Vol. 48: 29–36, 1988 | MARINE ECOLOGY – PROGRESS SERIES Mar. Ecol. Prog. Ser. | Published September 5

Chronic embryo-larval toxicity of tributyltin (TBT) to the hard shell clam *Mercenaria mercenaria*

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ABSTRACT: Fertilized clam eggs *Mercenaria mercenaria* were exposed to *bis* (tributyl)tin oxide (TBT) dissolved in seawater. Nominal initial exposure concentrations were 10, 25, 50, 100, 250 and 500 ng l^{-1}. Dosing protocol was daily renewal of static solutions. Chemical analysis indicated that initial dissolved TBT concentrations in seawater exposure solutions were ca 80 % of nominal ones, but after 24 h, they fell to 20 to 30 % of initial values. Effects of duration of exposure were also tested. One set of *M. mercenaria* larvae was exposed for 14 d. A second group ('recovery group') was exposed to all TBT concentrations for the first 5 d of development and then maintained in uncontaminated seawater for the next 9 d. Survival of TBT-exposed groups tended to be somewhat lower than that for controls, but declines were not consistently exposure dependent. At concentrations tested, TBT had 2 main effects: (1) mean valve length of veligers and postlarvae consistently declined with increasing TBT exposure, and (2) veligers in exposures of 100 ng l^{-1} and greater did not develop to pediveligers within the 14 d exposure period. Absence of metamorphosis in groups that failed to grow well implies a causality between gross growth and metamorphosis, with TBT acting primarily on the former to influence the latter. Responses of groups exposed for 5 or 14 d were statistically different after 14 d, suggesting that, although slow, recovery from adverse effects of TBT occurs after exposure ceases.

INTRODUCTION

In 1980, French scientists reported that tributyltin compounds (TBT), novel marine antifouling compounds, were responsible for decline of an oyster fishery based upon the introduced Pacific oyster *Crassostrea gigas*. Failure was a result of both poor larval recruitment over a period of several years, and abnormal growth of adults (Alzieu et al. 1981, 1982, 1986). There has been concern that other commercially significant mollusc species might be affected similarly by TBT exposure as use of these coatings continues to grow (Beaumont & Budd 1984, Bryan et al. 1986, Paul & Davies 1986). In North America, there are several estuarine bivalve species which support significant fisheries. The hard shell clam *Mercenaria mercenaria* is one of the most valuable. All life history stages occur in areas of estuaries where they might be exposed to TBT released from antifouling coatings, particularly from recreational craft. Concern for *M. mercenaria* has also been motivated by an unpublished report of a LC-50 (96 h) value of 15 ng l^{-1} for veligers exposed to TBT (Bacerra-Huencho 1984).

In the experiments reported here, we exposed embryos and veligers to low concentrations (nominally 10 to 500 ng l^{-1}) of TBT for up to 14 d. Exposure concentrations are environmentally relevant (Unger et al. 1986, Valkirs et al. 1986, Hall et al. 1987). In addition to survival, we measured cumulative growth and observed occurrence of metamorphosis to assess sublethal effects of TBT exposure.

MATERIALS AND METHODS

Adult clams *Mercenaria mercenaria* were collected from the Indian River Lagoon area of Florida (USA) during winter and spring 1987. This subtropical population spawns throughout the year, so there is no need to condition adults prior to inducing release of gametes in the laboratory by cyclical temperature changes between 20 and 30 °C of ~2 h duration. Induction of spawning occurred in a communal spawning trough. When gamete release commenced, females were identified, rinsed and moved to a separate aquarium so egg release could be completed in the absence of sperm until sperm was intentionally added to initiate fertilization. Eggs from several females, fertilized with sperm

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from one male, were pipetted into glass finger bowls containing ~50 ml of exposure solution so that the density of larvae would not exceed ~150 per bowl (3 larvae ml^{-1} or less in latter stages of the experiment).

