TOXICITY OF TRIBUTYLTIN TO MARINE BIVALVES

FINAL REPORT

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Program Officer: Mr. Harry Hodgkins

From

Harbor Branch Oceanographic Institution
Division of Applied Biology
5600 Old Dixie Highway
Fort Pierce, Florida 34946

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Principal Investigator: Roy B. Laughlin, Jr.
Research Associate: Peter Pendoley
Technicians: Gail Woon
Elizabeth Bokman
Thomas Chiles

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SUMMARY

Molluscs have been identified as sensitive nontarget organisms to tributyltin (TBT) released from antifouling coatings. Special concerns have focused on commercially important bivalves. The purpose of our research was to examine risks of TBT to the hard shell clam, Mercenaria mercenaria, and the oyster, Crassostrea virginica. We were particularly interested in clams because there had been no published information about effects of TBT on them.

Mercenaria mercenaria veligers were surprisingly resistant to acute effects of TBT; 2 and 4 day LC50 values were above 1 µg/L. Chronic effects studies indicated that clam veligers did not metamorphose to pediveligers if they were exposed to TBT concentrations of 100 ng/L and above. Growth of veligers was inhibited in TBT concentratios of 10 ng/L and above. Failure to metamorphose may be associated with decrements in growth. Growth inhibition in clams and presumably other bivalves, may be the most important effect of exposure to environmental levels of TBT

In a second set of experiments, we examined effects of TBT exposure on osmotic pressure and chloride ion concentrations in oyster hemolymph. TBT exposure did not significantly influence mean values of either, but variation increased with TBT exposure concentration. It appears that TBT does not directly influence osmotic pressure or chloride ion in oyster hemolymph but may interact with other factors, particularly high salinities, to secondarily influence the processes we examined.

In a third set of experiments, we examined bioaccumulation of TBT by clams, Mercenaria mercenaria. Clams took up dissolved TBT from exposures as low as 37 ng/L. No steady state occurred in 8 wk, but final observed burdens were proportional to exposure concentrations between 37 and 484 ng/L. Depuration half times were ~5 weeks. Bioaccumulation factors increased with increasing TBT exposure. This is in contrast to field studies and suggests food chain accumulation may be important in some situations.

To determine how food might act as a source of TBT for filter feeders, we examined bioaccumulation of TBT by 3 species of microalgae and one cyanobacterium. In 2 microalgae species and the cyanobacterium, burdens were linearly related to exposure concentration. This is consistent with a partitioning mode of action. One species, Isochrysis galbana, exhibited a distinctly non-linear relationship between dissolved TBT concentrations and cell burdens, implying that binding to a limited number of specific sites rather than partitioning controls burdens. Bioaccumulation from food organisms could be important regardless of the mode of association between cells and TBT. Bioaccumulation from food may be most important to filter feeders when dissolved TBT concentrations are below ~100 ng/L.

INTRODUCTION

During the last 15 years, tributyltin anitfouling paints have rapidly penetrated their market as effective antifouling materials. Approximately 80% of commercial shipping and perhaps somewhat less of the recreational craft in the US and Europe were protected by these paints. At one point, before analysis procedures for seawater samples became sufficiently sensitive, TBT coatings were viewed as environmentally preferable to those containing copper because release of active agent was thought to be insignificant to non-target organisms.

This view began to change approximately 8 years ago. First, French fishery biologists provided compelling circumstantial and experimental evidence that TBT released from coatings was responsible for declines in a fishery based on the introduced Pacific oyster, Crassostrea gigas (Alzieu et al., 1981, 1982, 1986). Subsequent experimental work by British investigators provided even stronger experimental evidence that it was in fact TBT, not necessarily other environmental factors, which caused poor larval recruitment, growth inhibition and shell malformations in C. gigas (Waldock and Thane, 1983). Both French and British investigators noted that recreational craft moored and used in estuaries appeared to be the primary source of TBT causing problems for shellfisheries.

A view of molluscs in general as a sensitive group began to emerge with publication by Bryan et al., (1986) of evidence for TBT's role in promoting imposex in dog whelks, Nucella lapillosus. This situation is interesting because the condition of 'imposex', the imposition of male sexual characteristics on functionally female snails is not directly lethal unless oviducts become blocked. Imposex does reduce fecundity, however, and this appears to be the most important mechanism of dog whelk population declines attributed to TBT exposure.

As of 1985, virtually all studies describing environmental toxicity of TBT to non-target molluscs had been done in Europe, and none had been performed, with any degree of rigor, using North American species, especially those important to commercial fisheries. Two commercially important species were the focus for special concern, the American oyster, Crassostrea virginica and the hard shell clam, Mercenaria mercenaria. The hard shell clam quickly became a species of special concern following publication of an LC50 value of 15 ng/L for veliger larvae (Bacerra-Huencho, 1984). It was with this backdrop that we began studies of clams, and secondarily of oysters, to determine acute and sublethal toxicity of TBT and to determine bioaccumulation characteristics for these filter feeders.

MATERIALS AND METHODS

Acute toxicity to clam veligers

Adult clams, Mercenaria mercenaria, collected from the Indian River Lagoon, Florida (USA), were induced to spawn in the laboratory by cyclic temperature exposures, first 20° C, followed by abrupt transfer to 30-32° C.

Eggs from several females were pooled, then fertilized with sperm from 1 male.

Veligers were 24-48 h old at initiation of exposure to TBT. Approximately 100 veligers were placed into beakers containing 1 L 30 °/oo seawater. Isochrysis galbana Tahiti strain (40-160 x 10^5 cells/mL) was added as food. At each sampling period indicated in the figures, the entire contents of a beaker were collected by straining through a fine mesh (Nitex, 51 µm)and preserved in buffered seawater-formalin until counting. Every other day, veligers in remaining beakers were similarly strained, reintroduced to freshly-prepared exposure solutions in clean glassware, and fresh microalgae added as food.

Clam postlarvae used in a second set of experiments were obtained from an experimental aquaculture project conducted by the Division of Applied Biology. They were approximately 4 wk old and 1 mm in valve length at initiation of the experiment. Approximately 40 post larvae were placed into a 4 cm finger bowl covered with nylon mesh. There were 3 of these bowls per exposure. All were placed into a larger (20 cm) finger bowl containing 500 ml of 30 °/oo seawater to which TBT had been added. Water in the larger bowl was renewed by siphoning TBT-seawater solutions from a larger reservoir so that flow rates were 2 L/day. Once each day, Isochrysis galbana were added as food for the post larvae. The 2 L/day flow rate was selected to renew toxicant solution while minimizing dilution of microalgal cells before they could be consumed by the postlarvae. Every third day, each small bowl containing clam postlarvae was examined to count living post-larvae and to remove dead ones. Bowls and mesh coverings were also wiped as necessary to remove slime.

Bis tri-n-butyltin oxide (Alpha Products) was first dissolved in acetone to make a series of stock solutions of sufficient strength so that addition of 10 µL/L seawater yielded desired concentrations. Exposure solutions in bioassays were analyzed for TBT by derivatization with borohydride, purge and trap of volatile butyltin hydrides followed by detection and quantification by atomic absorption spectroscopy (Hodge et al. 1979; Valkirs et al. 1985; 1987).

Chronic Mercenaria mercenaria Embryolarval Toxicity

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 hour 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 it was intentionally added to initiate fertilization. Eggs from several females, fertilized with sperm 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 or less in latter stages of the experiment).

Exposure to TBT, beginning within four hours of fertilization, was daily static renewal, except during the first 48 hours when water was not changed. One group composed of larvae in all TBT concentrations plus controls was exposed for 14 days, the duration of the experiment. A second, the "recovery" group, was exposed for the first five days of development, then grown for the next eight days in uncontaminated seawater. Each day after the second day, larvae were strained 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 final density in bowls) were added as food. For the duration of the experiment, temperature and salinity were 25° C and 32 °/oo, respectively. Clam larvae were exposed to bis-tri-n-butyltin 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 cause slime forming microbes to 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}. A seawater and acetone control (10 ng/L) were also tested.

Several times during the course of experimentation, exposure solutions, both freshly prepared, and pooled samples from bowls after 24 h were analyzed for TBT and other butyltins so that loss during the 24 hour interval between renewal could be calculated.

Butyltin compounds were detected and quantified using hydride derivatization, 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. Occassionally < 10 ng of dibutyltin was observed, but no TBT was observed in open lagoon waters (detection limit: ~2-5 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 ~4 ml seawater. Visual observations of the larvae were made, then they were killed by addition of two drops of formalin. Subsequently, larvae were pipetted to a Sedgwick-Rafter cell and counted under 100 x magnification. Valve length of 25 randomly selected individuals were determined at 400 x using an 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 days 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 by $\log_{10}$ prior to analysis to satisfy requirements that data be normally distributed. Data were tested with a general linear model analysis of variance because cell sizes were frequently unequal.

Hemolymph Osmotic and Ionic Regulation, Crassostrea virginica

Adult oysters, Crassostrea virginica, approximately 4-8 cm long, were collected from the Indian River Lagoon near Ft. Pierce, Florida. They were taken from waters known not to be contaminated by TBT. Their shells were cleaned of adhering macrofoulers before their use in any experiments.

For the steady state experiments, oysters were first acclimated to salinities of 5, 25 or 40 °/oo salinity. The two lower salinities were obtained by diluting Indian River water (salinity 30-32 o/oo) with well water. The higher one, 40 °/oo, was made by adding artificial sea salts to River water. All salinity determinations were made with a hand-held refractometer.

After salinity acclimation lasting 1 wk, oysters were subsequently subdivided into groups of 12 in aquarium tanks containing 20 L of acclimation salinity seawater for exposure to TBT. TBT was added to these tanks as an ethanol stock solution of sufficient strength so that 0.1 ml ethanol per liter of seawater would yield the desired TBT exposure concentration. Exposures were 0.5, 1.0 and 2.0 µg/L (expressed as TBTO) and lasted for 10 days. One set of controls was maintained in acclimation salinity seawater while another one was dosed with ethanol containing no TBT. Exposure was daily static renewal. Because we used ethanol with TBT, there was occasionally a problem with sliming in the tanks. Each day during water changes, the oysters' shells were scrubbed with a brush and the walls of the aquarium wiped clean with a paper towel.

To test for effects of TBT on short-term salinity acclimation, oysters were acclimated to 25 °/oo salinity and exposed for 10 days to the same TBT concentrations (0.5, 1.0 or 2.0 µg/L) used in the earlier experiment. On a staggered schedule during the tenth day, groups were transferred from a salinity of 25 to 5 or 40 °/oo. After transfer, hemolymph samples from 3 oysters was measured for total osmotic pressure and [Cl^-] at intervals during the next 24 hr.

During this experiment, TBT stock solutions were made by dissolving the neat material in deionized water so that 1 ml of stock per liter was sufficient to form the desired exposure concentration. Thus, there was no alcohol control.

To collect hemolymph samples, one valve was removed from an oyster. Then mantle tissue was dissected to expose the pericardial cavity, obvious because of ventricular contractions. The pericardial membrane was then pierced and removed. Immediately, two 5 µL samples were collected in calibrated capillary pipettes. One was then put onto the sample chamber of a Westcor vapor pressure osmometer without any attempt to remove hemolymph proteins. The other sample was added to reagent solution and analyzed for chloride ion content in a Buchler chloride ion titrator.

Bioaccumulation of TBT by clams

Hard shell clams, Mercenaria mercenaria, (3-5 cm valve length, 3-6 g tissue wet weight) were collected from the Indian River near Sebastian Inlet (Florida, USA) during September, 1987. Before use in experiments, they were

kept in concrete raceways containing running unfiltered seawater for approximately 2 months. Seawater we used was first drawn from the Indian River Lagoon into a settling tank to remove silt and large particulates. The seawater contained microalgae which was consumed by clams, but microalgal density was probably just sufficient to meet a maintenance ration.

Exposure System. Approximately 75 clams were placed into each of 5 aquaria containing 120 L of natural estuarine seawater (Salinity: 30 ± 3 °/oo; Temperature 25 ± 2° C). The exposure protocol during the accumulation phase was static renewal. Seawater in aquaria containing clams was changed every other day. Serial dilutions of an aqueous TBT stock solution were added daily to each tank to yield TBT exposure concentrations. On the days when water was not replaced, only 2/3 of the stock solution volume used when water was changed was added. This fractional addition was based on measurements of change in TBT concentration, which never declined to undetectable levels, in tanks over 20-24 h during the first week. The accumulation phase continued for 56 days.

