per millilitre. Five replicate cultures per concentration were set up and placed in an incubator at 22 °C. One culture was fixed with 10% neutralized formalin after 24 h, and the other four were fixed 48 h after fertilization. Three cultures were used to measure the length of the somatic rods of 180 four-armed echinoplutei,[32] and the fourth, and the one fixed after 24 h, to determine the frequencies of various developmental stages and growth anomalies in 200 individuals.

For statistical comparisons, analysis of variance (Anova) and the *G*-test were performed according to Sokal and Rohlf.[33]

RESULTS

The results of six spermiotoxicity experiments are shown in Fig. 1 as mean percentages of fertilized eggs at the various

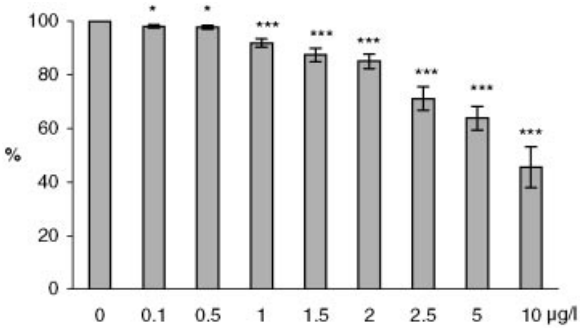

Figure 1. Percentages of fertilized eggs at various TPTA concentrations. Each bar represents the mean ± se of six experiments, expressed as percentage of controls. Anova: * $P < 0.05$; *** $P < 0.001$.

TPTA concentrations tested (0.1, 0.5, 1, 1.5, 2, 2.5, 5, 10 µg l^{-1}). The fertilization rate was significantly reduced ($P < 0.05$) in comparison with controls, even at the lowest concentrations of 0.1 and 0.5 µg l^{-1}. From 1 to 10 µg l^{-1}, a progressive, highly significant ($P < 0.001$) concentration-dependent decline in the percentage of fertilized eggs was observed, falling to 55.5% at the highest concentration tested (10 µg l^{-1}).

In embryotoxicity experiments, performed at the same TPTA concentrations as the spermiotoxicity ones, the frequency of the various developmental stages 24 h after fertilization showed no significant differences with respect to controls at 0.1 and 0.5 µg l^{-1} (Fig. 2). At 1 µg l^{-1}, development was significantly influenced by TPT exposure only in two experiments ($P < 0.01$ and $P < 0.001$). From 1.5 to 10 µg l^{-1}, slowing of development increased significantly with TPTA concentration in all experiments ($P < 0.001$). The percentage of young plutei decreased remarkably (mean reduction 64%) at 1.5 µg l^{-1}, whereas the young pluteus stage was never observed at concentrations from 2.5 to 10 µg l^{-1}, and only the gastrula stage was found at 10 µg l^{-1}.

No significant differences were observed in embryonic development 48 h after fertilization for TPTA concentrations of 0.1 to 1 µg l^{-1} (Fig. 3). The percentage of echinoplutei decreased significantly ($P < 0.001$) only in one out of four experiments at 1.5 µg l^{-1} and in three at 2 µg l^{-1}. At TPTA concentrations of 2.5 µg l^{-1} and above the frequency of developmental stages was always significantly different ($P < 0.001$) from that of controls; embryos never reached the pluteus stage at 5 µg l^{-1}, and development was blocked at the gastrula stage at 10 µg l^{-1}.

Larval growth, expressed as the mean length of pluteus somatic rods 48 h post-fertilization, is shown in Fig. 4. A significant reduction ($P < 0.05$) was observed in one out of