Exposure to TBT began within 4 h of fertilization. Static solutions were renewed daily, except during the first 48 h when water was not changed. One group of larvae with samples at all TBT concentrations plus controls was exposed for 14 d, the duration of the experiment. A second, the 'recovery' group, was exposed for the first 5 d of development, then cultured for the next 8 d in uncontaminated seawater. After the second day, larvae were strained daily through Nitex® mesh (55 µm mesh size) and gently rinsed into bowls containing freshly prepared exposure solution. Microalgae *Isochrysis galbana,* Tahiti strain, (> 40 000 cells ml^{-1} final density in bowls) were added as food. For the duration of the experiment, temperature and salinity were 25 °C and 32 ‰, respectively. Clam larvae were exposed to *bis* (tributyl)tin oxide (Cahn-Ventron). Stock solutions of TBT in acetone were prepared so that addition of 10 µl stock to 1 l seawater yielded the desired nominal concentration. Relatively low solvent additions were required since higher quantities display a marked tendency to encourage slime-forming microbes that foul bioassay containers. Growth of these bacteria is alone sufficient to kill all larvae within several days. Nominal exposure concentrations were 10, 25, 50, 100, 250 and 500 ng l^{-1}. Seawater and acetone controls (10 µl l^{-1}) were also tested.

Tributyltin concentrations were analyzed several times during each replicate bioassay. Initial concentrations were determined using seawater solutions freshly prepared from solvent stock solutions and analyzed within 4 h after preparation. Solutions that had been used for 24 h exposures were obtained by combining seawater from exposure bowls after clam larvae were removed to obtain sufficient water for analysis (up to 500 ml from the 10 ng l^{-1} treatment and controls).

Butyltin compounds were analyzed and quantitated using hydride derivitization – collection of volatile butyltin hydrides followed by quartz furnace atomic absorption spectroscopy (Hodge et al. 1979, Valkirs et al. 1985, 1987). Seawater for bioassays was analyzed for butyltin compounds prior to use. Occasionally < 10 ng l^{-1} of dibutyltin was observed, but no TBT was observed in open lagoon waters (detection limit: ca 2 to 5 ng l^{-1}).

On days when survival and growth were calculated, the entire contents of a bowl were sieved and collected in a small dish containing ca 4 ml seawater. Visual observations of the larvae were made, then they were killed by addition of 2 drops of formalin. Subsequently, larvae were pipetted to a Sedgwick-Rafter cell and counted under 100 × magnification. Valve length of 25 randomly-selected individuals was determined at 400 × using on ocular micrometer.

For purposes of statistical analysis of survival, each of the 3 spawns was treated as a replicate. There were up to 25 measurements per day of veliger valve length for each TBT exposure. Each measurement was treated as a replicate and the spawn treated as a block in the statistical analysis of growth on Day 14. A mean growth rate over 10 d of observation was calculated for each of the 3 spawns. Each mean was then treated as a replicate observation for statistical analysis.

Statistical analysis of data was performed using the SAS PC software package (Statistical Analysis System Institute, Cary, North Carolina, USA). Length data were transformed (log_{10}) prior to analysis to satisfy requirements that data be normally distributed. Data were tested using general linear model analysis of variance because cell sizes were frequently unequal.

RESULTS

Exposure concentrations

Analysis of TBT in freshly prepared exposure solutions yielded values 80 to 100 % of nominal ones (Fig. 1). At the end of 24 h, seawater used in the bioassay (to which larvae were added as well as microalgae as food) had TBT concentration values ca 20 to 30 % of nominal. Values reported at 24 h are for recoverable, dissolved TBT. Apparently microalgae bind appreciable quantities of TBT. In their presence at cell densities in bioassay containers, only about half of an added TBT quantity could be recovered compared to recovery from filtered seawater. Thus, initial concentrations were close to nominal, but the average for the 24 h duration was only about half the target value.