The depuration phase of the experiment was conducted in a single tank of flowing seawater. All remaining clams from each exposure aquarium (21-25 clams) were placed in 5 shallow plastic trays and placed in a large aquarium with flowing seawater. Flow rate of incoming seawater varied somewhat, but was always greater than 1 L m^{-1}. In addition to the 5 exposure groups, a 'control' group of 21 previously unexposed clams was placed into the depuration aquarium. The purpose of this group was to indicate whether TBT released by one set of clams, particularly higher exposure groups, would influence tissue burdens of those exposed to lower concentrations during the uptake phase.

TBT Exposure. Clams were exposed to [1-^{14}C]bis(tributyltin) oxide (custom synthesis, New England Nuclear; specific activity 3.67 mCi $mmol^{-1}$). An aqueous stock solution, approximately 1 µg L^{-1}, was prepared by adding neat TBT to sterile deionized water and stirring until it dissolved. Measurements of radioactivity were used to estimate TBT concentration. Composition of the stock solution was also checked by analysis using hydride derivatization, purge and trap of volatile hydrides followed by detection and quantification by atomic absorption spectrometry. There was a small proportion (~3-5%) of dibutyltin present, probably a redistribution product. Milliliter aliquots of stock solution were added to 1-2 L seawater in a stirred beaker and then this was added to the aquarium. This two part mixing protocol was used to insure adequate dispersion of TBT in the aquarium.

Measurement of TBT. We collected 125 ml aliquots of seawater from each aquarium and extracted ^{14}C-TBT with 3 ml toluene. We were careful to notice that the solvent phase separated from the aqueous one before decanting. We then placed the solvent phase in a vial and added liquid scintillation cocktail (Aquasol, New England Nuclear, 10 ml) for liquid scintillation counting. We collected water samples just after adding new TBT stock solution to each aquarium, and then 24 h later, just before refilling and/or redosing. Liquid scintillation fluid blanks were measured to obtain background counts for calculation of DPM values.

Twice each week during the uptake phase, and once each week during depuration, three clams were randomly selected from each exposure aquarium for dissection. Clams were dissected to yield muscle tissue (foot and aductor muscle), viscera (gut and gonads if developed) and remains (mantle, gills and gonad tissue if it extended into the upper mantle). Tissues from each clam were measured separately, yielding 45 samples per collection (3 clams x 3 tissues x 5 concentrations).

Upon dissection, wet weights were determined for each tissue. They were then air dried for several days, reweighed to determine dry weight, and processed in a Packard 306 tissue oxidiser. All ^{14}C is converted during combustion to $^{14}CO_2$, trapped by reaction with an organic base, and delivered to a vial containing liquid scintillation fluid. This method is very efficient at recovering all ^{14}C, but does not discriminate molecular identities of compounds originally labelled with radioisotope. In addition to tissues, 'machine blanks' were also measured to obtain background counts for subtraction from sample counts during calculations of DPM values.

Accumulation of TBT by Microlagae

Three species of marine microalgae were used: Isochrysis galbana (Tahiti strain), Nannochloris sp. and Chaetoceros gracilis. A cyanobacterium, Synechococcus sp. was also tested. Cultures, obtained from ones maintained in collections at HBOI, were unialgal, but not necessarily axenic. All microalgal species were grown in 32 $^{0}/_{oo}$ F/2 medium (Guillard & Ryther, 1957). Two liter Erlenmeyer flasks containing the microalgae were maintained in environmental chambers set at 24 ± 1° C with a 16:8 L:D photoperiod. Synechoccos, the cyanobacterium, was raised in 28 $^{0}/_{oo}$ ASP II (Provalsoli et al., 1957). Culture conditions were 35° C and continuous light in a system after Myers (1950).

Accumulation Tests. We prepared an ethanol solution of [1-^{14}C]bis (tributyltin) oxide (custom synthesis, New England Nuclear; specific activity 3.67 mCi $mmol^{-1}$). Serial dilutions of this ethanol ^{14}C-TBT stock solutions were made by microliter additions of ethanol stock to filtered (0.45 µm membrane filters) seawater (30-35 $^{0}/_{oo}$). Eleven exposure concentrations ranged from 20 ng/L to 2 mg/L. The highest concentration is approximately the water maximum seawater solubility of TBT. Seawater solutions were prepared in polycarbonate centrifuge tubes prior to addition of cells. Solution volume for exposures of 1 µg L^{-1} and above was 50 ml. Due to relatively low specific activity, 250 ml volumes were used with the 4 concentrations below 1 µg L^{-1}. Following completion of the dilution steps, sufficient microalgae culture was added to give a final cell density of 1.5 x 10^5 cells ml^{-1}. Cell density of Synechoccus was 1.0 x 10^6 due to its significantly smaller size.

All microorganisms were incubated in the TBT-seawater solutions for 2 h at 25° C. Then they were centrifuged at 10,000g for 10 m at 4° C. We rinsed the cell pellet with 5 ml filtered seawater, isolated it in a scintillation vial and added 0.2 ml Soluene 350 tissue solubilizer (Packard). After 2 h disgestion, we added 0.2 ml acetic acid to neutralize the solubilizer, followed by liquid scintillation fluid (Aquasol, New England Nuclear).

Competition Experiments. A series of TBT-ethanol stock solutions were prepared so that each contained identical quantities of radioisotope (nominally 1 ng/L, 600 dpm in 45 ml). The remainder of the TBT added to produce desired nominal concentration contained non-radioactive carbon isotopes. Nominal concentrations were verified using chemical analysis techniques based on hydride derivatization, purge and trap of volatile butyltin hydrides and detection/quantification with atomic absorption spectrometry as explained previously). The specific activity of the ^{14}C-TBT we used set a lower concentration of 1 µg L^{-1}; thus, only 5 TBT concentrations were tested. In this exercise, 2 microalgal species, Isochrysis galbana and Nannochloris sp. were tested. Other details of the protocol were identical to those described above to test TBT accumulation to phytoplankton.

RESULTS

Acute Toxicity to Clam Larvae

All clam-post larvae in 10 µg/L were dead at the end of 25 days exposure to TBT (Fig. 1). Survival of remaining exposure groups was between 40 and 50%, typical of laboratory and hatchery survival rates of this species. Within this narrow range, survival was not consistently exposure dependent. These data do not lend themselves to rigorous estimation of an LC50 value, but it falls within the range of 7.5 to 10 µg/L for exposure durations of 25 days. Experience with TBT suggests that a dose-dependent pattern would likely be exhibited during longer exposure periods (Laughlin et al. 1982). The one used here, 25 days, is long enough, however, to support the conclusion that postlarvae are not a particularly sensitive stage.

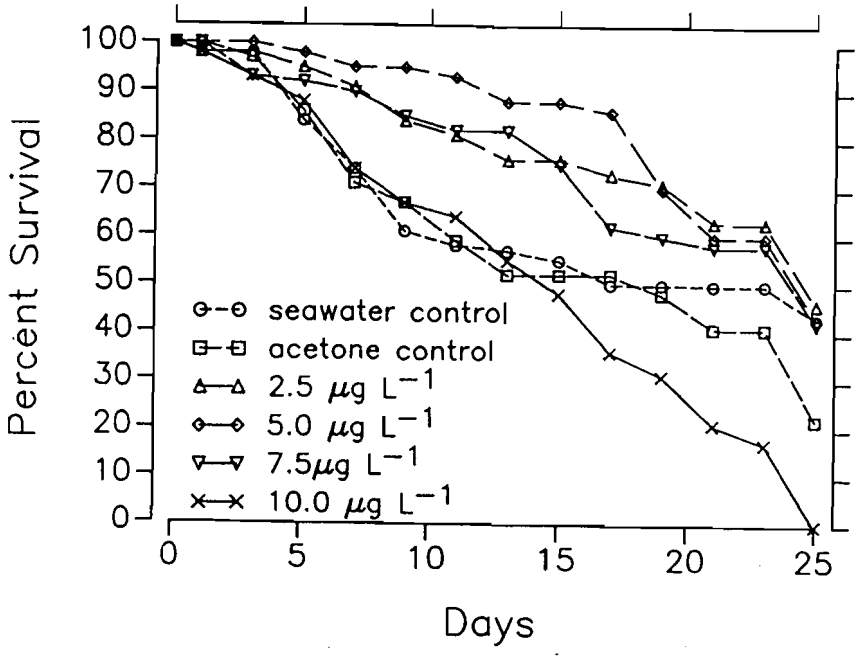

Figure 1. Mercenaria mercenaria. Survival of clam postlarvae exposed to tributyltin.

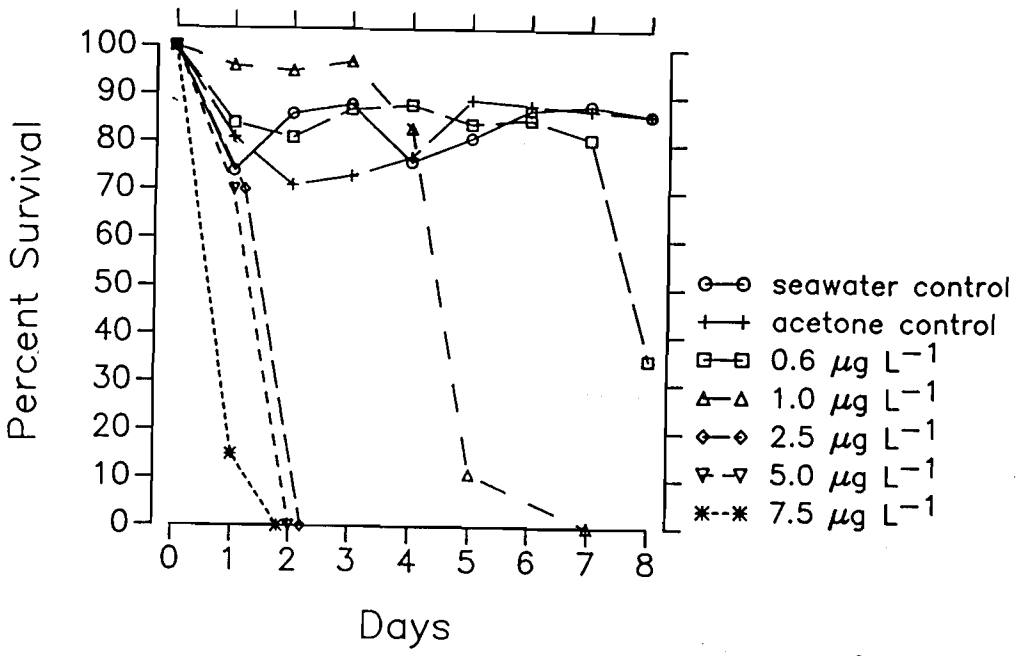

Figure 2. Mercenaria mercenaria. Survival of clam veligers exposed to tributyltin for 8 days.

Compared to postlarvae, mortality of clam veligers, the planktonic larval stages, occurred at lower concentrations and after shorter duration exposure to TBT (Fig. 2). All veligers were dead by day 2 in 2.5, 5.0 and 7.5 µg/L. Those in 1 µg/L displayed very little mortality relative to controls until day 4. By day 7, all were dead. Veligers exposed to 0.6 µg/L had survival similar to controls until day 8, then it fell to 36%. Like data for post-larvae, those for veligers do not lend themselves to typical LC50 calculations because of lack of partial kills necessary for rigorous determinations of statistics. It is, nevertheless clear that the 48 hr LC50 value is between 1 and 2.5 µg/L. Roberts (1987) recently published a 48 hr LC50 estimate of 1.65 µg/L for M. mercenaria veligers from Virginia. Our value and his are consistent. We conclude from our data that an LC50 for the duration of larval development is less than 0.6 µg/L.

Highly variable and occasionally low survival of acetone controls was a characteristic of both postlarval and veliger bioassays. This situation has been repeatedly observed in a number of bioassays we have conducted. Addition of acetone or ethanol in excess of 100 µL/L seawater leads to microbial growth which fouls test containers and kills larvae outright. Reduction of carrier volume to 10 µL/L was not, apparently, sufficient to completely stop microbial induction by added carbon sources. That its effects were not observed in TBT solutions may be due to toxicity of the organotin compound to some bacteria. Other explanations of carrier control mortality are also plausible. Subsequent experience has suggested

that use of an aqueous stock, if possible, is preferable to one with acetone, ethanol or acetate.

Growth of veligers was markedly reduced by exposure to TBT (Fig. 3). Valve length of controls increased from 101.2 ± 1.6 to 232 ± 8.8 µm (mean ± 1 standard deviation). Veligers exposed to 0.6 µg/L TBT, the only concentration with any survival for the duration of the experiment, increased their mean valve length to only 119 ± 4.7 µm (measurement on day 8). Given the standard deviation of mean valve length during the week of rearing, it is difficult to assume any significant growth occurred after the first day in veligers exposed to TBT.