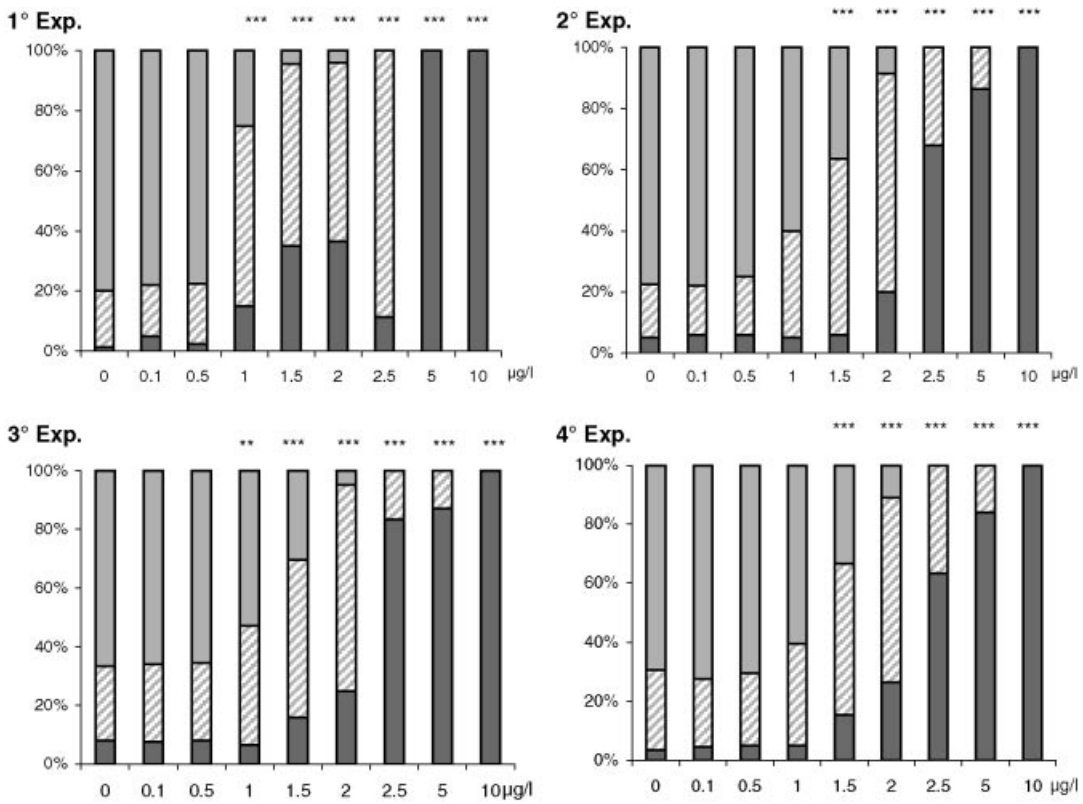

Figure 2. Percentages of gastrulae (dark grey), prisms (oblique lines) and young plutei (light grey) 24 h post-fertilization in four experiments at various TPTA concentrations. *G*-test: ** $P < 0.01$; *** $P < 0.001$.