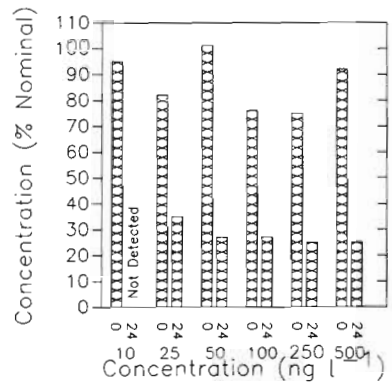

Fig. 1. Initial and final TBT concentrations in bioassay solutions. Values are percentage of nominal values initially and after 24 h in bioassay containers

Survival

The most noticeable trend seen among clam larvae in the experiment was a gradual decline in survival over the 2 wk observation period (Figs. 2 and 3). Survival in seawater and acetone controls appeared to be somewhat greater than in any groups exposed to TBT, but differences were not large. Survival differences for TBT-exposed groups were not exposure dependent with respect to concentration or duration. An analysis of variance using a general linear model showed that of 3 factors tested, 'TBT concentration', 'day' or 'duration of exposure', only 'day' was statistically significant (Table 1). The correlation coefficient of this statistical

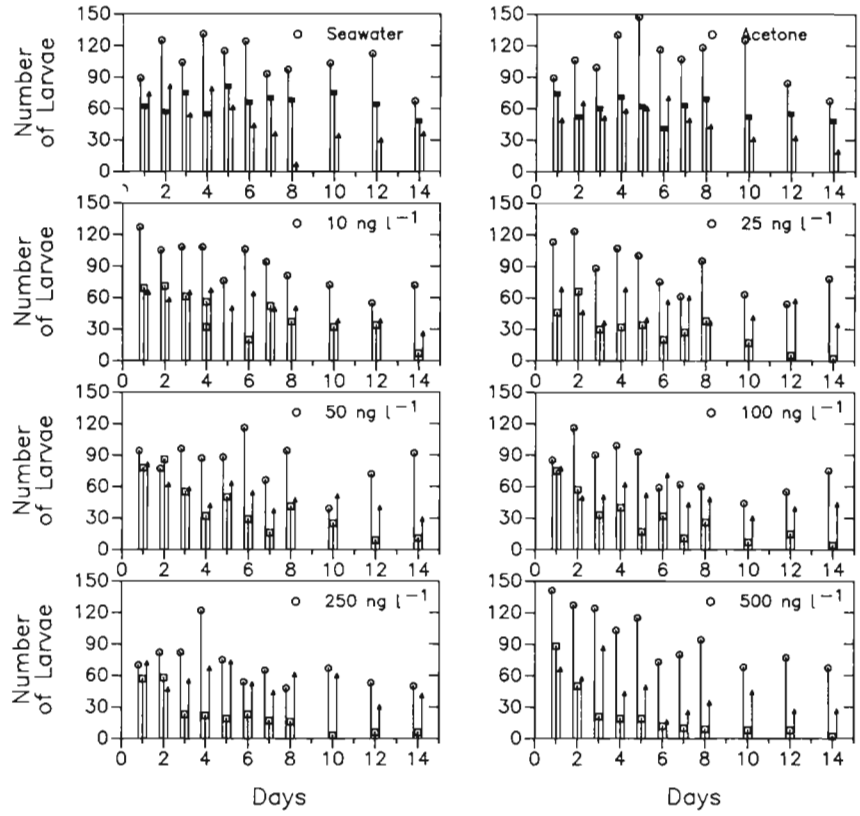

Fig. 2. *Mercenaria mercenaria*. Survival of clam veligers exposed for 14 d to TBT. Each symbol designates 1 of 3 replicates