Analysis of TBT in exposure solutions from experiments showed that measured concentrations were within 87% of nominal (Fig. 4). After 24 hr, exposure solutions for the postlarval experiments were ~50% of nominal and those in the veliger experiments (static renewal) were 28% of nominal. Analysis of 24 h solutions was performed without filtration or any other treatment to remove microalgae. In their presence, recovery of added TBT (0.120 µg/L) was only half that for oceanic seawater with virtually no suspended particulates. Thus our analyses report dissolved TBT and are likely to underestimate TBT bound to microalgae and potentially available by ingestion. These data also indicate that time averaged mean exposure concentrations are lower than nominal values by approximately 50%. For purposes of clarity, however, nominal values will be used for reference throughout this report.

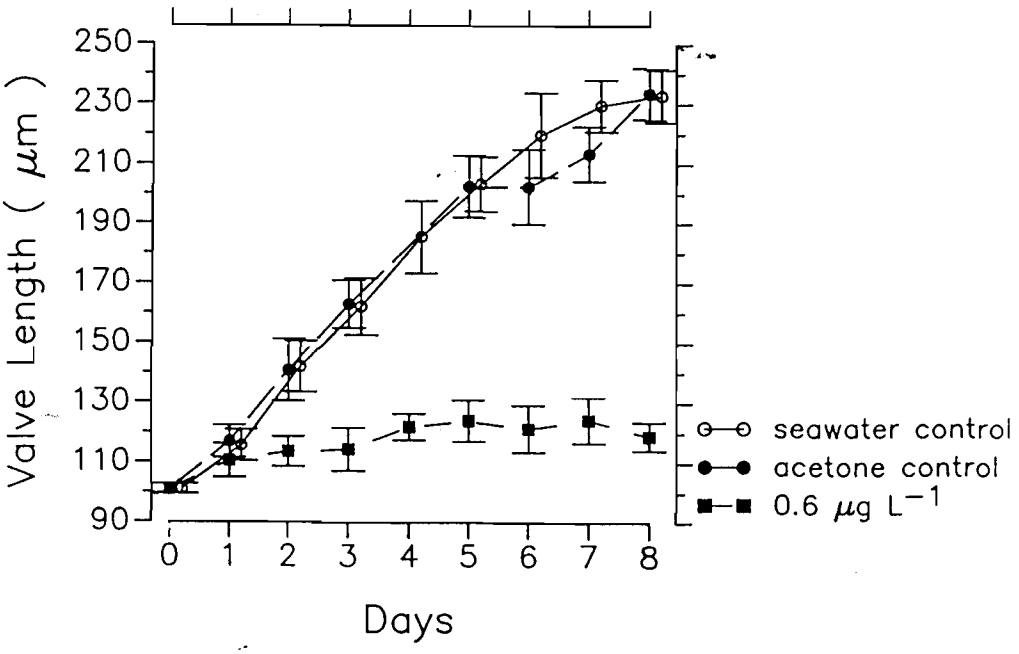

Figure 3. Mercenaria mercenaria. Cumulative growth of clam veligers exposed for 8 days to tributyltin.

Chronic Embryolarval Toxicity to Mercenaria mercenaria

Analysis of TBT in freshly prepared exposure solutions yielded values 75-102% of nominal ones (Fig. 4). At the end of 24 h, seawater used in the bioassay (to which larvae and microalgae were added as food) had TBT concentration values approximately 20-30% 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 present in bioassay containers, only about half of an added TBT spike 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.

The most noticeable trend in survival of clam larvae in the experiment was a gradual decline over the 2 wk observation period (Figs. 5 and 6). 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 three factors tested, 'TBT concentration', 'day' or 'duration of exposure', only 'day' was statistically significant (Table 1). The correlation coefficient of this statistical model is only 0.174, reflecting a very poor exposure dependence, as well as a highly variable daily count of living larvae.

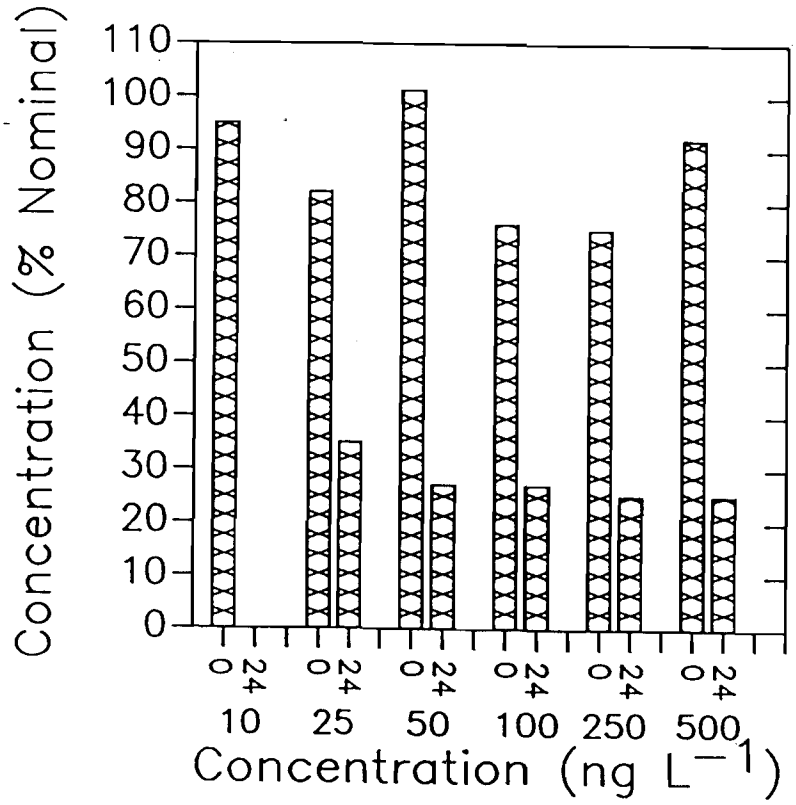

Figure 4. Initial and final TBT concentrations in bioassay solutions. Values are percentage of nominal values initially and after 24 h in bioassay containers.

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. Abbreviations: Day, number of larvae on each day of observation; Duration: 5 or 14 day exposure to TBT, treated as a class variable.

Source	df	SS	MS	F	p > F
TBT Concentration	1	946	946	1.31	0.2533
Day	1	44831	44831	62.11	0.0001
Duration	1	4	4	0.01	0.9427
Error	302	217990			
Total	305	263771			

$R^2 = 0.174$

Growth of clam larvae in both controls was dramatically rapid (Fig. 7). On the first day, mean valve length was ~100 µm in the 3 groups, but increased to between 210 and 250 µm, depending on the hatch. Growth of TBT exposed groups consistently declined as exposure concentrations increased. In 500 ng/L TBT, there was virtually no growth. Mean valve length increased only ~30 µm during the entire 14 day 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. 8). Differences between the two groups, although small, were statistically significant (Table 2a).

The decline in valve length shown in Fig. 8 is relatively steep. It is not obvious that a 'no effect' level occurred even at the lowest exposure concentration, 10 ng/L. The Student-Newman-Keuls test showed all TBT exposed groups to be significantly smaller than 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 Figure 5. 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 exposure groups were performed. Each of the comparisons showed the TBT group was statistically smaller than controls (Table 2c). Seawater and acetone controls were not significantly different from each other so data in these two groups were pooled to obtain the control group for t-tests.

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

A. Mercenaria mercenaria. Results of analysis of variance of valve length data of clam larvae on day 14. The dependent variable is the measured length transformed by log 10. Abbreviations: Duration, 5 or 14 day exposure, 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-Kuels tests. Means of two 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. Abbreviations: 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			

In order to better visualize how TBT influences growth of clam larvae, daily growth rates were calculated from data shown in Figure 7. Daily growth rates are calculated as the difference between the mean size of each group and the mean on the previous day. Daily growth rates are not

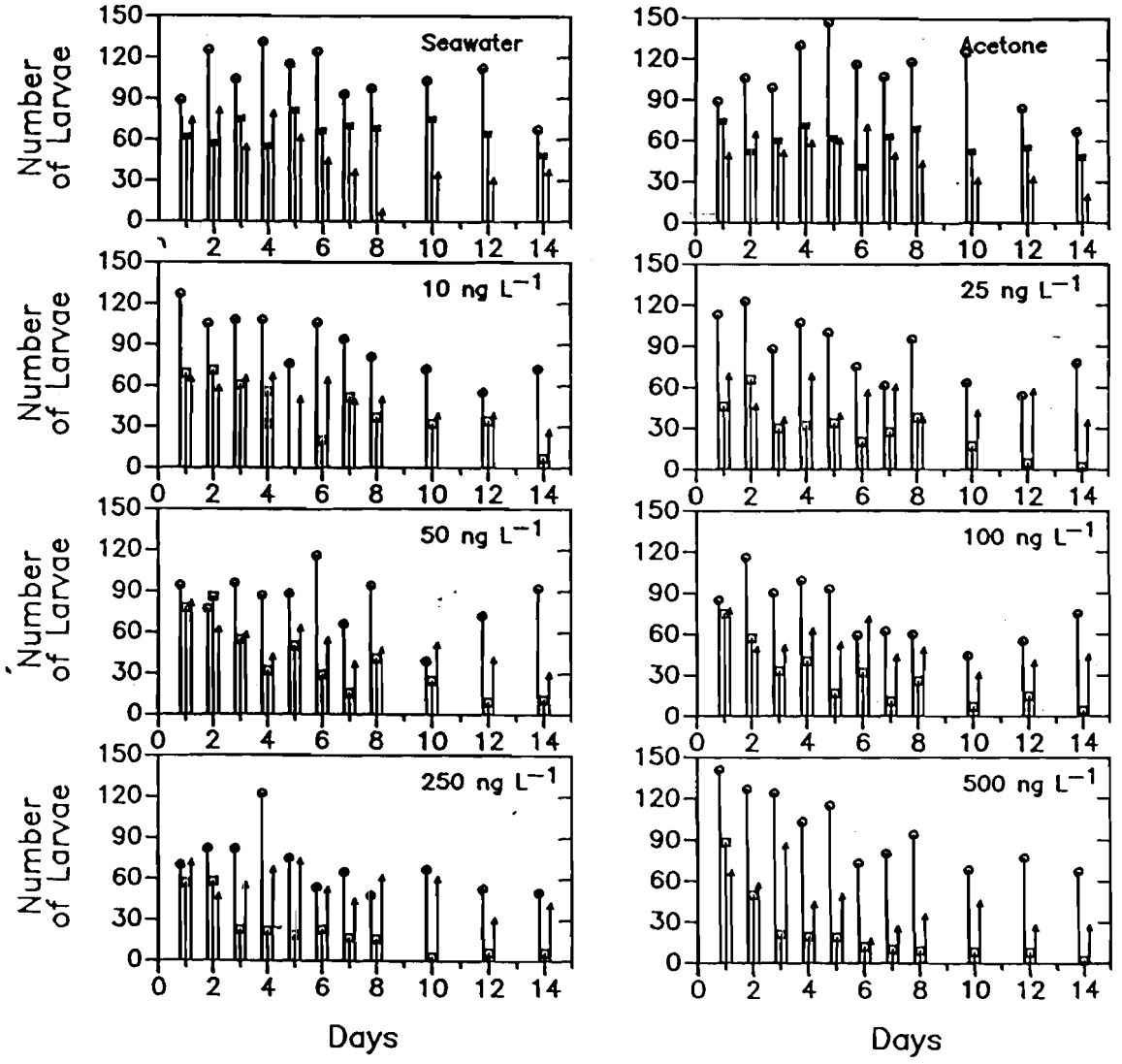

Figure 5. Mercenaria mercenaria. Survival of clam veligers exposed for 14 days to TBT. Each symbol designates 1 of 3 replicates.

