Bull. Environ. Contam. Toxicol. (1987) 39:1012–1019

Bulletin of Environmental Contamination and Toxicology

Acute Toxicity of Tributyltin Chloride to Embryos and Larvae of Two Bivalve Mollusks, *Crassostrea virginica* and *Mercenaria mercenaria*

Morris H. Roberts, Jr.

Virginia Institute of Marine Science, School of Marine Science, College of William and Mary, Gloucester Point, Virginia 23062

Tributyltin (TBT) has been used in antifouling paints since the early 1960s. TBT is highly effective for control of macrofouling invertebrates on boat hulls in marine waters, and as a result has gradually come to dominate some segments of the market (Evans and Karpel 1985). TBT has also been considered for use in cooling systems of electric power generating plants (Burton 1980). In both applications, TBT is leached from antifoulant coated surfaces. While TBT degrades to less toxic forms, it persists for significant periods in natural waters (Maguire and Huneault 1981).

French researchers were the first to relate a biological effect to the presence of TBT. Pacific oysters (Crassostrea gigas) in the Baie d'Arcachon exhibited shell thickening resulting from exposure to TBT leaching from antifoulant coatings on boats (Alzieu et al. 1980, 1982). Similar abnormalities observed in Pacific oyster shells along the English coast, initially attributed to high sediment concentrations (Key et al. 1976), were shown to result from TBT (Waldock and Thain 1983) There is no evidence of shell thickening American oysters, C. virginica.

Tributyltin (TBT) is reported to be acutely toxic to many marine organisms at extremely low concentrations, with 96-h LC50 values ranging from 0.5 ug TBTO/L for a mysid shrimp to 20-60 ug TBTO/L for a mussel (US Navy 1983). About half the 7 day-old larvae of the mussel (Mytilus edulis) exposed to 0.1 ug TBTO/L were dead after 15 days, and all those exposed to 10 ug/L were dead after 5 days (Beaumont and Budd 1984). Acute mortality of oyster (C. gigas) larvae exposed to TBT acetate occurs at concentrations as low as 1 ug/L (as TBT acetate) (His and Robert 1980; Robert and His 1981). These data suggest a 48-h LC50 >5 ug/L, a 96-h LC50 of 4 ug/L, and a 216-h LC50 of about 1 ug/L. Thain (1983) reported a 48-h LC50 for C. gigas of 1.6 ug/L (age of larvae unspecified). In sharp contrast, the 96-h LC50 for hard clam larvae (Mercenaria mercenaria) is reported to be 0.015 ug TBTO/L) (Becerra-Huencho 1984), by far the lowest acute concentration yet reported.

Contribution No. 1396 from the Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA 23062.
Send reprint requests to M.H. Roberts at the above address.

The objectives of the present study were to verify the results for M. mercenaria larvae, to extend the observations to fertilized clam embryos and to determine the acute toxicity of tributyltin oxide (TBTO) to embryos and larvae of the native oyster, C. virginica.

MATERIALS AND METHODS

Clams were obtained from two sources; one group was purchased from Biosphere, Inc. (New Brunswick, NJ) and a second group was collected locally from the York River, VA. Oysters were collected variously from the James and Rappahannock Rivers, VA. Broodstocks of both species were conditioned by maintaining them in heated flowing York River water (24-26 ^{o}C supplemented with cultured algae until they developed ripe gametes and then held at 19-20 ^{o}C until spawned.

Broodstock clams or oysters were induced to spawn by thermal shock and sperm introduction (Loosanoff and Davis 1963). Eggs from each female were fertilized with sperm from one or more males. For embryo tests, the fertilized gametes were maintained at the spawning temperature (28^{o}C) until the zygotes reached the 4-16 cell stage at which time an accurate count of fertilized embryos could be made. For larval tests, embryos were diluted to about 50/mL and held at the spawning temperature for 24 h by which time the embryos had developed to the straight-hinge stage. About 35% of the hard clam larvae were trochophores or shell-less veligers at the start of the larval tests, and did not develop further under any treatment including the control. While no explanation can be offered for this failure to develop further, such is regularly observed even in large cultures (Castagna, Virginia Institute of Marine Science, 1987, personal communication).