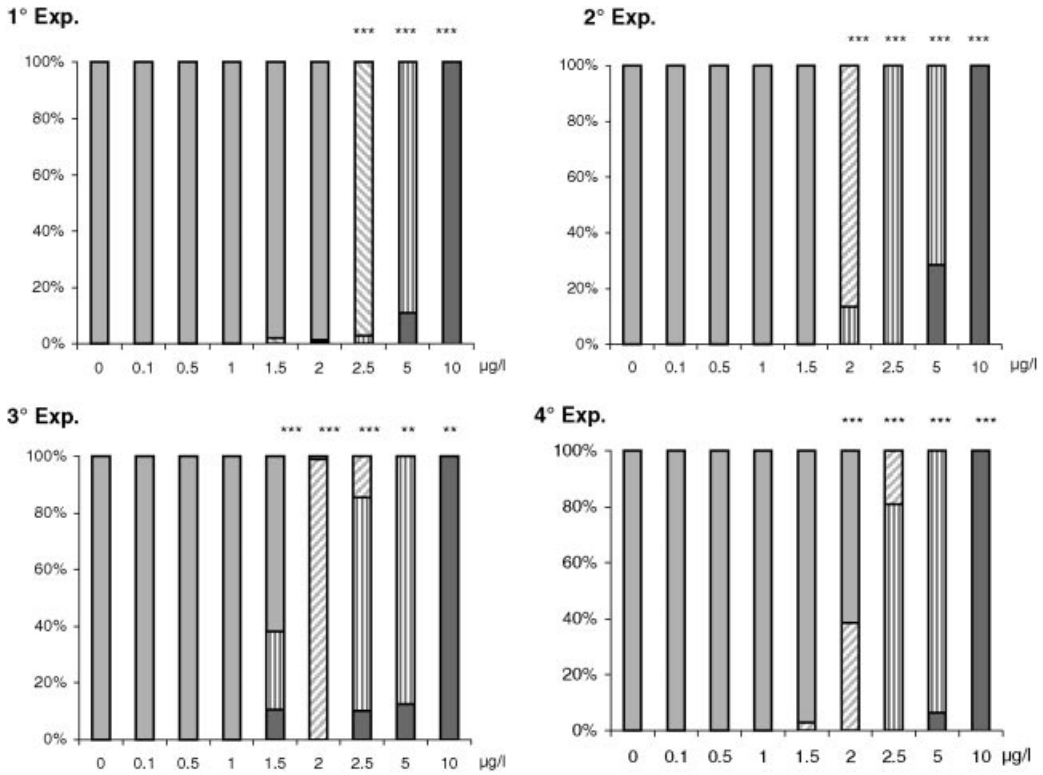

Figure 3. Percentages of gastrulae (dark grey), prisms (vertical lines), young plutei (oblique lines) and plutei (light grey) 48 h post-fertilization in four experiments at various TPTA concentrations. *G*-test: *** $P < 0.001$.

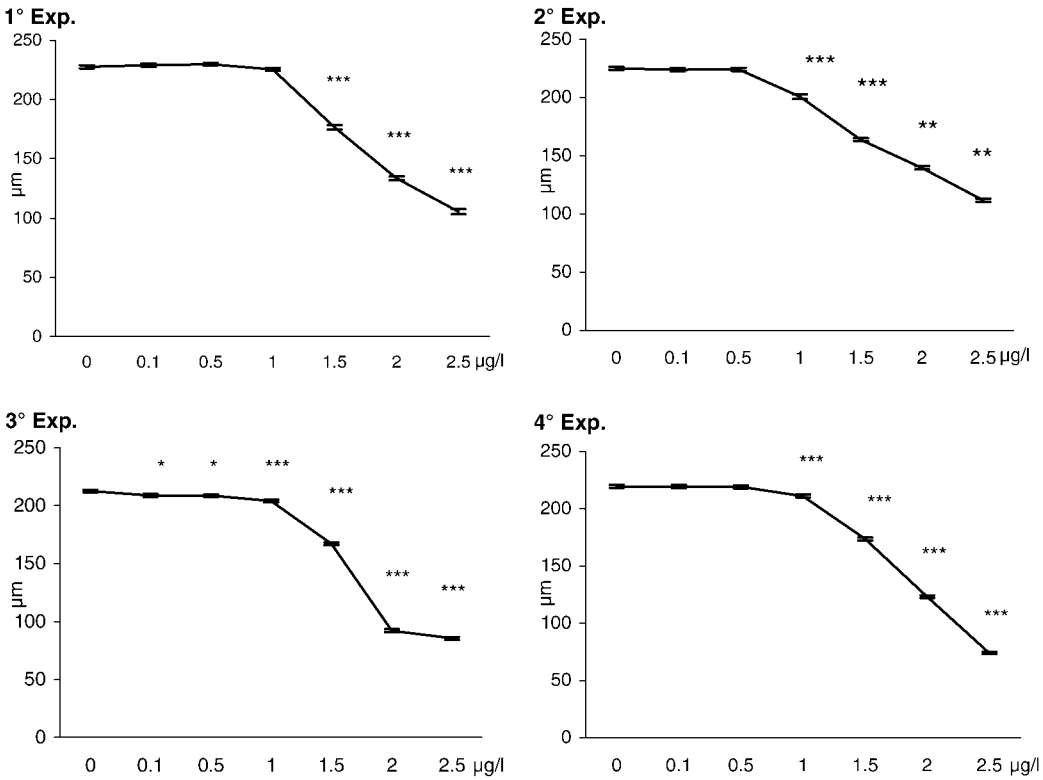

Figure 4. Mean lengths ± se of pluteus somatic rods 48 h post-fertilisation in four experiments at various TPTA concentrations ($n = 180$). Anova: * $P < 0.05$; *** $P < 0.001$.