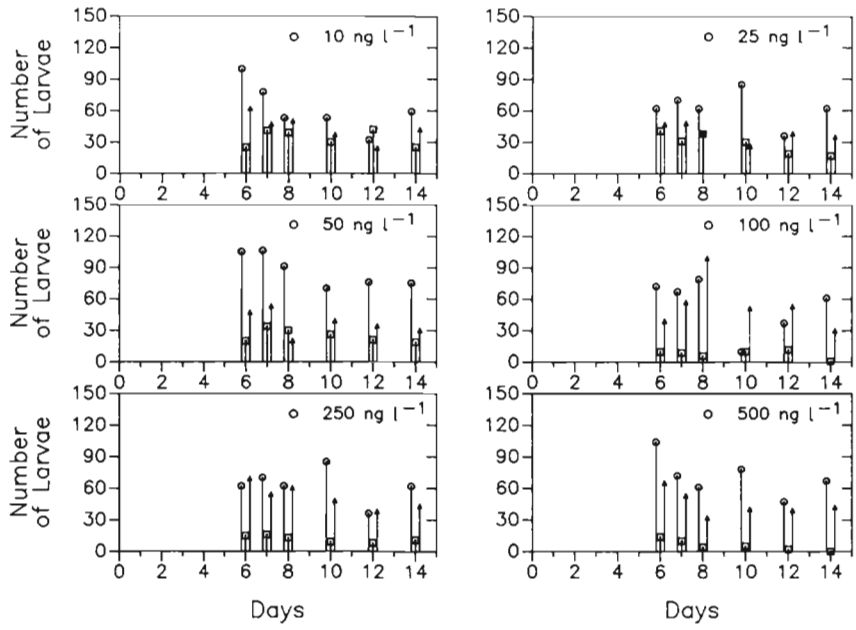

Fig. 3. *Mercenaria mercenaria*. Survival of clam veligers exposed to TBT for the first 5 d, then kept in uncontaminated seawater for the next 9 d. Measurements began after TBT exposure ended. Each symbol designates 1 of 3 replicates and corresponds with usage in Fig. 2

Table 1. *Mercenaria mercenaria*. Analysis of variance of survival data for clam larvae exposed to TBT The dependent variable is the number of living clam larvae. Duration refers to 5 or 14 d exposure to TBT and is treated as a class variable

Source	df	SS	MS	F	$p > F$
TBT concentration	1	946	946	1.31	0.2533
Day	1	44 831	44 831	62.11	0.0001
Duration	1	4	4	0.01	0.9427
Error	302	217 990			
Total	305	263 771			
$R^2 = 0.174$					

model is only 0.174, reflecting a very poor exposure dependence, as well as a highly variable daily count of living larvae.

Growth

Growth of clam larvae in both controls was rapid (Fig. 4). Mean valve length of controls increased from ~100 µm on Day 1 to between 210 and 250 µm on Day 14, varying with hatch. Growth of TBT-exposed groups consistently declined as exposure concentrations increased. In 500 ng l^{-1} TBT, there was virtually no growth. Mean valve length increased only ~30 µm during the entire 14 d period.

Clam larvae recover slowly from exposure to TBT. In most cases, the mean valve length of recovery groups exceeded that of their respective continuous exposure match by only a few percent (Fig. 5). Differences between the 2 groups, although small, were statistically significant (Table 2a).

The decline in valve length shown in Fig. 5 is relatively steep. It is not obvious that a 'no effect' level occurred even at the lowest exposure concentration, 10 ng l^{-1}. The Student-Newman-Keuls test showed that mean valve length of all TBT exposed groups was significantly smaller than for either control (Table 2b). Results of multiple comparison tests on growth data must be interpreted cautiously if size differences are large because variance is proportional to the mean. This is intuitively obvious from inspection of Fig. 5 because smaller means display smaller variances. Pooling variance to derive a value for multiple comparisons may therefore give a biased estimate of differences. As a further check, therefore, *t*-tests of controls paired with 10, 25 or 50 ng l^{-1} exposure groups were performed. Each of the comparisons showed that mean valve length of TBT group were statistically smaller than controls (Table 2c). Seawater and acetone controls were not significantly different from each other so data in these 2 groups were pooled to obtain the control group for *t*-tests.