Tests with both stages of oyster and clam larvae were performed using a static replacement procedure. For each test a 7-step logarithmic concentration series (0.056 to 1.8 ug/L or 0.180 to 5.6 ug/L) was used. In all but one experiment, glacial acetic acid was used as a carrier for the tributyltin chloride. The amount of carrier was in all cases 16 ul/L. Both a diluent water and a carrier control were included in the experimental design. The pH was reduced approximately 0.6 pH units in all cases by the glacial acetic acid, with a final pH of 7.1 or greater.

Embryo tests were initiated with 4- to 16-cell stage embryos, larval tests with straight-hinge larvae. In embryo tests, the density was about 40/mL and in larval tests, about 10/mL. Triplicate samples of embryos or larvae were counted prior to dilution to establish the initial larval concentration. For embryo tests, only fertilized eggs were counted. For larval tests, trochophores and straight-hinge larvae were counted and recorded separately. Embryos or larvae were then introduced into the test medium and exposed at room temperature for 24 h. At that time, the larvae in each test solution were collected on a 35 um screen and resuspended in 100 mL of water, counted, and returned to

freshly prepared medium. A sample of used medium from two test concentrations was set aside for chemical analysis. This procedure was repeated after the second 24 h exposure. For larval tests, cultured algae were added to the test medium to yield 10^4cells/mL using a mixture of Pavlova lutheri and Isochrysis galbana.

York River water was the diluent for all experiments. Water was passed through a 1-um filter and extracted by passage through an activated carbon filter. Carbon filtration was used for two reasons: removal of TBT which is known to be present at times in the water delivered to the laboratory (R.J. Huggett, Va. Inst. Mar. Sci., Gloucester Pt., Va., personal communication), and removal of any bacterial or algal toxins which may have been present. Measured TBT concentration in control water samples was < 10 ng/L (as TBTCl).

Each day, temperature, salinity, dissolved oxygen and pH were measured. On day 0, water samples were collected for TBT analysis from the diluent control plus four test concentrations and acidified. On day 1 three samples were collected from the new diluent control and two freshly prepared test concentrations and two samples from day-old test solutions. At the end of each test, samples were obtained from the 24-h old control and 4 test concentrations. All samples were analyzed for TBT by the method of Unger et al. (1986). Measured concentrations were regressed against applied concentrations calculated from stock TBT concentrations. Exposure concentrations, used to calculate LC50s, were calculated from the regression for each applied concentration.

For each treatment, the triplicate counts were averaged for each day and the number per milliliter calculated. Percent survival for each treatment was then calculated for each day. Since control mortality was greater than zero (21 to 38%), mortality resulting from TBT at each dose was calculated as

$$\text{corr \% survival}_{E,d} = \frac{\text{\% survival}_{E,d}}{\text{\% survival}_{C,d}}$$

where $\text{survival}_{E,d}$ = survival in treatment E on day d and $\text{survival}_{C,d}$ = survival in the control treatment on the same day. The LC50 was estimated by plotting $\sin^{-1}(\text{corr \% response}_{E,d})^{1/2}$ against calculated exposure concentration. The best LC50 estimate was derived by nonlinear interpolation (Stephan 1977).

The degree of oxygen saturation was calculated from measured temperature, salinity and dissolved oxygen. All physicochemical parameters are expressed as means during exposure.