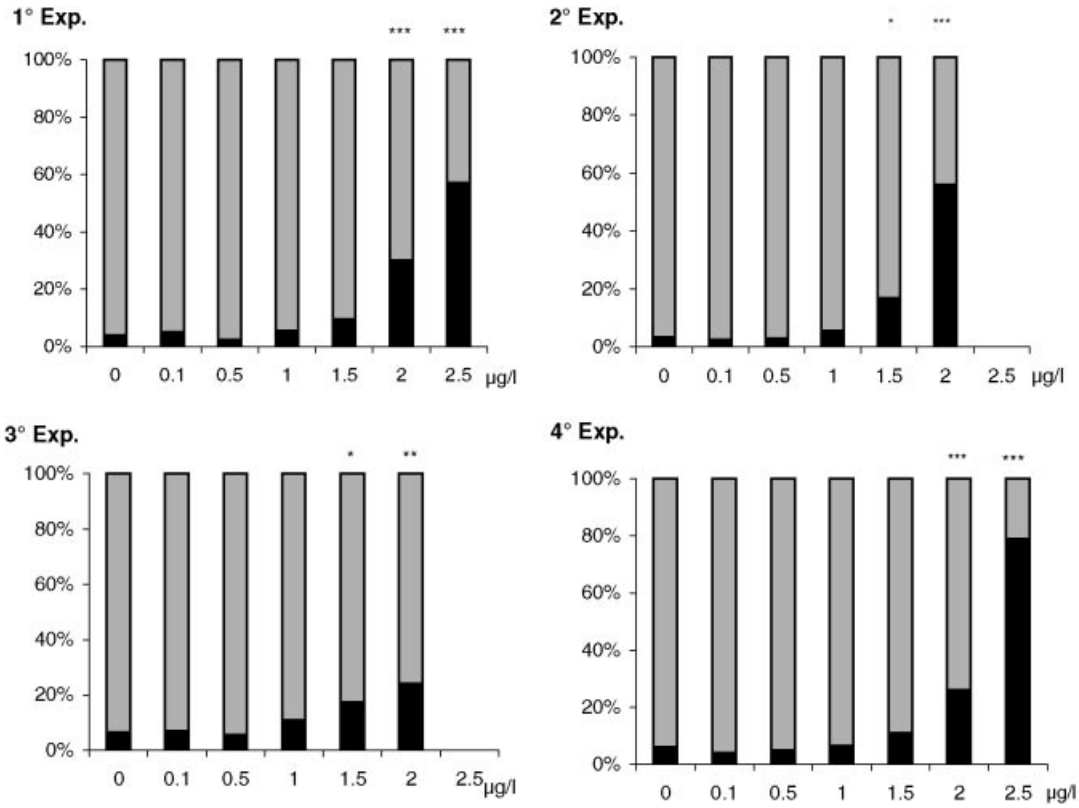

Figure 5. Frequencies of plutei without (grey) and with (black) skeletal anomalies 48 h post-fertilization in four experiments at various TPTA concentrations. *G*-test: * $P < 0.05$; *** $P < 0.001$.

four experiments at 0.1 and 0.5 µg l^{-1} and in three experiments at 1 µg l^{-1} ($P < 0.001$). Higher TPTA concentrations always caused a significant reduction ($P < 0.001$) with respect to controls; above 2.5 µg l^{-1} the embryos were not able to reach the pluteus stage.

Skeletal anomalies were also detected (Figs 5 and 6): no significant differences were reported with respect to controls from 0.1 to 1 µg l^{-1}. The frequency of anomalies increased progressively at concentrations higher than 1 µg l^{-1} up to 2.5 µg l^{-1}, the maximum concentration that still allowed embryos to reach the pluteus stage.

DISCUSSION

This study was performed to evaluate the effects of TPT on sperm activity and embryonic/larval development of the sea urchin *P. lividus*, which is widespread along Mediterranean coasts.

