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A Portable Environment Test System: A Field Assessment of Organotin Leachates

Test and Evaluation

S. M. Salazar
M. H. Salazar
Naval Ocean Systems Center

B. M. Davidson
P. M. Stang
K. Meyers-Schulte
Computer Sciences Corporation

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NAVAL OCEAN SYSTEMS CENTER
San Diego, California 92152-5000

E. G. SCHWEIZER, CAPT, USN
Commander

R. M. HILLYER
Technical Director

ADMINISTRATIVE INFORMATION

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A PORTABLE ENVIRONMENT TEST SYSTEM: A Field Assessment of Organotin Leachates—Test and Evaluation

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S.M. Salazar, M.H. Salazar, B.M. Davidson, P.M. Stang, and K. Meyers-Schulte

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A Portable Environmental Test System (PETS) was evaluated with tributyltin (TBT) antifouling leachates in San Diego Bay over a 7-month period. Overall mean test concentrations were 0.065, 0.077 and 0.193 $\mu g/\ell$ TBT. Treatments were tested against ambient seawater controls with three replicates of each using 340-ℓ tanks. Unfiltered seawater was pumped over a TBT-coated panel, creating a TBT-leachate diluted with ambient seawater in dilution/mixing units and distributed to test tanks.

The following parameters were measured: abundance and species diversity on fouling panels; condition and gonad indices, and bioaccumulation in adult mussels (Mytilus edulis); condition indices and bioaccumulation in clams (Macoma nasuta); and growth rates in juvenile mussels and juvenile oysters (Crassostrea gigas, Crassostrea virginica, Ostrea edulis, Ostrea lurida). There appeared to be several indications of effects at the highest concentration tested, although statistically significant differences were not consistently measured. At all TBT concentrations, adult mussels and clams accumulated TBT and adult mussel growth was reduced. However, growth of juvenile mussels and oysters in PETS tanks was significantly slower than growth of control animals held in the bay near the system seawater intake. These results suggest test animals in PETS tanks may have been under stress from the test system. It is not clear whether results represent affects to be expected in nature; the particular bioindicators selected may have been resistant to TBT and insensitive to measuring significant effects, or variability and system inadequacies reduced the ability to measure significant effects.

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EXECUTIVE SUMMARY

A Portable Environmental Test System (PETS) was evaluated with tributyltin (TBT) antifouling leachates in San Diego Bay for 7 months. Site-specific bioassays were performed to determine the effects of TBT leachates on individual species and communities of organisms. TBT leachates were produced in the leachate tank by circulating ambient seawater around a Plexiglas panel coated with TBT antifouling paint. The leachate was diluted in dilution/mixing units to obtain treatment solutions of 0-, 10-, 25-, and 100-percent leachate. These dilutions represented nominal TBT concentrations of 0 (Control), 0.02, 0.05, and 0.20 μg/l. Actual mean TBT concentrations were 0.065, 0.077, and 0.193 μg/l for the respective treatments.

The individual species monitored were mussels (*Mytilus edulis*), clams (*Macoma nasuta*), and oysters (*Crassostrea gigas, C. virginica, Ostrea edulis, O. lurida*). Adult mussels were measured for gonad and condition indices and TBT bioaccumulation in soft tissues. Adult clams were measured for condition indices and TBT bioaccumulation in soft tissues. Juvenile mussels and oysters were monitored for growth as measured by weight. Communities of organisms were studied by censusing prefouled and unfouled settling panels.

Several indications of effects appeared at the highest concentration tested, although statistically significant differences were not consistently measured. Major biological effects observed included the following:

1. No significant differences were observed for mussel condition indices, gonad indices, or clam condition indices when controls were compared to treatments.

2. The TBT accumulated in the soft tissues of mussels and clams was proportional to the concentration in seawater. Under similar exposures, mussels accumulated more TBT than clams.

3. No significant effect was found on juvenile oyster growth at any exposure condition.

4. Growth of juvenile mussels was significantly reduced at TBT concentrations as low as 0.070 μg/l when exposure times extended beyond 53 days.