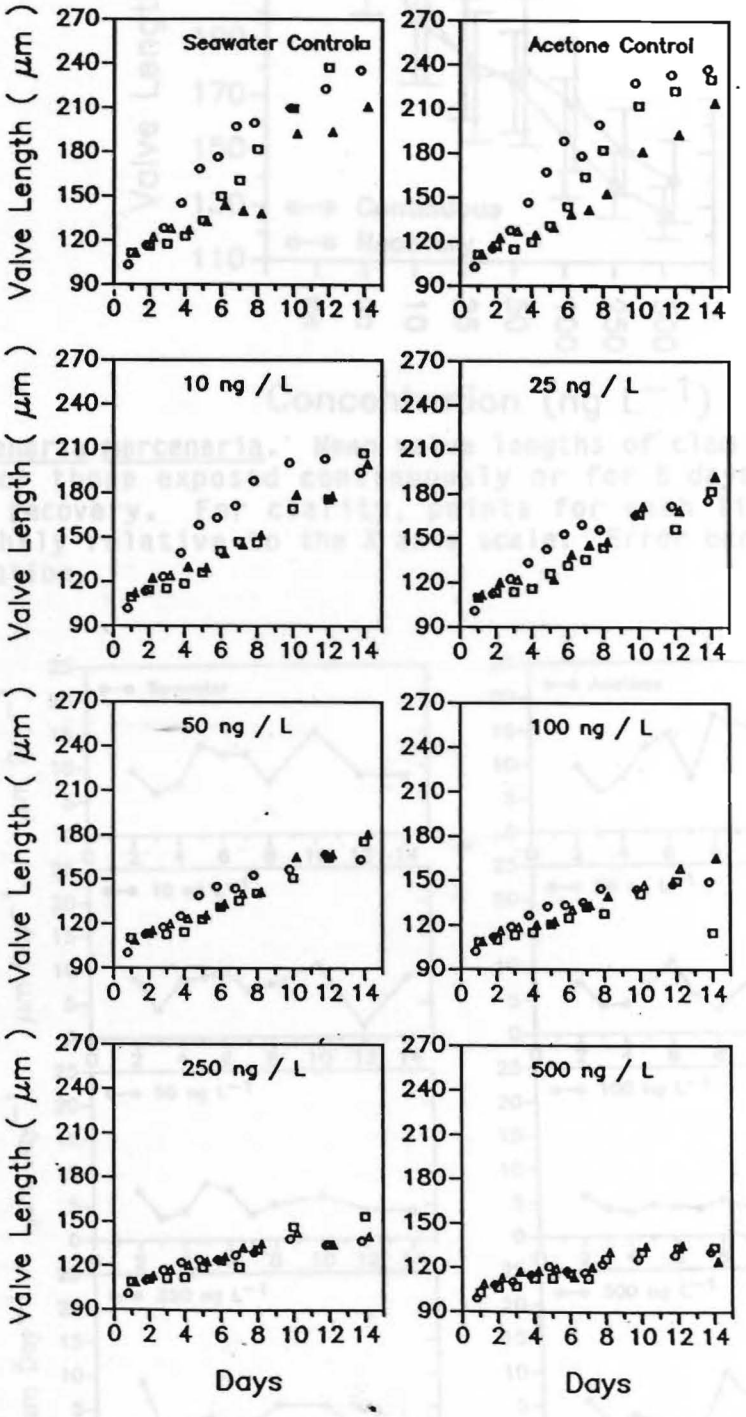

Figure 7. Mercenaria mercenaria. Growth of clam larvae exposed for 14 days to TBT. Each symbol represents 1 of 3 separate groups of clams. Each point is the mean of up to 25 larvae. Points are offset slightly for clarity on each day mark.

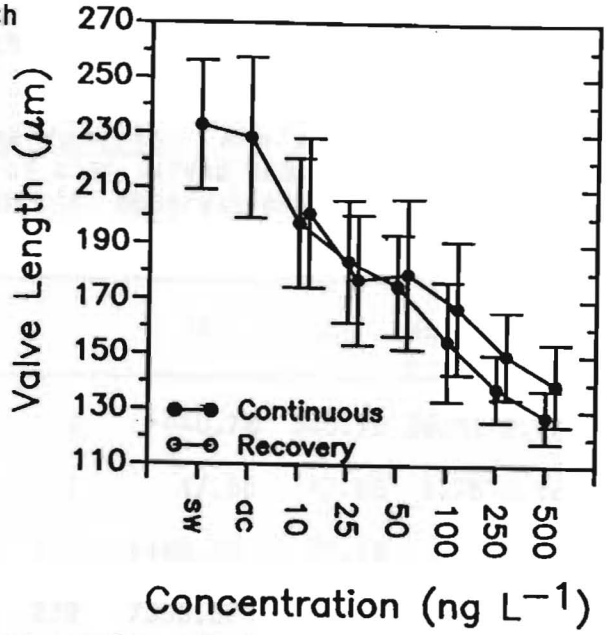

Figure 8. Mercenaria mercenaria. Mean valve lengths of clam larvae on day 14 for those exposed continuously or for 5 days followed by 9 days recovery. For clarity, points for each line are offset slightly relative to the X axis scale. Error bars: 1 standard deviation.

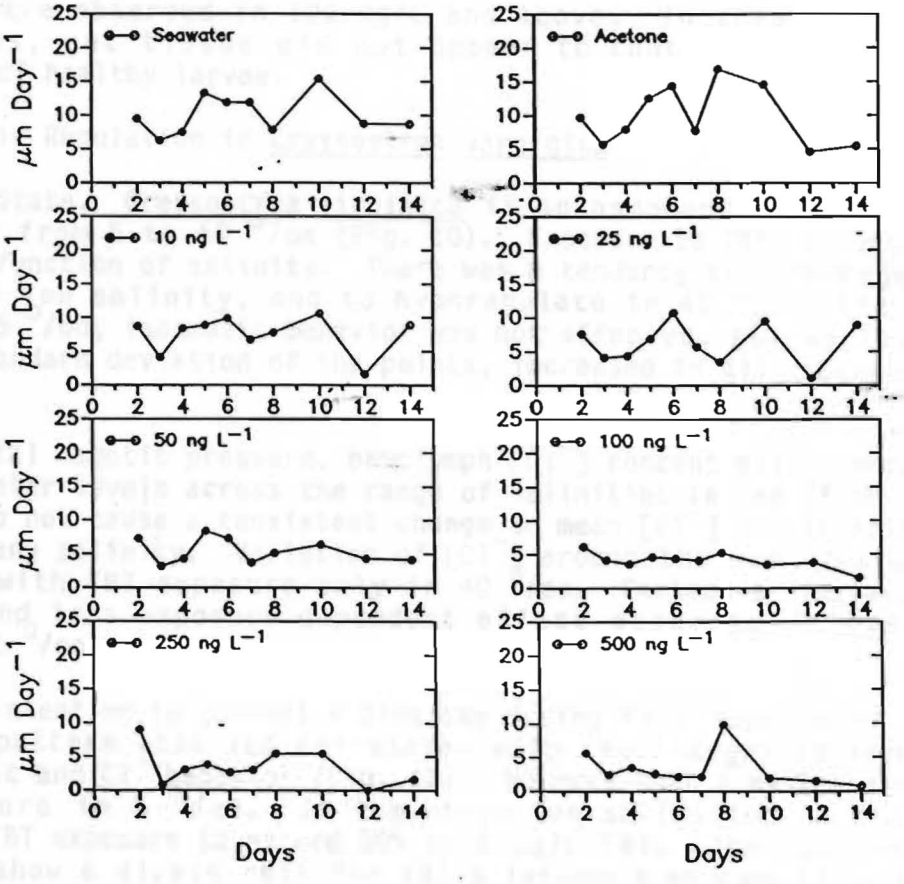

Figure 9. Mercenaria mercenaria. Daily growth rates of clam larvae exposed to TBT for 14 days. Each point is the mean of 3 replicates.

Table 3. Mercenaria mercenaria: Analysis of variance for data of daily growth rates of clam larvae exposed to TBT. Abbreviations: Day, day of experimental observation.

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			

$R^2 = 0.121$

Visual observations during daily counting and measurements did not reveal any 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 and above. In these exposure concentrations, gut tissue did not appear to contain microalgae, a characteristic of healthy larvae.

Osmotic and Ionic Regulation in Crassostrea virginica

Steady State. Crassostrea virginica is an osmoconformer over the salinity range from 5 to 40 °/oo (Fig. 10). Exposure to TBT produced two responses as a function of salinity. There was a tendency to hyperregulate in 5 °/oo, the low salinity, and to hyporegulate in 40 °/oo, the high salinity. In 25 °/oo, isosmotic behavior was not affected, but variation, shown by the standard deviation of the points, increased in all exposures to TBT.

Like total osmotic pressure, hemolymph [Cl^-] concentration conformed to ambient seawater levels across the range of salinities tested (Fig. 11). TBT exposure did not cause a consistent change on mean [Cl^-] levels relative to controls at any salinity. Variation of [Cl^-] around the mean increased substantially with TBT exposure only in 40 °/oo. Perhaps a similar, but less obvious and less exposure dependent effect occurred in oysters acclimated to 25 °/oo.

It was not our intention to conduct a bioassay during this experiment, but the mortality pattern observed correlates with the changes observed by hemolymph osmotic and Cl^- behavior (Fig. 12). No more than 1 oyster died in any TBT exposure in 5 °/oo. In the other two salinities, mortality increased with TBT exposure to exceed 50% in 2 µg/L TBT. The data do not unequivocally show a direct role for TBT's influence on osmotic or ionic regulation as a factor in mortality. The correlation between salinity and mortality indicates a significant interaction between TBT and salinity whose physiological basis warrants further investigation.

Kinetics of Osmotic and Ionic Regulation. When oysters were transferred from 25 to 5 °/oo salinity, there was little change in hemolymph osmotic pressure within the 24 hr. observation period (Fig. 13). Adjustment was not influenced by exposure to TBT. The same is true of Cl^- regulation (Fig. 14). In general, it appeared that oysters remained shut most of the time following the salinity decline, only occasionally opening to take in small volumes of water into the mantle cavity.

Hemolymph osmotic and Cl^- change much more rapidly when oysters are transferred from 25 to 40 °/oo (Figs. 15 and 16, respectively). When control oysters were transferred to higher salinities, hemolymph osmotic pressure and Cl^- both show a change within 0.5 hr. Values for both continued to increase during the next 24 hr to values of ~1000 mosm for osmotic pressure and 320 meq/L for Cl^-. This is at least 90% of the steady state value measured in the first part of the experiment, indicating that acclimation to salinity increases occurs within 24 hr, a very rapid rate.

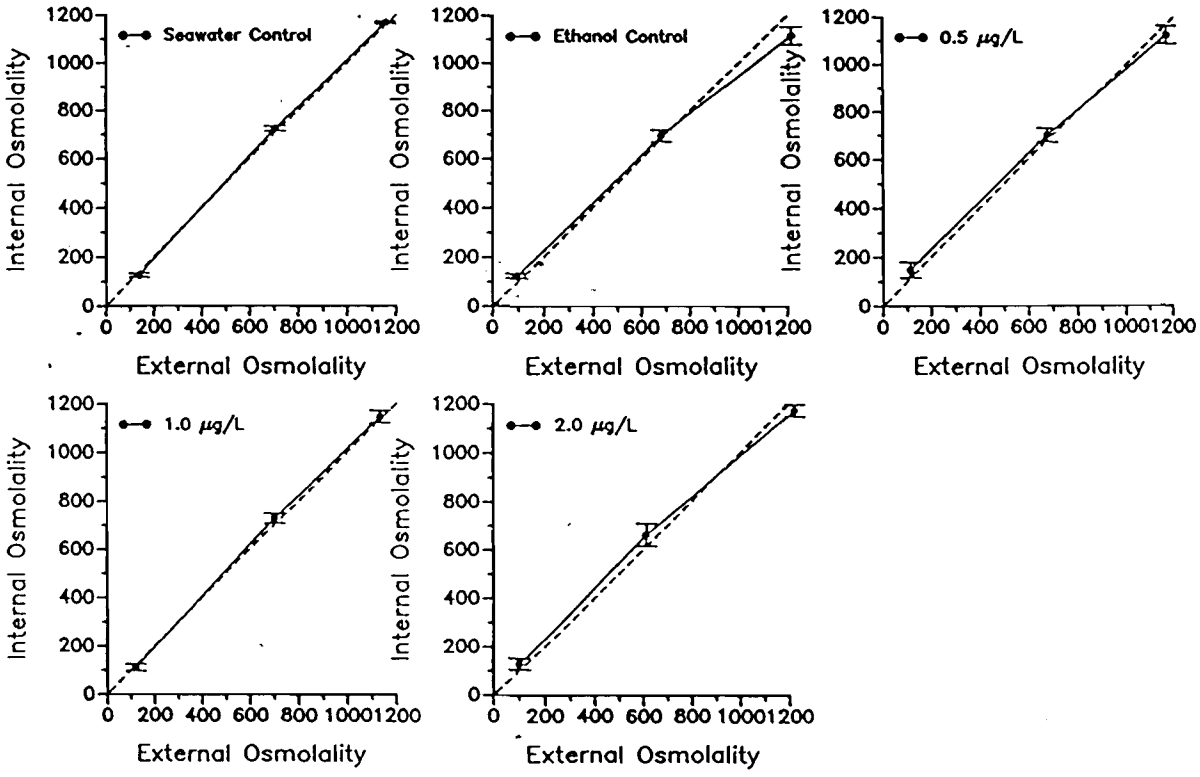

Figure 10. Crassostrea virginica. Hemolymph total osmotic pressure. Dotted line: isosmotic line. Each point is the mean of 5-12 oysters (cf. Fig 12). Error Bars: 1 standard deviation. Oysters are osmoconformers across the salinity range 5 to 40 °/oo. TBT exposure tends to lead to hyperregulation in 25 °/oo and hyporegulation in 40 °/oo.