In spermiotoxicity experiments, TPT caused a concentration-dependent decrease of fertilization success. The effects of exposure were observed at concentrations as low as 0.1 µg l^{-1}, and fertilization was inhibited in more than 50% of eggs tested at the highest concentration of 10 µg l^{-1}. TPT may act by causing a decrease in fertilization capability and/or sperm viability. However, it did not affect embryonic development until 1 µg l^{-1}. Both early development and larval growth were significantly reduced in all experiments at 1.5 µg l^{-1}, embryos did not reach the pluteus stage at 5 µg l^{-1}, and they were blocked at the gastrula stage at 10 µg l^{-1}. On the whole, TPT produced increased slowing in embryonic development in a concentration-dependent manner.

The toxic action of TPT on the embryonic development of marine invertebrates is not so easy to explain, considering the lack of information available in the literature, which mainly focuses on the more toxic TBT compounds. Nevertheless, it is hypothesized that the mechanisms of action of TPT are very similar to those of TBT. Both compounds can block the embryonic development of the ascidian *Styela plicata*, giving rise to anomalous embryos, with irreversible effects, probably due to inhibition of microtubule polymerization during mitosis.[34] Moreover, in the early life stage of the European minnow *P. phoxinus*, the toxicity of both TBT and TPT is essentially similar, as revealed by survival, morphological and histopathological data.[7]

TBT also alters the activity of the hepatic microsomal cytochrome P-450 and associated enzyme in the scup *Stenotomus chrysops*,[35] and a similar effect is induced by TPT in rat liver cells.[36] Furthermore, TBT affects first and

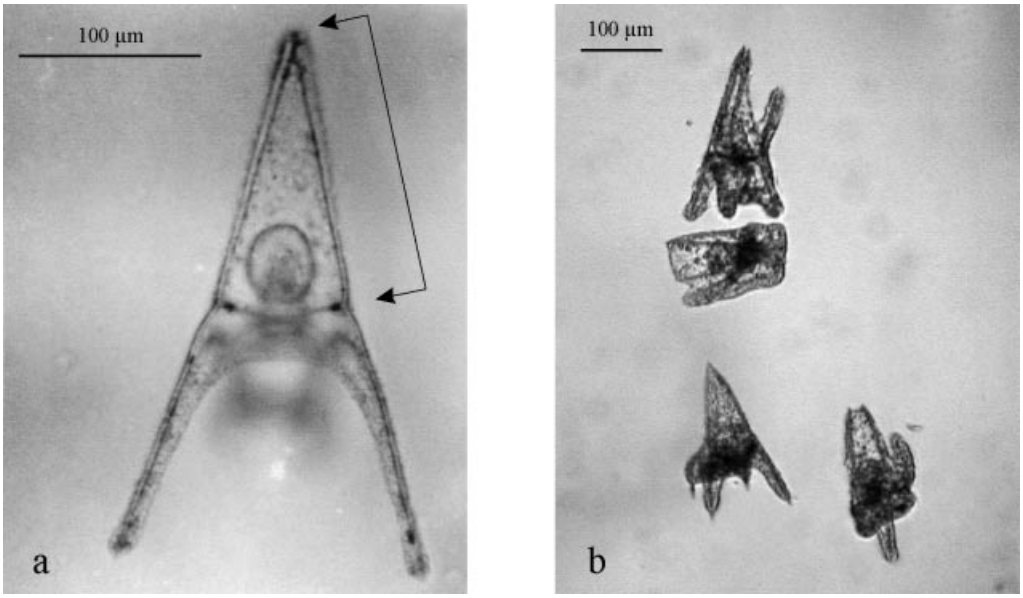

Figure 6. Plutei of *P. lividus* at 48 h post-fertilization: (a) control pluteus (arrows indicate the length of the somatic rod); (b) plutei exposed to TPTA concentration of 1.5 µg l^{-1} showing severe skeletal anomalies.

second cleavages in the eggs of the sea urchin *P. lividus*, inhibiting intracellular sequestration of Ca^{2+} into the reticular compartment at low concentrations. However, high TBT levels increase egg plasma membrane permeability to Ca^{2+} and Na^{+} ions.[37] In gametes of the ascidian *Phallusia mammillata*, TBT inhibits the Na^{+} currents of unfertilized eggs and has a deleterious effect on the transduction mechanism of the sperm signal into eggs or on the sperm-activated channels of the egg membrane.[38]

TPT probably acts in a similar manner on *P. lividus* sperm, preventing the cellular mechanism of the sperm signal and decreasing sperm viability because of its cytotoxicity. Similar toxicity levels were observed in fertilization tests on *P. lividus* after sperm exposure to TBT. However, the percentage of fertilized eggs falls more rapidly with increasing TBT concentrations, with fertilization being almost completely inhibited at 10 µg l^{-1} (Marin, unpublished data).