RESULTS AND DISCUSSION

The 48-h LC50 for clam embryos was 1.13 ug/L and that for oyster embryos was 1.30 ug/L (0.71 ug/L with acetone carrier). There was no marked difference between survivorship in diluent and solvent

Table 1. Toxicity (mean and 95% confidence limits) of tributyltin to embryos and larvae of the eastern oyster, Crassostrea virginica, and the hard clam, Mercenaria mercenaria. (GAA = glacial acetic acid as solvent carrier)

Species	Life Stage	LC50 (as ug/L TBTCl) 24 h	48 h
Crassostrea virginica	Embryo	>1.38	1.30 (GAA) (0.78-1.38)
			0.71 (Acetone) (0.53-1.20)
	Larva	>4.21	3.96 (2.42-4.21)
Mercenaria mercenaria	Embryo	>1.31	1.13 (0.72-1.31)
	Larva	>4.21	1.65*

* 95% confidence limits cannot be specified in this case.

controls for either species. The maximum exposure concentration was insufficient to produce a 24-h LC50 in embryo tests with either species, i.e. the 24-h LC50 exceeded 1.3 ug/L (Table 1).

Clam embryo development was delayed at some TBT doses below the measured 48-h LC50. At 0.77 ug/L, development to straight-hinge was recognizably delayed, although the experimental design does not allow one to calculate a delay time. Despite the delay, most resultant larvae were normal in appearance. Some larvae failed to develop to the straight-hinge stage in all conditions including diluent control.

Oyster embryos, in addition to a slight delay in development at high doses, also exhibited abnormal shell development. All surviving larvae developed into straight-hinge larvae during the test. However, at 0.77 ug TBT/L and above, some larvae developed shells which were flattened rather than convex resulting in inability of larva to withdraw all meat and velum into the shell. It was not possible to enumerate the abnormal animals separately from the normal straight-hinge larvae, since in some positions one could not readily evaluate the condition of the shell. At the highest doses, the meats of still motile larvae often consisted of only portions of the mantle, heart, and velum, leaving a large "vacuolated" space within the shell.

Clam and oyster larvae were slightly more tolerant of TBT than embryos. The 24-h LC50 exceeded 4.1 ug/L in both cases. The 48-h

Table 2. Toxicity of TBT to bivalve molluscan embryos and larvae. (S-h = Straight-hinge larvae).

Species	Stage	Comment	Source
Crassostrea virginica	embryo	48-h LC50 = 1.30 ug/L	this study
	S-h larva	48-h LC50 = 3.96 ug/L	this study
Crassostrea gigas	embryo	24-h LC50 < 5 ug/L	His & Robert 1980
	embryo	(incomplete formation of S-h larva at 1 ug/L at 24 h; mortality after 48 h)	Robert & His 1981
	S-h larva	48-h LC50 < 5 ug/L	His & Robert 1980
	S-h larva	~50% dead: at 5 ug/L after 192-240 h at 3 ug/L after 123-144 h at 1 ug/L after 96-120 h	Robert & His 1981
	S-h larva(?)	48-h LC50 = 1.61 ug/L	Thain 1983
Mercenaria mercenaria	embryo	48-h LC50 = 1.13 ug/L	this study
	S-h larva	48-h LC50 = 1.65 ug/L	this study
	post S-h larvae	96-h LC50 = 0.015 ug/L*	Becerra-Huencho 1984
Mytilus edulis	S-h larva(?)	48-h LC50 = 2.3 ug/L	Thain 1983
	7-day larva	360-h LC50 ~ 0.1 ug/L 240-h 0.1 < LC50 < 1.0 ug/L 120-h 1.0 < LC50 < 10.0 ug/L	Beaumont & Budd 1984
Mytilus galloprovincialis	embryo	48-h 1.0 < LC50 < 3 ug/L 96-h LC50 = 1.0 ug/L	Robert & His 1981
	S-h larva	1 20-h LC50 = 5 ug/L	Robert & His 1981

* Expressed as TBTO; reported originally as 0.006 ug/L as Sn.