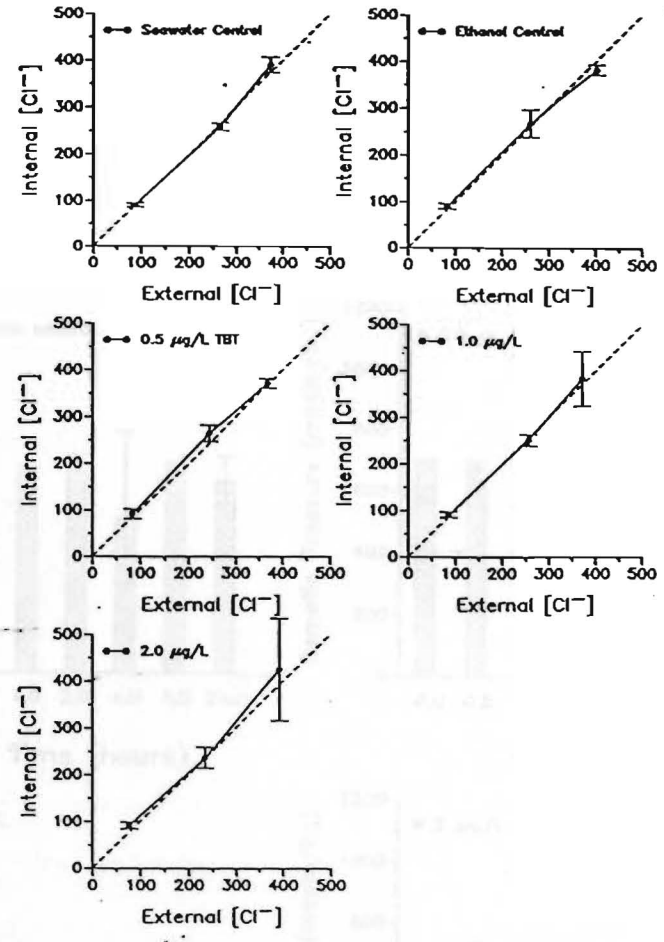

Figure 11. Crassostrea virginica. Hemolymph Cl^- ion regulation. Dotted line: isochloride line. Each point is the mean of 5-12 determinations (cf. Fig. 11). Units on axes are meq Cl^-/L. Error Bars: 1 standard deviation. In 25 and particularly in 40 °/oo, variation around the mean hemolymph [Cl^-] increases with increasing TBT exposure.

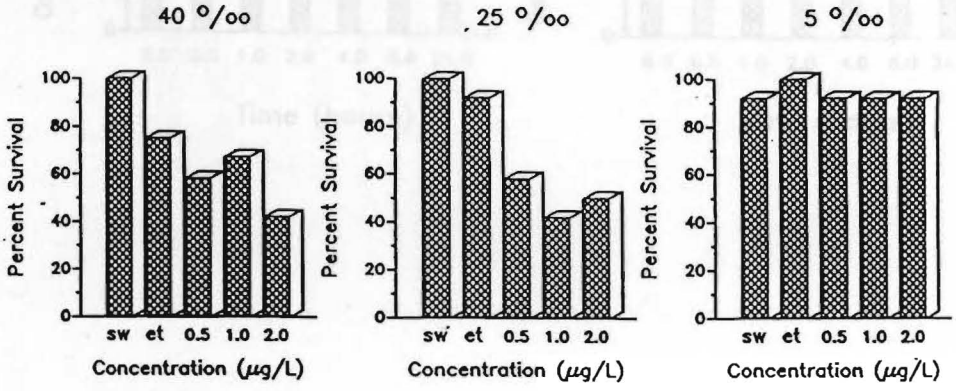

Figure 12. Crassostrea virginica. Survival of oysters acclimated to 3 different salinities and then exposed to TBT dissolved in seawater. Percent values are based on survival of 12 oysters originally present in test aquaria. Mortality was significant and exposure dependent in salinities of 25 and 40, but not in 5 °/oo.

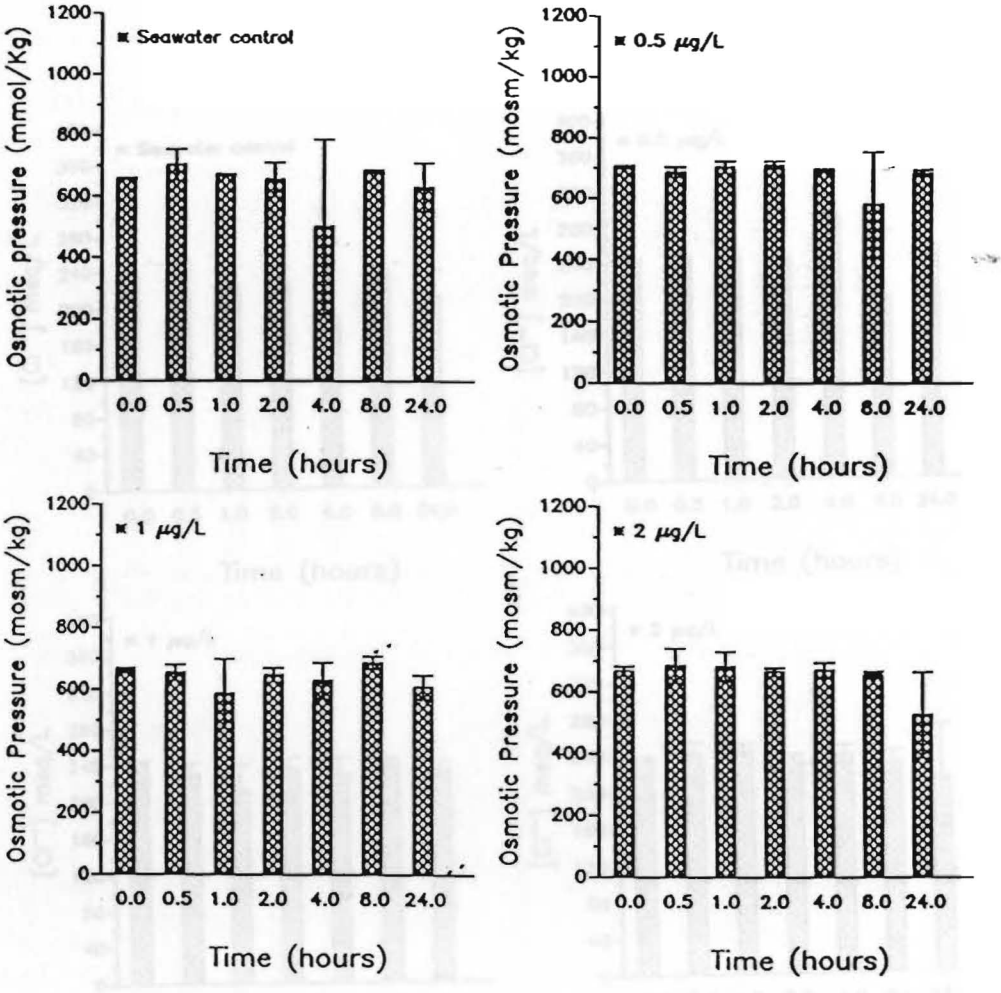

Figure 13. Crassostrea virginica. Hemolymph osmotic pressure, transfer from 25 to 5 °/oo. Error bars: 1 standard deviation. During 24 hr after transfer, there was very little change in osmotic pressure in controls or in oysters exposed to TBT.

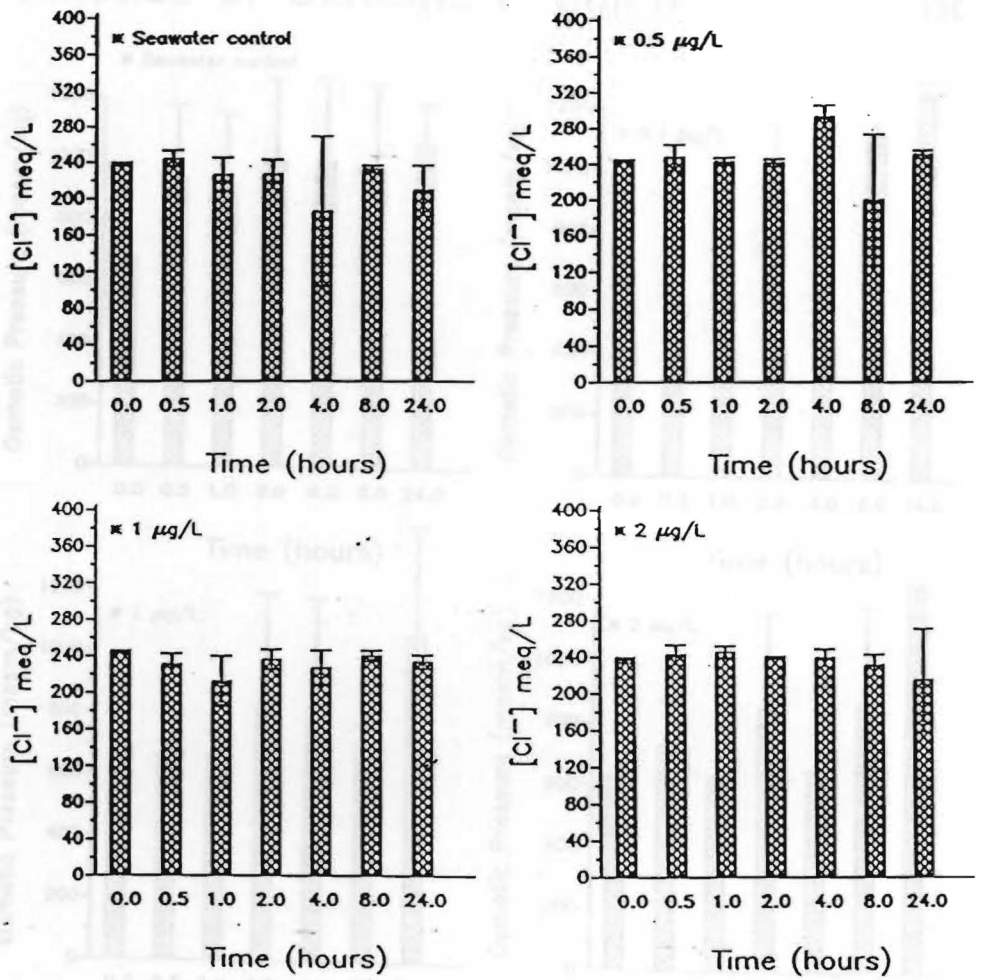

Figure 14. Crassostrea virginica. Hemolymph [Cl^-] during transfer from 25 to 5 °/oo. Error bars: 1 standard deviation. Like osmotic pressure, there was very little change after 24 hr either in controls or in oysters exposed to TBT.