These findings may be attributed to problems in the PETS rather than TBT exposure. An overall evaluation of the system included the following:

1. Producing the TBT leachate in a single, large source provided less chance of variability between replicates.

2. The design of the dilutor/mixing unit precluded a separation of the lower two concentrations. The unit could not consistently produce the volumes of control and leachate water for the required ratios.

3. Since the test tanks were fed by gravity flow, the height of the receiving tank and size of delivery lines determined the maximum available flow rate. The flow rate was inconsistent due to fouling and sedimentation in the delivery lines.

4.   The arrangement of tanks is very critical in obtaining true replicates. Slight differences in exposure to sun or wind may significantly alter the conditions within the experimental tanks.
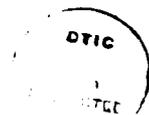
5.   The PETS is more representative than laboratory studies and permits meaningful environmental studies over extended periods. However, direct extrapolation of results cannot be made until the system has been field-validated and it has been clearly shown that organisms in the test tanks respond similarly as field-maintained animals.

Our suggestions for improving this particular system are as follows:

1.   Increase the flow rate to provide seawater containing its complete particulate load and to reduce tank effects.

2.   Configure the test system and use shading to minimize effects of atmospheric conditions.

3.   Reconfigure dilutor system to deliver more accurate volumes of seawater.

4.   Always include a field control to verify that the measured biological parameters are really tracking natural variation.

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INTRODUCTION

The flowthrough microcosm facility at the Naval Ocean Systems Center (NOSC) in Ulupau, Hawaii, described by Evans (1977) has been used for several years to study the effects of pollutants on harbor organisms. Anticipating Navy Fleet use of organotin-based antifouling (AF) coatings, NOSC researchers used this facility to study the effects of tributyltin (TBT), the primary toxic component of organotin AF paints, on selected benthic organisms and fouling communities (Henderson, 1985).

Direct extrapolation of results from these Hawaii-based organotin studies to the many U.S. ports and harbors used by the Navy was not possible because each of these harbors is environmentally unique and different than Hawaii. The fate and effect of any toxicant is highly dependent upon the interaction of biological, physical, and chemical processes at each site. Therefore, to accurately determine the impact of organotin use, the assessments should be performed with the seawater, biota, and environmental conditions indigenous to the particular site, i.e., site-specific bioassays.

A Portable Environmental Test System (PETS), comparable to the Hawaii microcosm facility, was developed for these site-specific organotin assessments and tested in Hawaii (Henderson, 1985). A second version, which was tested in San Diego and is reported here, included improvements such as removing test panels from treatment tanks, using a bin-dumping dilutor system, and larger tanks, and placing greater emphasis on generalized needs and portability.

An evaluation of the efficacy of PETS is presented with suggestions for improving the system. The most important criteria are the ability of PETS to adequately represent the environment, its utility in all environments, tropical to arctic, and the ability to extrapolate results to nature. The test and evaluation of PETS was performed in San Diego Bay using organotin-based leachates as the toxicant. The purpose of the organotin study was to collect as much environmentally realistic effects data as possible on organisms of economic and recreational value exposed to sub-part-per-billion concentrations of TBT.

METHODS

EXPERIMENTAL DESIGN AND PROCEDURES

Site Location

The PETS experiments were conducted at the end of a pier at the Naval Amphibious Base, San Diego (figure 1). This area was selected because it is in the central portion of San Diego Bay near Naval Station, San Diego, and is representative of the areas within the bay which may be influenced by Navy use of organotin AF coatings. In addition, the site offers security and utilities.