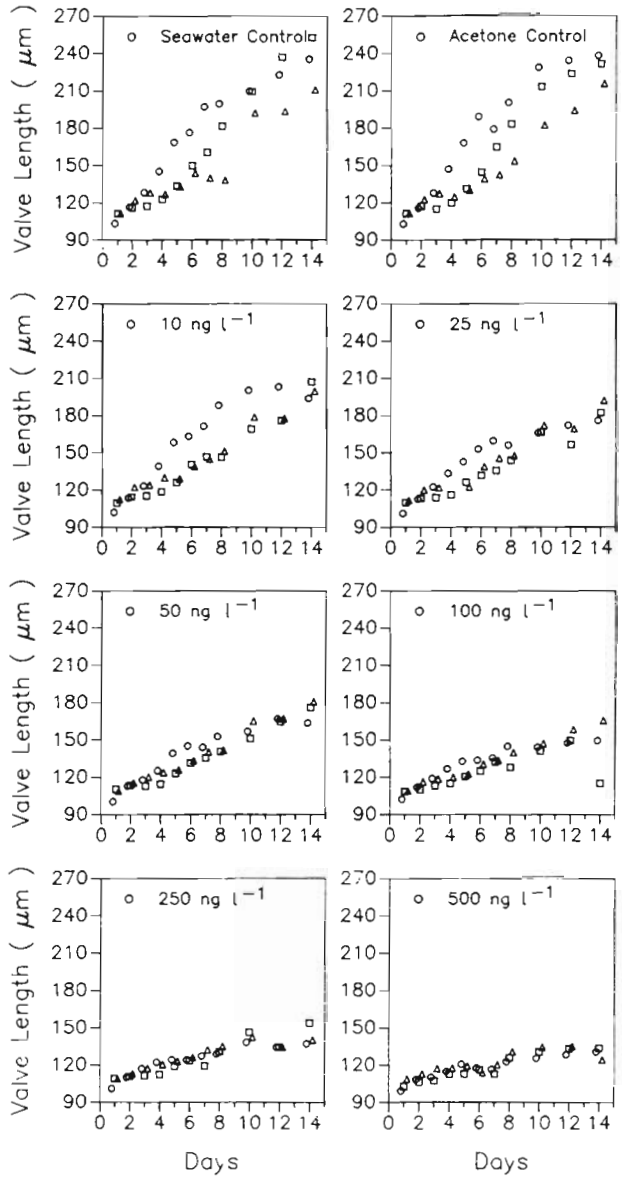

Fig. 4. *Mercenaria mercenaria*. Growth of clam larvae exposed for 14 d to TBT. Each symbol represents 1 of 3 separate groups of clams. Each point is the mean of up to 25 larvae. Points are offset slightly for clarity on each day mark

Daily growth rates

To better visualize how TBT influences growth of clam larvae, daily growth rates were calculated from data shown in Fig. 4. Daily growth rates are calculated as the difference between the mean valve length size of each group on a given day and the mean valve length of the previous day. Daily growth rates are not consistent throughout larval development (Fig. 6). Highest growth rates of controls tended to increase until metamorphosis (around Days 8 to 10) and then dropped somewhat thereafter. Highest and average growth