As for embryotoxic effects, we hypothesize that TPT interacts with calcium homeostasis during embryonic and larval development of *P. lividus*, causing alteration of intracellular Ca^{2+} levels at low concentrations and inhibiting the ion flow during skeletal deposition. Higher TPT concentrations can slow embryonic development further to the point of total inhibition, which causes death of exposed embryos.

Similar toxic effects have been observed in TBT-exposed embryos of *P. lividus*:[39] larval growth is significantly affected at 0.01 µg l^{-1}, and 1 µg l^{-1} is the maximum concentration allowing embryos to reach the pluteus stage at 48 h post-fertilization. At the highest TPT concentration used in the present work (10 µg l^{-1}), all embryos were at the gastrula stage, whereas in TBT treatment at the same concentration they were blocked at the earliest morula stage.[39]

The results obtained for both TPT in this study and for TBT by Marin *et al.*[39] confirm the relative toxicity levels of these organotin compounds as reported by other authors,[7,34] with TBT being more toxic than TPT. Moreover, TPT is revealed to be more spermotoxic than embryotoxic, as fertilization is significantly inhibited at a concentration ten times lower than that affecting larval growth.

Although few data are available concerning environmental contamination by TPT, Alzieu *et al.*[8] found concentrations up to 0.1 µg l^{-1} along the Mediterranean coast of Spain, whereas TPT concentrations up to 0.2 µg l^{-1} were detected by Fent and Hunn[10] in boat harbours.

However, it was observed that environmental TPT levels can increase up to 1.5 µg l^{-1} in areas adjacent to agricultural fields,[40] where this organotin compound is commonly employed as a fungicide and herbicide. In the Lagoon of Venice, a progressive contamination of sediments and water may reasonably be inferred owing to the excessive and uncontrolled use of TPT as repellent against *Cercospora beticola* and lepidopteran larvae on sugar beet leaves.[41] As a consequence, an environmentally relevant impact of TPT is hypothesized, and investigations are in progress to evaluate TPT levels in the Lagoon.

Since, in our study, the lowest concentration tested (0.1 µg l^{-1}) significantly reduced the fertilization capability and then reproductive success of the sea urchin *P. lividus*, a condition of potential risk for the preservation of coastal biocenoses is highlighted, as well as the need for more restrictive regulation of TPT use.