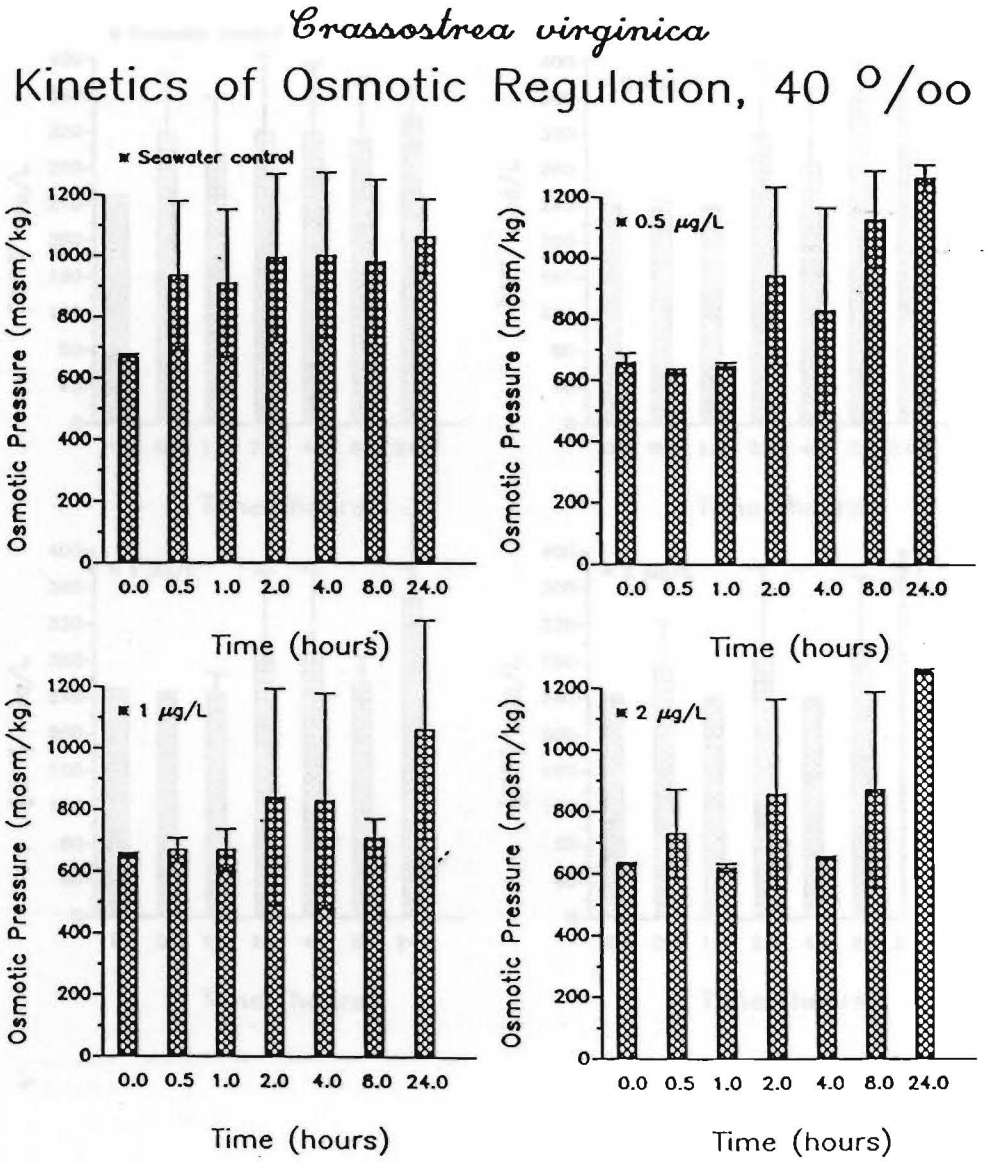

Figure 15. Crassostrea virginica. Hemolymph osmotic pressure during transfer from 25 to 40 °/oo. Error bars: 1 standard deviation. In controls acclimation to the new salinity began within 30 min, but was delayed until after the first hour in oysters exposed to TBT.

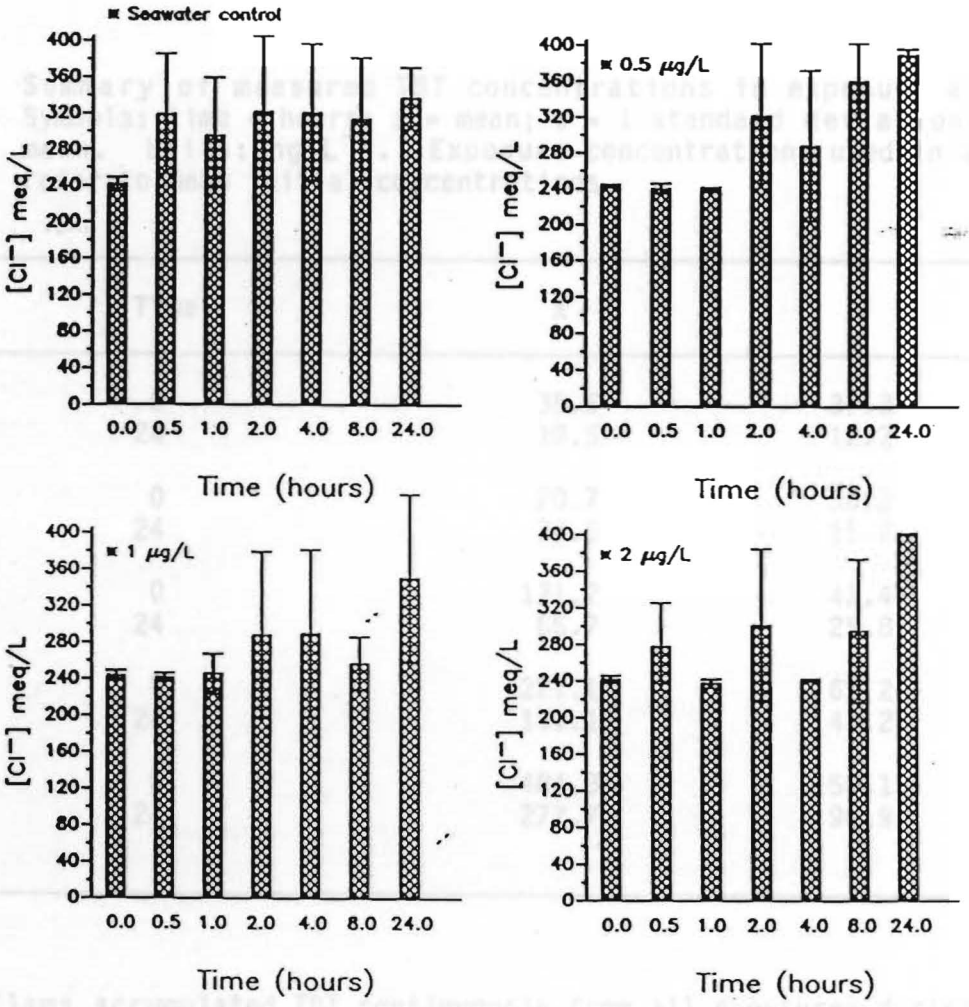

Figure 16. Crassostrea virginica. Hemolymph [Cl^-] during transfer from 25 to 40 °/oo. Error bars: 1 standard deviation. Changes in [Cl^-] closely mimic those of total osmotic pressure during acclimation. Oysters exposed to TBT appear to begin the process more slowly, but then come to steady state more quickly.

Bioaccumulation of TBT by Clams.

Extracts of seawater from aquaria indicated large apparent variability in exposure concentrations, frequently by as much as a factor of 3 (Figs. 17 and 18). In all cases, final concentrations were lower by 40-50% than initial ones and displayed high variability (Table 4).

Table 4. Summary of measured TBT concentrations in exposure aquaria. Symbols: time = hours; x = mean; s = 1 standard deviation of the mean. Units: ng L^{-1}. Exposure concentrations used in the text refer to mean initial concentrations.

Tank	Time	x	s
1	0	36.6	37.3
	24	19.5	12.2
2	0	70.7	30.2
	24	34.5	15.2
3	0	121.2	42.4
	24	65.7	25.8
4	0	221.1	62.2
	24	131.1	43.2
5	0	484.3	150.1
	24	277.7	96.9

Clams accumulated TBT continuously from all exposures during the 56 day observation period (Figs 17 and 18). Viscera had highest weight specific burdens, followed by remains and muscle tissue. During the uptake phase, relative differences in tissue burdens were not greater than a factor of 3 among the 3 tissue types, and frequently much less. There was no apparent steady state in accumulation in any exposure. Final tissue burdens increased in proportion to exposure concentration. For example, no weight specific TBT burden in tissue of clams exposed to 37 ng L^{-1} exceeded 250 ng g^{-1}. Highest burdens increased to 3600 in 484 ng L^{-1}. Half times for doubling tissue burdens appeared in most cases to be slightly greater than 2 wk.

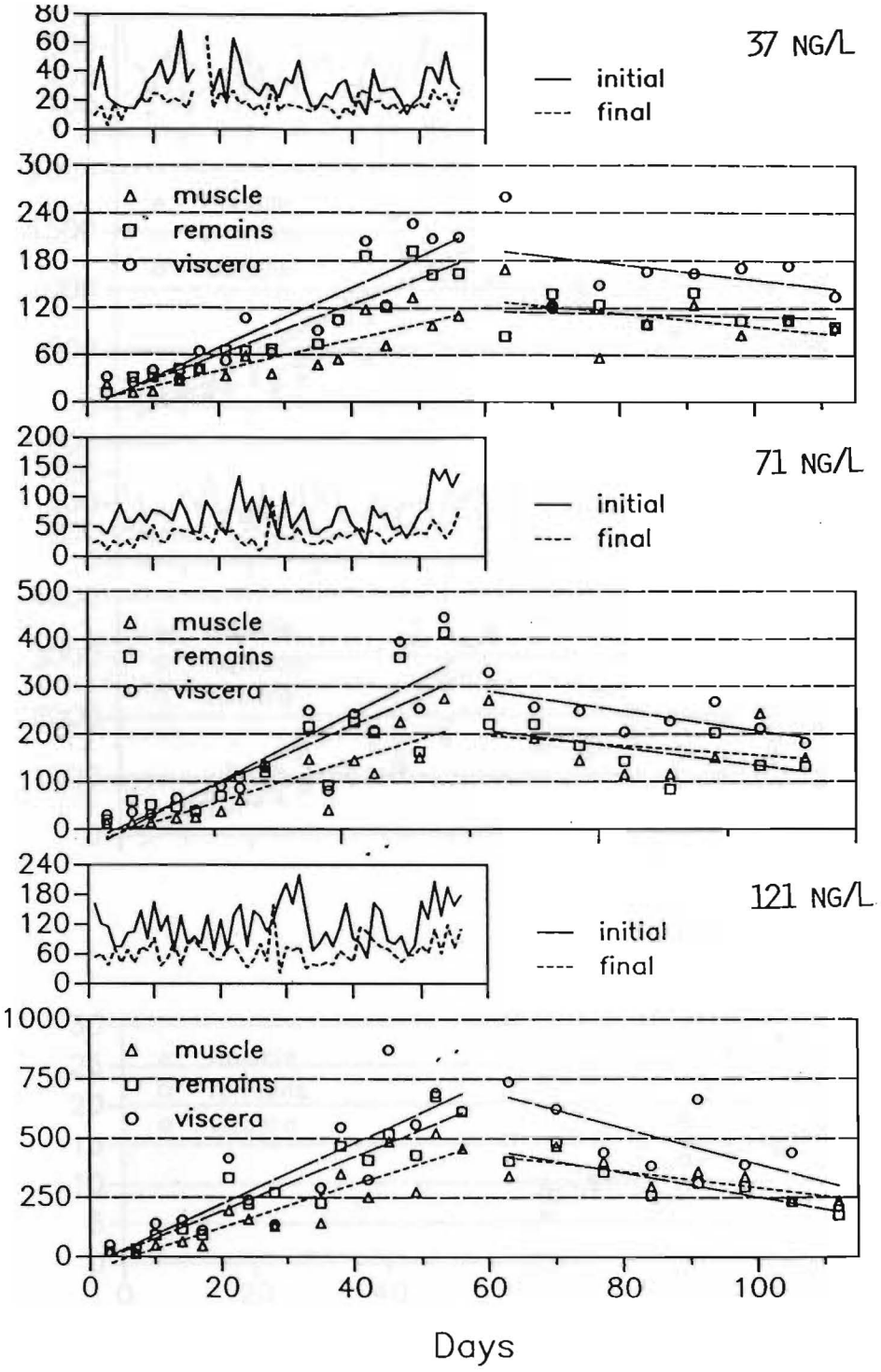

Figure 17. Mercenaria mercenaria. TBT exposures of 37, 71 and 121 ng/L. Upper panels: Initial and final TBT concentrations in aquaria during exposure phase. Lower panels: Accumulation and depuration of TBT. Lines are least square regressions on tissue burdens against time.