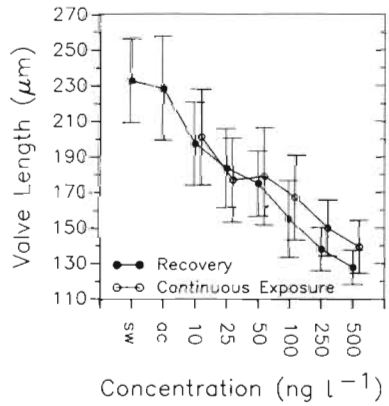

Fig. 5. *Mercenaria mercenaria*. Mean valve lengths of clam larvae on Day 14 for those exposed continuously or for 5 d followed by 9 d recovery. For clarity, points for each line are offset slightly relative to the X-axis scale. Error bars: 1 standard deviation

rates of TBT-exposed groups failed to equal those of controls and these reductions were exposure dependent. Groups exposed to TBT displayed declining growth rates as duration of exposure to TBT increased, and it appeared that in 500 ng l^{-1}, virtually no growth occurred by the end of 14 d. An analysis of variance showed that TBT concentration, but not the day of larval development, significantly influenced growth rates (Table 3).

Effects on morphology

Visual observations during daily counting and measurements revealed no morphological abnormalities other than occasional non-symmetrical valves on the veligers. The most significant finding was that no pediveligers were observed in 100 ng l^{-1} and above. In these exposure concentrations, gut tissue did not appear to contain microalgae, a characteristic of healthy larvae.

DISCUSSION

Toxicity of TBT to molluscs has been a primary motivation for research on its environmental effects. Several bivalve species have been the subject of larval bioassays. Beaumont & Budd (1984) estimated mussel larvae, *Mytilus edulis*, had a LC-50 (15 d) of 100 ng l^{-1}. Other researchers have attempted to estimate short-

Table 2. *Mercenaria mercenaria*. Analysis of growth of clam veligers exposed to TBT

(A) Analysis of variance of valve length data of clam larvae on Day 14. The dependent variable is $\log_{10}$ of measured length. In this analysis, duration refers to 5 or 14 d exposure and is treated as a class variable

Source	df	SS	MS	*f*	$p > F$
Duration	1	0.1357135	0.1357135	30.06	0.0001
Concentration	1	3.2057969	3.2057969	710.12	0.0001
Error	864	3.90045574			
Total	866	7.24196611			
R^2 =0.461					

(B) Results of Student-Newman-Keuls tests. Means of 2 groups underscored by the same line are statistically indistinguishable. Abbreviations: SW, seawater control; AC, acetone control

SW	AC	10	25	50	100	250	500

(C) Results of *t*-test comparisons of valve length between controls and 10, 25, and 50 ng l^{-1} TBT exposures. C: pooled seawater and acetone controls. Numbers refer to TBT concentrations (ng l^{-1})

Variable	*n*	*t*	df	$p > t$
SW	74	1.0665	112.5	0.2885
AC	67			
C	141	7.6590	197	0.0001
10	58			
C	141	11.1083	195	0.0001
25	56			
C	141	11.4485	182	0.0001
50	43			