LEGEND

- **●** PETS Site (Phase I Juvenile Mussels)
- **▲** Shelter Island Site
- **■** Phase I Scallops
- **⊙** Phase I Adult Mussels
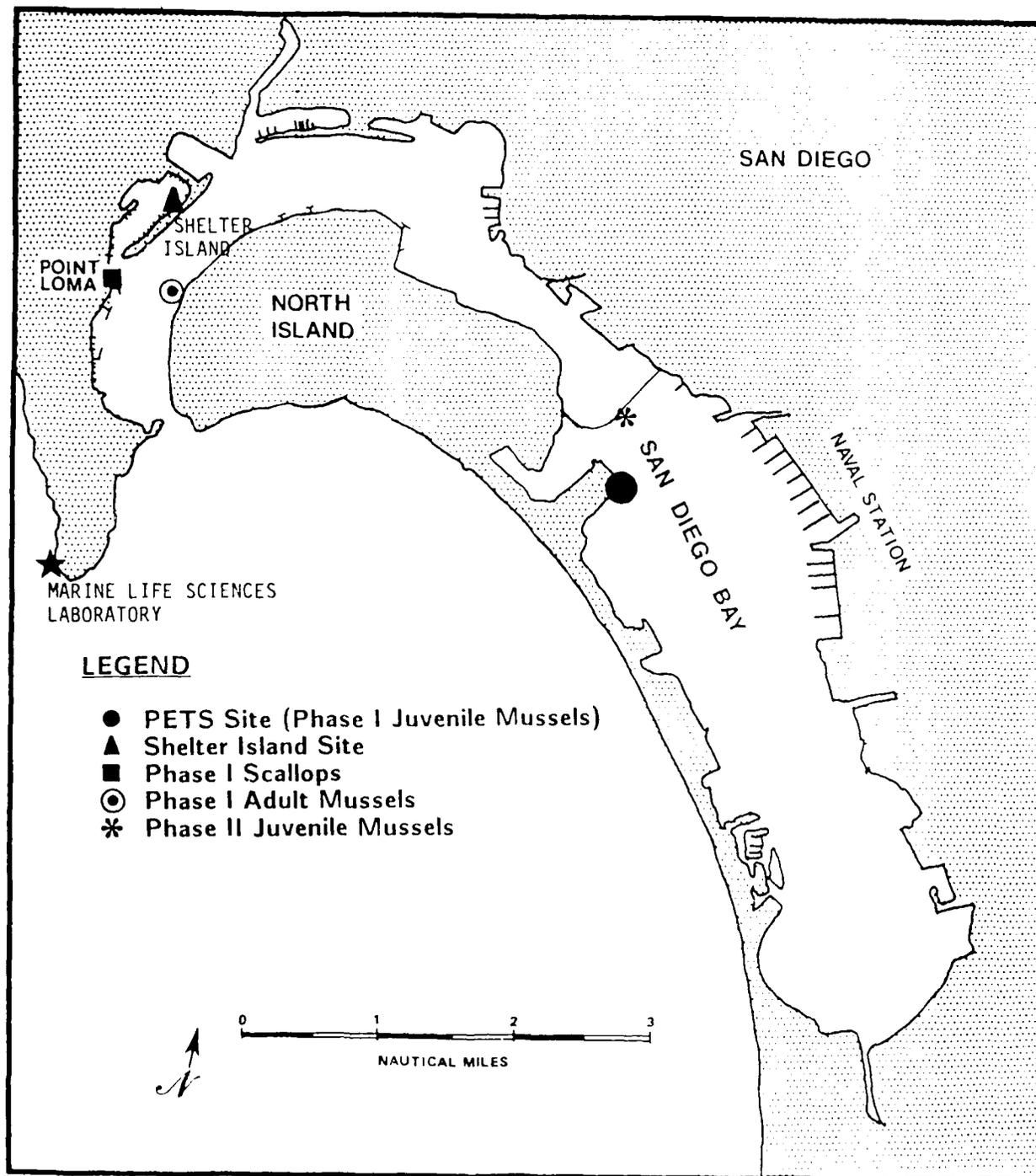- **✳** Phase II Juvenile Mussels

Figure 1. San Diego Bay site locations. PETS, Shelter Island, and animal collection sites.