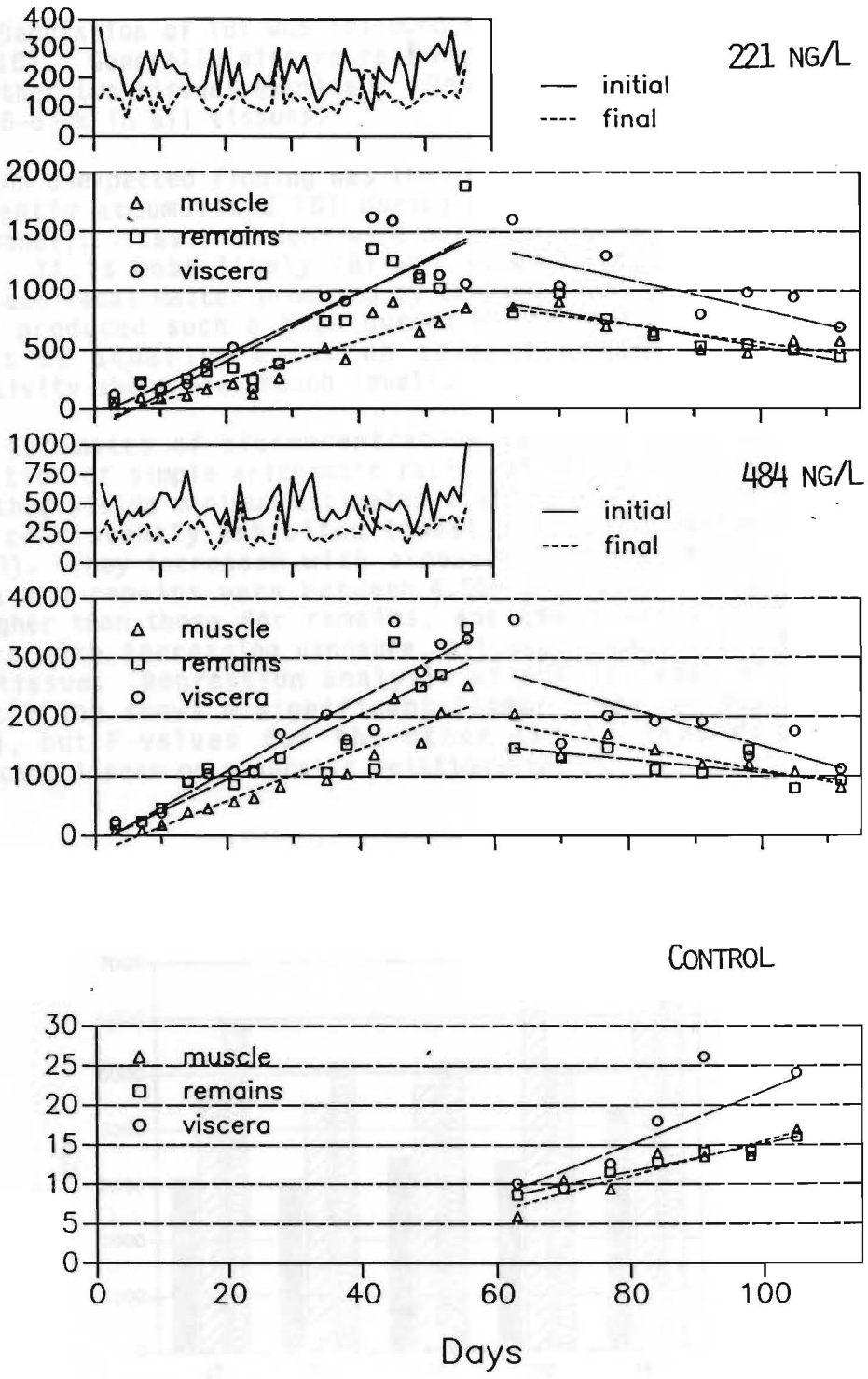

Figure 18. Mercenaria mercenaria. TBT exposures of 221, 484 and depuration controls. Upper panels: Initial and final TBT concentrations in aquaria during exposure phase. Lower panels: Accumulation and depuration of TBT. Lines are least square regressions on tissue burdens against time.

Depuration of TBT was followed for 7 wk following accumulation (Figs 17 and 18). Generally viscera retained highest TBT concentrations relative to the other two tissues examined. Depuration was not rapid with a half time of 5-6 wk in all tissues.

An unexpected finding was that control clams in the depuration tank consistently accumulated TBT during a 6 wk observation period (Figure 18, bottom panel). Tissue burdens were not high, never exceeding 26 ng g^{-1} wet weight. It is most likely TBT was taken up from water. Ingestion of particulate fecal matter produced by exposed clams is possible, but unlikely to have produced such a high burden in the controls. We measured solvent extracts of aquarium water on several occasions, but never found radioactivity above background levels.

Estimates of bioconcentration factors (BCF) were obtained by calculation of simple arithemtic ratios of tissue and water concentrations. This method yields minimum estimates since no steady state occurred. Muscle tissue consistently exhibited lowest BCF values, between 3,000 and 4,250 (Fig. 19). They increased with exposure concentration. BCF values for viscera and remains were between 4,500 and 6,500. Usually viscera values were higher than those for remains, and there was a tendency for BCF to increase with increasing exposure, although trends are not as clear as for muscle tissue. Regression analysis of BCF for each tissue on exposure concentration shows a significant linear trend for muscle ($F = 38.5$; $p < 0.0084$), but F values for the other two tissues do not indicate a significant linear or quadratic relationship.

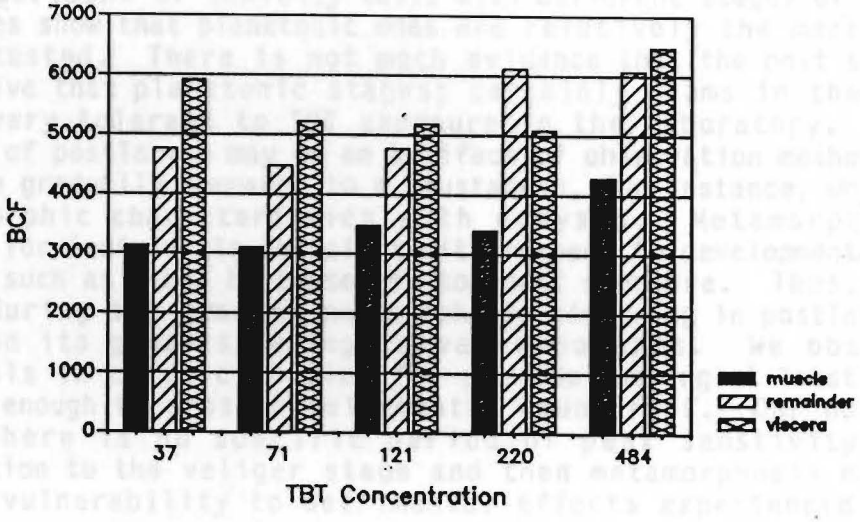

Figure 19. Mercenaria mercenaria. Bioconcentration factors for clams exposed to 5 different TBT concentrations.

Accumulation of TBT by Microalgae

Marine microalgae can be distinguished both by the extent and pattern of their TBT bioaccumulation. Chaetoceros gracilis displayed the highest accumulation, Nannochloris sp. was intermediate and Isochrysis galbana was lowest (Fig. 20). Quantitatively, I. galbana and Nannochloris had burdens only ~1/20 and ~1/7, respectively, of those of C. gracilis. Perhaps as interesting as the difference in burdens is the pattern with respect to concentration. Both Chaetoceros gracilis and Nannochloris display a linear relationship between exposure concentration and accumulation. This pattern is consistent with a partitioning mode of action. Isochrysis galbana, however, exhibits a distinctly non-linear trend that is consistent with a binding mode of accumulation. The cyanobacterium, Synechococcus, had the lowest uptake TBT relative to cell density and there was a linear relationship between exposure concentration and cell burdens (Fig 21).

Competitive binding experiments also indicate a difference between Isochrysis galbana and Nannochloris (Fig. 22). Differences in cell burdens of ^{14}C-TBT in Nannochloris were independent of the amount of ^{12}C-TBT present and differences between mean counts at any concentration were not statistically significant ($F = 0.64$; $p = 0.44$). Reductions in ^{14}C-TBT accumulated consistently and nonlinearly decline with increasing proportion of ^{12}C-TBT present. This pattern is compelling evidence for a mechanism dominated by hydrophobic partitioning in Nannochloris and ionic or covalent binding in I. galbana. In the latter case, the number of active sites is relatively easily saturated, limiting the extent of bioaccumulation and thus cell burdens.

DISCUSSION

Comparisons of toxicity tests with different stages of clam larvae and juveniles show that planktonic ones are relatively the most sensitive stages we tested. There is not much evidence that the post set stage is more sensitive that planktonic stages; certainly clams in the 1 mm size class are very tolerant to TBT exposure in the laboratory. Reports of sensitivity of postlarvae may be an artifact of observation methods. Clams metamorphose gradually compared to a crustacean, for instance, which assumes post metamorphic characteristics with ecdysis. Metamorphosis is a bottleneck for individuals deficient with respect to developmental rates or deformities such as might be caused by toxicant exposure. Thus, mortality expressed during the gradual metamorphosis occurring in postlarval stages may have had its genesis during larval exposures. We observed our experimentals in chronic studies for periods lasting at least 14 days, a period long enough to cross developmental boundaries. Our observations indicate there is no specific period of peak senstivity, although differentiation to the veliger stage and then metamorphosis may provide windows of vulnerability to detrimental effects experienced at earlier periods.

These studies focused primarily on larval stages of clams, Mercenaria mercenaria because of a report of a 5 day LC50 value of 15 ng/L for veligers (Huencho-Bancerra, 1984). None of the experiments conducted with larval clams support such a low value. Simply on the basis of

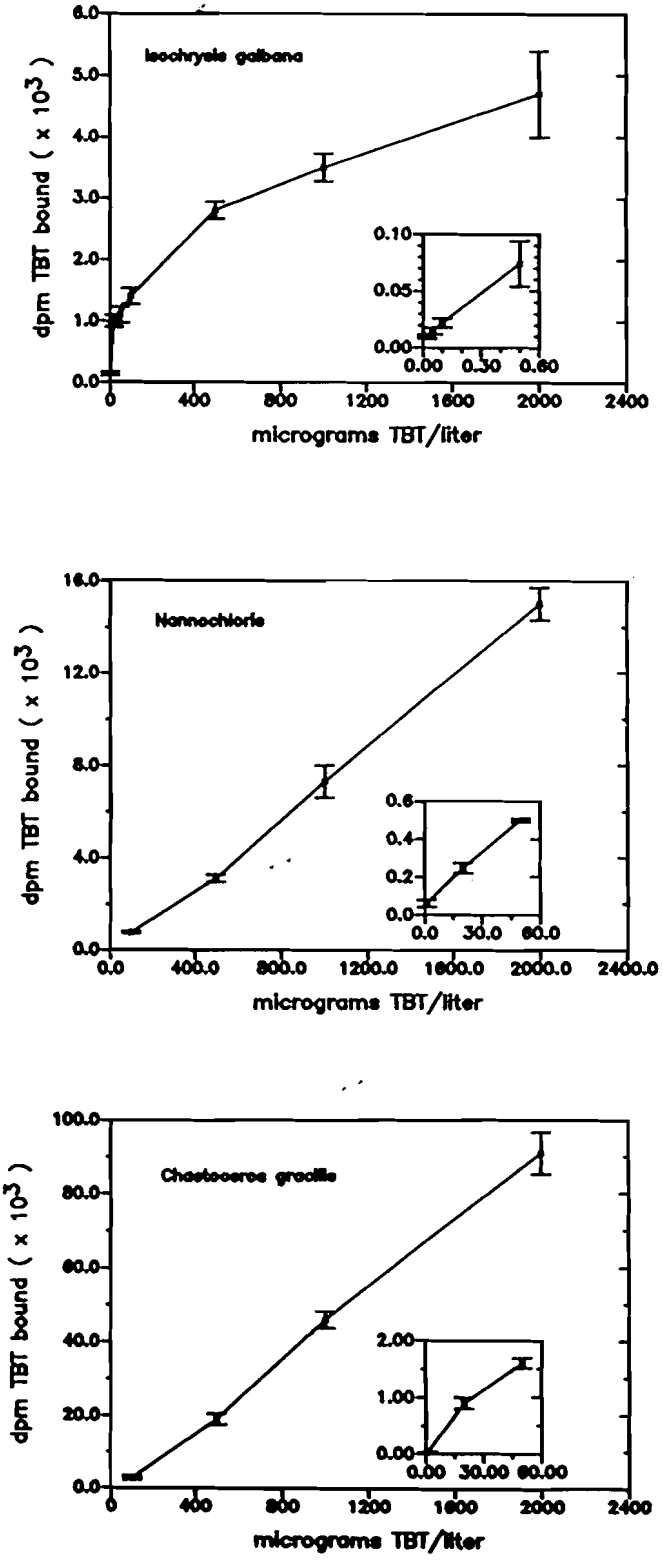

Figure 20. Accumulation of TBT by 3 species of marine microalgae. Insets show expansion of scales for data at low exposure concentrations. units on these axes are the sasme as for major axes.