REFERENCES

1. Mercier A, Pellettier E and Hamel JF. *Aquat. Toxicol.* 1994; **28**: 259.
2. Tolosa I, Readman JW, Blaevoet A, Ghilini S, Bartocci J and Horvat M. *Mar. Pollut. Bull.* 1996; **32**(4): 335.
3. Morcillo Y and Porte C. *Environ. Pollut.* 2000; **107**: 47.
4. Balls PW. *Aquaculture* 1987; **65**: 227.
5. Nichols JA. *Environ. Manag.* 1988; **12**: 243.
6. Schramel P, Samsahl K and Pavlu J. *Int. J. Environ. Stud.* 1973; **5**: 37.
7. Fent K and Meier W. *Arch. Environ. Contam. Toxicol.* 1994; **27**: 224.
8. Alzieu C, Michel P, Tolosa I, Bacci E, Mee LD and Readman JW. *Mar. Environ. Res.* 1991; **32**: 261.
9. Biselli S, Bester K, Huhnerfuss H and Fent K. *Mar. Pollut. Bull.* 2000; **40**(3): 233.
10. Fent K and Hunn J. *Environ. Sci. Technol.* 1991; **25**: 956.
11. Stäb JA, Traas TP, Stroomberg G, van Kesteren J, Leonards P, van Hattum B, Brinkman UAT and Cofino WP. *Arch. Environ. Contam. Toxicol.* 1996; **31**: 319.
12. Harino H, Fukushima M and Kawai S. *Arch. Environ. Contam. Toxicol.* 2000; **39**: 13.
13. Horiguchi T, Shiraishi H, Shimizu M and Morita M. *J. Mar. Biol. Assoc. U.K.* 1994; **74**: 651.
14. Horiguchi T, Shiraishi H, Shimizu M and Morita M. *Environ. Pollut.* 1997; **95**: 85.
15. Shim WJ, Kahng SH, Hong SH, Kim NS, Kim SK and Shim JH. *Mar. Environ. Res.* 2000; **49**: 435.
16. Solé M, Morcillo Y and Porte C. *Environ. Pollut.* 1998; **99**: 241.
17. Bryan GW, Gibbs PE and Burt GR. *J. Mar. Biol. Assoc. U.K.* 1988; **68**: 733.
18. Jarvinen AW, Tanner DK, Kline ER and Knuth ML. *Environ. Pollut.* 1988; **52**: 289.
19. Fent K, Lovas R and Hunn J. *Naturwissenschaften* 1991; **78**: 125.
20. Horiguchi T, Imai T, Cho HS, Shiraishi H, Shibata Y, Morita M and Shimizu M. *Mar. Environ. Res.* 1998; **49**: 469.
21. Phillips BM, Anderson BS and Hunt JW. *Environ. Toxicol. Chem.* 1997; **3**: 453.
22. Larrain A, Riveros A, Silva J and Bay-Schmith E. *Bull. Environ. Contam. Toxicol.* 1999; **62**: 749.
23. Kobayashi N. *Publ. Seto Biol. Lab.* 1977; **24**: 9.
24. Pagano G, Anselmi B, Dinnel PA, Esposito A, Guida M, Iaccarino M, Melluso G, Pascale M and Trieff NM. *Arch. Environ. Toxicol.* 1993; **25**: 20.
25. Marin MG, Da Ros L, Moschino V and Campesan G. *Aquat. Ecosys. Health Manag.* 2001; **4**: 215.
26. Connor PM. *Mar. Pollut. Bull.* 1972; **3**: 190.
27. Ringwood AH. *Arch. Environ. Contam. Toxicol.* 1990; **19**: 338.
28. Bougis P. *Helgol Wiss. Meeresunters.* 1967; **15**: 59.
29. Tyler A. *Collect. Net* 1949; **19**: 19.
30. Marin MG, Bressan M and Brunetti R. *Acta Embryol. Morphol. Exper.* 1987; **8**: 31.
31. Dinnel PA, Link JM and Stober QJ. *Arch. Environ. Contam. Toxicol.* 1987; **16**: 23.
32. Bressan M, Marin MG and Brunetti R. *Hydrobiologia* 1995; **304**: 175.
33. Sokal RR and Rohlf FJ. *Biometry*. WH Freeman & Co: New York, 1981.
34. Cima F, Ballarin L, Bressa G, Martinucci G and Burighel P. *Ecotoxicol. Environ. Saf.* 1996; **35**: 174.
35. Fent K and Stegeman J. *Aquat. Toxicol.* 1991; **20**: 159.
36. Nebbia C, Ceppa L, Dacasto L and Carletti M. *Toxicol. Environ. Health* 1999; **56**: 433.
37. Girard JP, Ferrua C and Pesando D. *Aquat. Toxicol.* 1997; **38**: 225.
38. Franchet C, Goudeau M and Goudeau H. *Aquat. Toxicol.* 1999; **44**: 213.
39. Marin MG, Moschino V, Cima F and Celli C. *Mar. Environ. Res.* 2000; **50**: 231.
40. Stäb JA, Cofino WP, van Hattum B and Brinkman UAT. *Fresenius J. Anal. Chem.* 1993; **347**: 247.
41. Cima F, Ballarin L, Bressa G, Sabbadin A and Burighel P. *Mar. Chem.* 1997; **58**: 267.