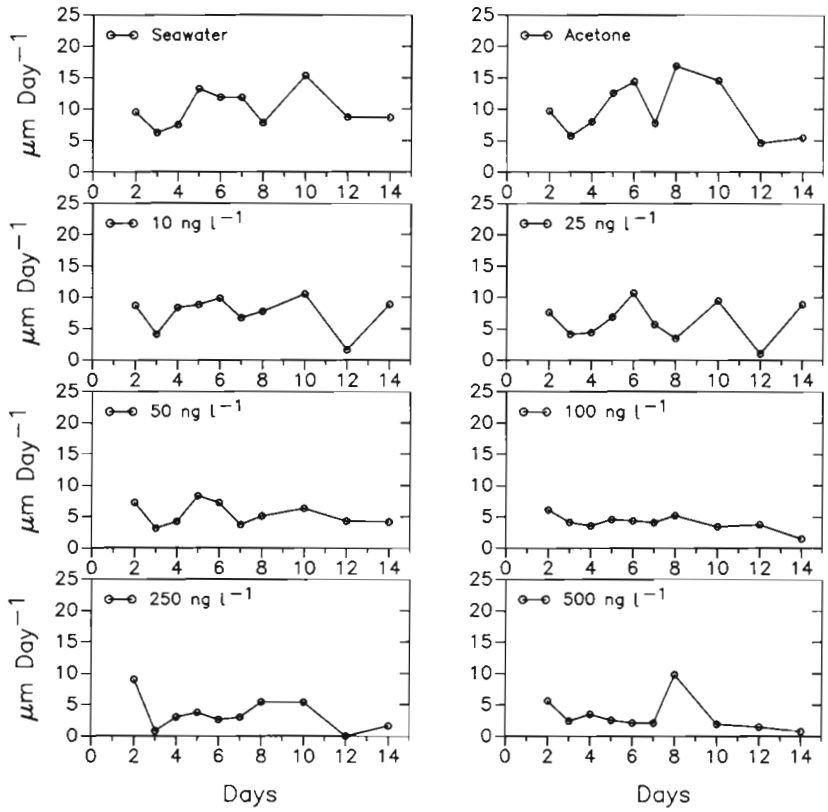

Fig. 6. *Mercenaria mercenaria*. Daily growth rates of clam larvae exposed to TBT for 14 d. Each point is the mean of 3 replicates

Table 3. *Mercenaria mercenaria*. Analysis of variance for data of daily growth rates of clam larvae

Source	df	SS	MS	F	$p > F$
TBT concentration	1	840.78	840.78	30.81	0.001
Day	1	47.66	47.66	1.75	0.1876
Error	237	6468.22	27.29		
Total	239	7356.67			
$R^2 = 0.121$					

term acute toxicity values. For the Pacific oyster *Crassostrea gigas*, mortality occurs within several days in TBT exposures of 1 to 100 µg l^{-1} (His & Robert 1980). Estimates of similar toxicity to American oysters *Crassostrea virginica* and clams *Mercenaria mercenaria* were greater than 1 µg l^{-1} in all tests (Roberts 1987). Experiments reported in this paper are notable in that they continued for 14 d, used low, more environmentally-relevant TBT concentrations, and continued through a biological endpoint, metamorphosis. They indicate that acute toxicity of TBT is not the most significant environmental effect of this compound. Even at 500 ng l^{-1}, there was fairly good survival of experimentals specimens in 3 different replicates.

Results of these tests do not support a reported LC-50 (96 h) value of 15 ng l^{-1} TBT for *Mercenaria mercenaria* (Bacerra-Huencho 1984). There is no statistical basis for an exposure-dependent reduction in survival in TBT groups. Values reported here for growth inhibition by TBT are among the lowest reported for any bivalve. Given that no metamorphosis occurred in exposures of 100 ng l^{-1} and greater, it is likely that these exposures would, in the field, pose an unacceptable hazard to this fishery resource. Additional field studies are needed to determine the threat of lower exposures in the environment.

Growth reductions were the most sensitive index of TBT action on clam larvae. Statistically significant reductions in valve length were observed in 10 ng l^{-1}. Reductions in growth of bivalves exposed to TBT have been reported for *Mytilus edulis* (Beaumont & Budd 1984, Stromgren & Bongard 1987), scallops *Pecten maximus* (Paul & Davies 1986) and oysters *Crassostrea gigas* (Thain & Waldock 1985, Paul & Davies 1986, His & Robert 1987). Reduced growth of larvae from exposed adults was reported by His & Robert (1987). In none of the laboratory experiments cited above were lowest exposure concentrations as low as those tested here.

Significance of growth reductions can be viewed from 2 different perspectives. Stebbing (1982) and Stebbing & Brinsley (1985) have suggested that growth inhibition is a response to non-specific action on metabolic homeostatic processes. Cybernetic mechanisms partition energy equivalents between metabol-

ism and growth. In experiments reported here, there was a high inverse correlation between growth and exposure to TBT. Notable is the small quantity of TBT capable of acting to reduce growth under chronic exposures and slow recovery from fairly short exposure to TBT. Laboratory experiments can give fairly accurate descriptions of the action of growth control mechanisms and it is clear that effects of TBT on them are significant.