The Bioassay System

The system consisted of the following: (1) seawater intake, (2) receiving tank (286 l), (3) leachate tank (1000 l), (4) two dilution/mixing units, (5) twelve 340-l flowthrough polyethylene test tanks arranged in two rows of six tanks, and (6) a van modified for power and laboratory space (figure 2). The test tanks were shaded with a 70-percent sunscreen to reduce some adverse effects of direct sunlight. Two intake pumps were situated on a floating dock approximately 30 meters from the test tanks. Unfiltered seawater was pumped from a depth of 2 meters to the elevated receiving tank. The TBT-dosed seawater (leachate) was produced in the leachate tank by circulating aerated ambient seawater around a Plexiglas panel coated with a self-polishing, co-polymer antifouling paint (International Paint Co., BFA 956 Pink SPC-9 HiSol). Flow rate and AF paint surface area were adjusted to yield a leachate concentration of -0.2 μg/l. Unfiltered seawater and TBT leachates were distributed by gravity flow. Overflow from the test tanks emptied into a spillway which drained into the bay approximately 50 meters from the seawater intake. Self-contamination was not considered a problem because (1) the overflow water was passed through charcoal filters to remove TBT from solution, and (2) the strong currents in that area provided rapid mixing of discharge water with clean bay seawater.
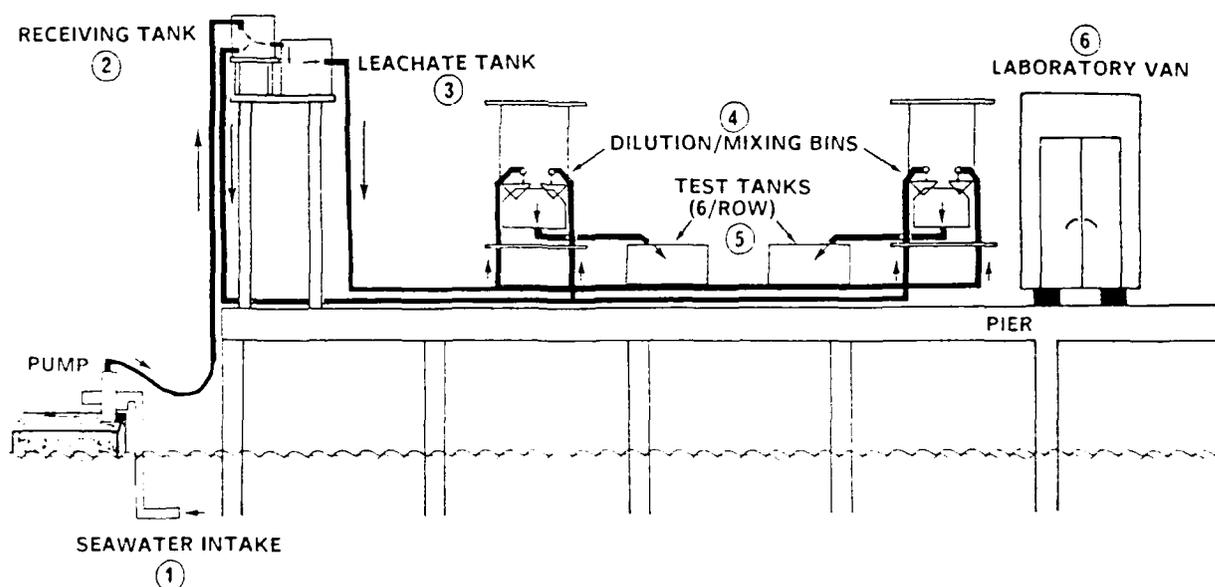
Figure 2. PETS schematic.

Treatment solutions were generated in the dilution/mixing units by combining unfiltered ambient seawater with leachate water at different volume ratios. The treatments were 100-, 25-, and 10-percent leachate solutions, representing nominal TBT concentrations of 0.200, 0.050, and 0.020 μg/l, respectively. Unfiltered ambient seawater was used for the controls (0 percent leachate). These nominal organotin

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concentrations were selected because (1) toxic effects have been observed at the 0.20-μg/l concentration in laboratory studies for a variety of species, (2) the 0.05-μg/l concentration is the "no effect" concentration suggested by the U.S. Naval Sea Systems Command (1984) and, (3) the 0.02-μg/l concentration was an anticipated no-effect concentration.

Two dilution/mixing units were used, each servicing six tanks. Each unit consisted of two opposing sets of six adjacent and interconnected bins, each bin in the shape of a wedge. One set of bins received control water directly from the receiving tank while the other received leachate water from the leachate tank. Each test tank was fed water from one pair of bins (control plus leachate). The bins were attached to a timer-controlled cam. Every 30 seconds the contents of opposing bins were dumped into a common trough that delivered test water to individual tanks. The treatment assigned to each tank is shown in figure 3.