LC50 was 1.65 ug/L for clam larvae and 3.96 ug/L for oyster larvae. There was no obvious flattening of valves of oyster larvae which already had well formed valves at the start of the experiment. Some subtle changes in shell shape were observed at the two highest doses including a notching of the valves opposite the hinge line. Concentrations of TBT have been shown to reach 1 ug/L seasonally

near marinas in the Chesapeake Bay (Huggett et al. 1986; Hall et al. 1987). In most bay waters, the concentration of TBT is below 20 ng/L.

Water quality conditions were uniform for all tests. Within each test, temperatures varied less than 1°C. Mean test temperatures were between 20 and 24°C. Salinity within any experiment varied less than 1 $^{o}/_{oo}$. Mean test salinity was between 18 and 22 $^{o}/_{oo}$. Initial oxygen concentrations were approximately equal to 100% of saturation. In later experiments, dissolved oxygen was also determined on the used culture medium; dissolved oxygen was at saturation in the diluent control and progressively depressed with increasing dose of TBT. Oxygen saturation was never below 66%.

The salinity range in these experiments was approximately optimal for C. virginica larvae, but below the optimum for M. mercenaria larvae which is reported to be 27 $^{o}/_{oo}$ (Davis and Calabrese 1964). The minimum dissolved oxygen allowing normal development of M. mercenaria larvae is reported to be 0.5 mg/L or 6.9% of saturation (Morrison 1971). No adverse effect on clam larvae would be expected from the reduction in dissolved oxygen to 66% of saturation in present experiments. No report of a minimum oxygen concentration has been found for oyster larvae.

Anomalous development has been reported previously in bivalve embryos exposed to TBT. After exposure to 5 ug/L TBT for 24 h, all C. gigas larvae were "trochophore monstrueuses" (His and Robert 1980). It is not clear precisely what this means anatomically, but obviously no larvae reached the straight-hinge stage. At 10 ug/L, trochophores were rarely observed to develop. In the present study, all fertilized embryos developed into straight-hinge larvae without observed formation of extremely anomalous trochophores, but in no case were embryos exposed to concentrations as high as 5 ug/L TBT. Straight-hinge larvae of C. gigas exposed to 1 ug/L were often markedly reduced in size (Robert and His 1981). No such effect was recognized in the present study. The shell flattening observed in C. virginica has not been reported previously.

Clearly there was no significant difference in LC50 for clam and oyster embryos or for clam and oyster larvae within this study. The LC50 in each case was within the range of values reported for many other invertebrate species. The data also seem to compare favorably with nearly all results reported for other bivalve embryos and larvae despite differences in exposure methods and data analysis. The only exceptional value seems to be that of Becerra-Huencho (1984) who reported a 96-h LC50 for M. mercenaria of 0.015 ug/L, much lower than that from the present study or any other with bivalve larvae. In larval experiments reported herein, data for 72- and 96-h exposure periods were not used to calculate a 72- or 96-h LC50. After 48 h, there was obvious bacterial growth, especially in the treatments receiving glacial acetic acid, and survival declined in all dosed treatments. There was no way to determine the extent to which the mortality resulted from TBT versus bacteria. Whether the experimental results of Becerra-

Huencho (1984) reflect an impact of bacterial contamination or some other intervening factor cannot be determined.

One must be cautious in comparing species responses because of differences in methodology or uncertainty about specific details in some experiments. Nevertheless, it seems fair to conclude that embryos and larvae of the 5 species of bivalve mollusks which have been tested are similar in sensitivity to TBT and that tolerance increases slightly with increasing larval age.

Acknowledgments. Animals were counted by P. DeLisle, H. Sutton, E. Wilkins, D. Sved, P. Balcom, and B. Sweeney. Water samples were analyzed by D. Westbrook, and F. Espourteille of the Chemical Oceanography Department (now Division of Chemistry and Toxicology). M. Castagna, K. Kurkowski, and R. Bohn provided clam and oyster larvae for some experiments. This research was partially supported by the Virginia State Water Control Board.

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Received March 9, 1987; accepted July 29,1987.