A second perspective on growth inhibition is with respect to ecological consequences. In this case, failure of veligers to metamorphose and recruit into benthic populations due to growth inhibition would have significant effects on the population. Concentrations causing inhibition of metamorphosis were above 50 ng l^{-1}. There is a much higher degree of uncertainty in estimates of ecological effects of reduced growth in TBT exposures below 50 ng l^{-1}. It is possible only to assume that smaller postlarvae are at a competitive disadvantage in the field because there have been few studies showing this in the case of clams. Field studies are needed to corroborate ecological effects of sublethal responses such as growth reductions.

The interval between TBT concentrations which cause acute mortality (above 1 µg l^{-1}) and those causing significant chronic effects (10 to 50 ng l^{-1}) is quite large. The mode of action may be different, with high concentrations acting as a narcotic and low ones acting as metabolic inhibitors of energy metabolism (Laughlin 1987, Lawler & Aldrich 1987). In addition, the route of exposure may play a significant role. It is quite plausible that the primary route of exposure of clam larvae was through consumption of phytoplankton rather than by partitioning of dissolved TBT from water. If accumulation from water was the dominant route, one would expect that TBT toxicity would be more rapid because these small organisms would quickly come to steady state with dissolved TBT. Accumulation through consumption of tainted microalgae would be slower and potentially lead to higher tissue burdens (Laughlin et al. 1986), but cumulative effects on growth would not be apparent until after several days. The latter scenario was the one observed.

Measurements of tributyltin concentrations in the environment may allow estimates of probable risk of this chemical to clam populations. The primary source of TBT is from antifouling paints containing it as an active agent. In Europe, small boat harbors have been shown to be the primary threat to oyster fisheries (Alzieu et al. 1981, Waldock & Thain 1983) In the USA, harbor areas tend to have TBT concentrations below 30 ng l^{-1} (Grouvhoug et al. 1986). Similar data are available for some further United States sites. Hall et al. (1987) found 72 % (102/142) of their values from sites in the Chesapeake Bay (USA) to be in a similar range. In San Diego Bay (California, USA), 41 % (13/32) of the values found during a year's observation were at or below 50 ng l^{-1}. In the Indian River Lagoon (Florida, USA), TBT can routinely be detected in most marinas, but not in open lagoon waters (unpubl. obs.). In all these studies, marinas or concentrated boating activity were shown to be a primary source of TBT inputs to the environment where concentrations exceeded 100 ng l^{-1}. There should not be much concern about TBT concentrations within marinas per se because this substance is only one of a host of factors which could act to exclude marine bivalves. The risk to shellfisheries, however, depends upon how significant a point source such facilities are to larger areas outside marinas. There are only a few examples from areas outside Europe demonstrating a TBT threat from these sources (for example, Wolniakowski et al. 1987). Absence of evidence should not be considered evidence of absence of a problem. Critically focused field studies are warranted.

In summary, TBT produces a concentration-dependent hierarchy of effects on clam larvae. Exposures of 500 ng l^{-1} prevented growth. Although limited growth occurred in 100 ng l^{-1}, no metamorphosis occurred during 14 d. Growth inhibition occurred in TBT concentrations as low 10 ng l^{-1}, but it was not sufficient to completely inhibit metamorphosis. These results may serve as a guide in the design and evaluation of field studies to examine effects of TBT release from antifouling paints.

Acknowledgements. We thank P. Wall, D. Leek and J. Taylor for help collecting adult clams spawned in experiments. This research was supported by the Chief of Naval Research, Energy Research and Development Program through the US Office of Naval Research, contract number N0004-86-K-0184. Harbor Branch Oceanographic Institution Contribution no. 652.

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This article was presented by Dr D. Alderdice; it was accepted for printing on July 8, 1988