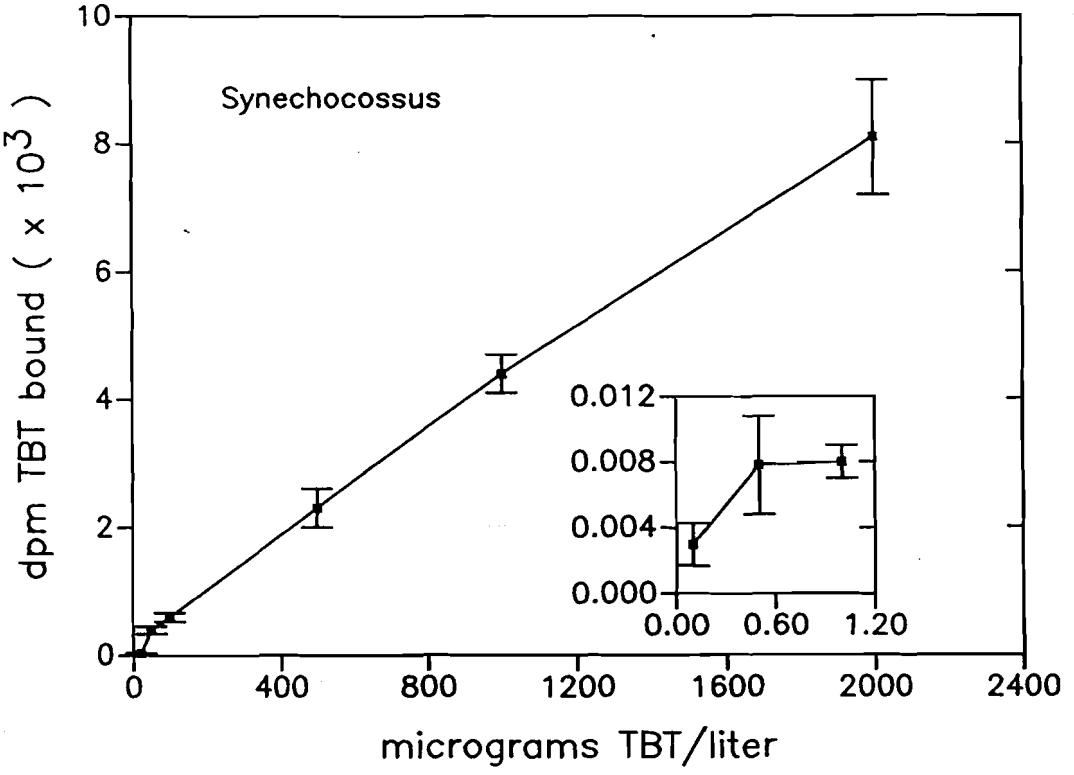

Figure 21. Accumulation of TBT by the cyanobacterium, Synechocossus.

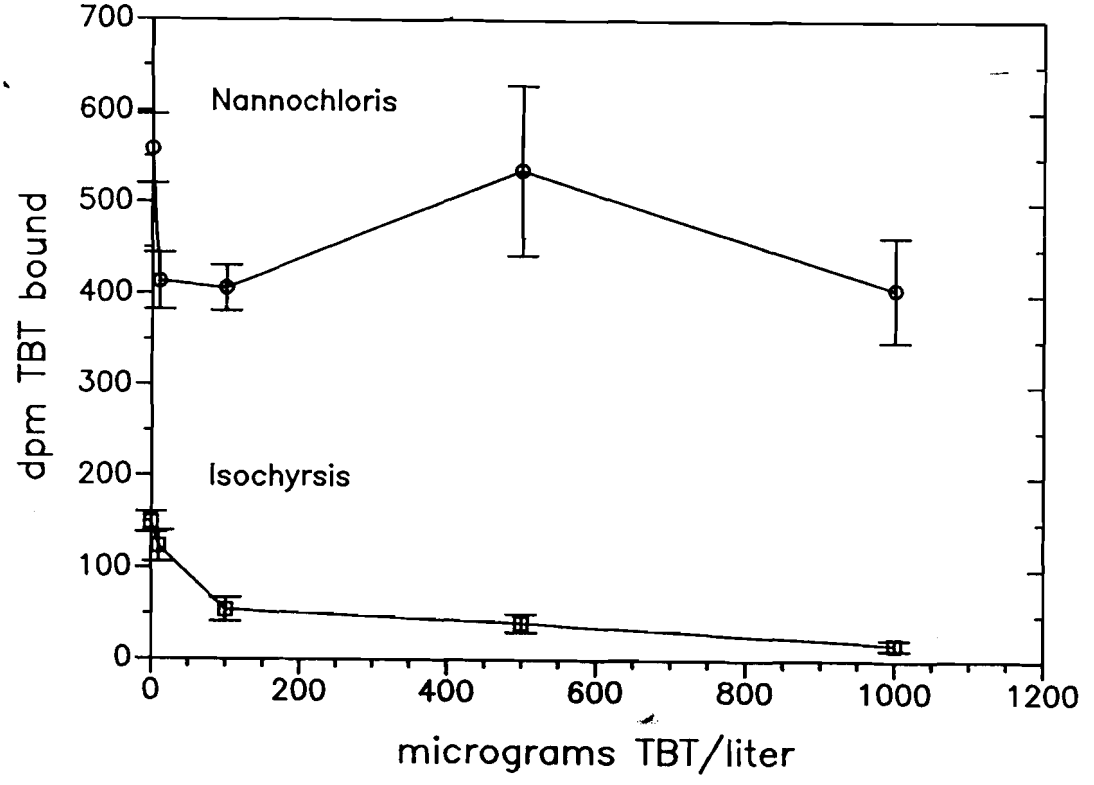

Figure 22. Accumulation of ^{14}C-TBT in the presence of ^{12}C-TBT.

experimental evidence, clam larvae will survive 500 ng/L for 14 days without experiencing acute mortality within this period. Reductions in growth of clam larvae were perhaps a more significant index of sublethal stress with potentially important effects. Growth reductions were consistently exposure dependent in concentrations between 10 and 500 ng/L and in the highest exposure, there was virtually no growth during 14 days. In TBT concentrations of 100, 250 and 500 ng/L, we observed no metamorphosis from veligers to pediveligers, the settlement stage. There is a common perception among workers who study marine bivalve larvae that adequate growth is a necessary (but not sufficient) prerequesite for metamorphosis. Thus, lack of metamorphosis is likely not a specific effect of TBT, but rather a generalized result of poor growth, a trait typical of many non-specific narcotic toxins.

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. Reductions in growth of bivalves exposed to TBT have been reported for Mytilus edulis (Beaumond and Budd, 1984, Stromgren and Bongard, 1987), scallops, Pecten maximus, (Paul and Davies, 1986) and oysters, Crassostrea gigas (Thane and Waldock, 1983, Paul and Davies, 1986, His and Robert, 1987). Reduced growth of larvae from exposed adults was reported by His and 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 two different perspectives. Stebbing (1982, Stebbing and Brinsley, 1985) has suggested that growth inhibition is a response to non-specific stress on metabolic homeostatic processes. Cybernetic mechanisms partition energy equivalents between metabolism and growth. In experiments reported here, there was a high negative 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 exposures 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 is 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. There is a much higher degree of uncertainty in estimates of ecological effects of reduced growth in TBT exposures below 50 ng/L. It is possible to only 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) and those causing significant chronic effects (10-50 ng/L) 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 (Lawler and Aldrich, 1987, Laughlin, 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.

Bioaccumulation is the link between the environment and the organism. It controls the dose responsible for acute and sublethal effects. It is, unfortunately, one subject where we still have insufficient knowledge on which to base informed judgements about the relationship between exposure and biological response. We undertook two sets of experiments to try to better understand important phemomena. The first involved accumulation of TBT by adult clams, the second, accumulation by microalgae consumed by clams.

Clams accumulated TBT in a pattern reflecting exposure concentrations. The highest burdens we observed were ~4 $\mu g\ g^{-1}$ wet weight, a value similar to mussels (Laughlin et al., 1986) and oysters (Waldock et al., 1983; Salazar et al., 1987). At concentrations between 30 and 200 ng L^{-1}, those more characteristic even of impacted environments, tissue burdens were correspondingly lower. Bioconcentration factors varied between ~3000 and ~6500, and increased with increasing exposure concentration.

There may be an interesting paradox in these data. On the one hand, both laboratory and field studies of marine bivalves produce similar values for tissue burdens of TBT, between undetectable and 10 $\mu g\ g^{-1}$ wet weight. The upper limit is apparently produced by an interaction between physico-chemical properties of TBT--its fugacity--and the proportional amount of hydrophobic material within bivalves which receives and contains bioaccumulated TBT. Tissue with largest lipid contents are capable of sequestering highest amounts of hydrophobic chemicals. BCF estimates from field and laboratory studies are similar, from 1,000 - 10,000 when exposure concentrations are above ~100 ng L^{-1}, values characterizing areas with direct TBT inputs from antifouling paints. Estimates of BCF values deviate considerably between laboratory and field studies when apparent exposure are low, below ~100 ng L^{-1}. In this case, BCF values range from 10,000 up to 100,000. It is possible that such high BCF values, based on water analysis, is an artifact of sampling, but they have been reported in several studies by different investigators at different sites and sampling different species. Thus, it seems possibile that sampling artifacts can be discounted. A more likely explanation is that where dissolved TBT concentrations are high (above ~100 ng L^{-1}), accumulation from water dominates the uptake route resulting in agreement between laboratory and field studies. Other routes or mechanisms appear to operate under some conditions of lower exposure. The most likely is food chain accumulation.

Food chain routes were initially suspect to us because some microalgae are known to sequester relatively high burdens of TBT (Magure et al., 1985). The processes illustrated by these investigators in one microalaga, Ankistrodesmus falcatus, are clearly not entirely due to

partitioning processes. Thus, high burdens in food may be translated into relatively high tissue burdens in filter feeders either because mass transfer processes via food invalidate assumptions of steady-state betweem organism and environment, or because water concentrations do not directly influence tissue burdens by partitioning.

Our investigations of microalgae and cyanobacteria show that food organisms may in fact be capable of accumulating high TBT burdens to be passed on to filter feeders. Ability to accumulate TBT varies by a factor of 20 between the best and worst accumulators. Also, some microorganisms tested accumulate TBT by a binding mechanism, while others appear to partition TBT from solution. Paradoxically, the best accumulator of those we tested appeared to partition TBT, while the worst bound TBT. These results indicate that food chain accumulation may be important route of TBT accumulation for marine bivalves. It is however, difficult to predict which microorganisms will be notable accumulators. It turns out that most of our larval rearing experiments were performed using Isochrysis galbana as food, the species exhibiting the lowest accumulation potential of any of the 3 we routinely feed bivalves.

There has been much speculation about the mode of action of toxic action of TBT. There is good evidence that its mode is concentration dependent (Lauglin, 1987). At high concentrations, it may act as an anionophore to disrupt ionic gradients across membranes in mitochondria (Aldridge, 1976; Selwyn, 1976). In principle, the same action could occur across any epithelia where there was an ionic gradient. Experiments with adult oysters, Crassostrea gigas were an attempt to test some aspects of this hypothesis.

Our experiments with oysters suggest that exposure to TBT levels as high as any reported from environmental analyses will not directly affect osmotic or ionic regulation. The increase in variation around a mean value for chloride ion regulation in particular, and salinity correlated mortality suggests that TBT acts indirectly on physiological mechanisms associated with osmotic regulation. This interaction will most likely be important in marginal habitats where physical conditions are already near the limits tolerated by oysters.

Risk assessment from laboratory studies relies on a knowledge of TBT concentrations in the field, and factors affecting fate and persistence. Environmental monitoring has indicated that boating activity is the primary identified source of TBT in the marine environment (Valkirs et al., 1986; Unger et al., 1986; Grovhoug et al., 1986; Hall et al., 1987). Marinas appear to be a point source for water containing high TBT concentrations. Such areas display TBT concentrations which will at least reduce growth of clams and possibly other bivalves, and may cause measurable bioaccumulation in bivalves. The influence of TBT released from densely moored boats and marinas on surrounding estuarine areas has yet to be conclusively demonstrated. The further the distance from the source of TBT, the likelier that uptake from food is important. This route is particularly difficult to trace directly; its recognition is usually through circumstantial evidence. Degradation balances release and bioaccumulation of TBT. Degradation processes includes both biotic and abiotic mechanisms.

In summary, research reported here supports findings that bivalves may be sensitive taxa to TBT, but sublethal effects, particularly reduced growth, are the likeliest ones under environmental levels usually reported. Bioaccumulation of TBT, both directly from water and through food remains a process of potential significant about which more information would be helpful, particularly to explain why steady state occurs so slowly and burdens are much higher than predicted from accepted bioaccumulation models.

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