Volumes required for the 100-, 50-, 10-, and 0-percent dilutions were set by fixing a false bottom in each bin. A total volume of 1.8 liters (combined control water plus leachate water) was delivered to each tank during each 30-second dumping cycle providing a complete volume exchange every 1.5 hours.

The PETS experiments were performed in two phases (figure 4). Phase I was conducted for 110 days (16 May to 3 September 1986) and examined TBT effects on fouling communities, adult mussels, scallops, clams, and juvenile mussels. Phase II was conducted for 56 days (21 October to 16 December 1986) and examined TBT effects on juvenile mussels and four species of juvenile oysters. The juvenile mussel study portion of Phase I was not terminated after 110 days. Measurements continued in conjunction with the Phase II study. The Phase I juvenile mussel study ran for 196 days. All tests included a Tank Control and three TBT treatments with three replicates of each. To evaluate tank effects, animals were suspended in the bay immediately adjacent to the seawater intake (Pier Control). Adult mussels were used for the Phase I Pier Control. Juvenile mussels and oysters were used for the Phase II Pier Control. During Phase I adult mussels were also suspended at a TBT-contaminated site in a Shelter Island marina (figure 1) to compare the effects of TBT on animals maintained under natural conditions with those maintained in PETS. Plastic holding bags and trays for mussels, clams, and oysters were leached for at least 2 weeks in flowing seawater to remove toxic compounds.

System Maintenance

Routine maintenance procedures were established to minimize fouling and eliminate accumulated sediment inside the seawater pumps, receiving tank, leachate tank, pipes, and dilution/mixing units. Many of these procedures were created as the need for them became apparent. The following is a brief outline of weekly maintenance procedures.

1. Seawater Pumps. Only one pump was used to deliver seawater to the receiving tank. Each week, one system was shut down and cleaned; the other was started, flushed, and put on-line. The intake pipe of the down pump was removed, scrubbed, and kept dry for a week to eliminate fouling organisms. During the week of downtime, the seawater within the lines of the off-line pump became anoxic and killed most fouling organisms within the pump housing and delivery hoses.
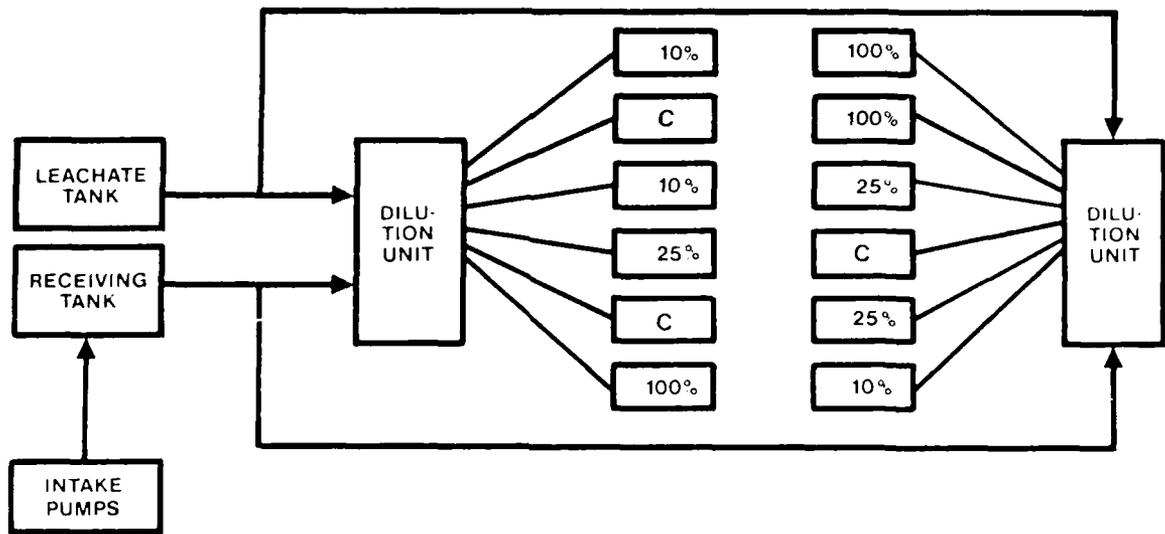
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Figure 3. Top view of PETS showing relative position of seawater intake pumps, receiving and leachate tanks, dilution units, and treatment tanks.
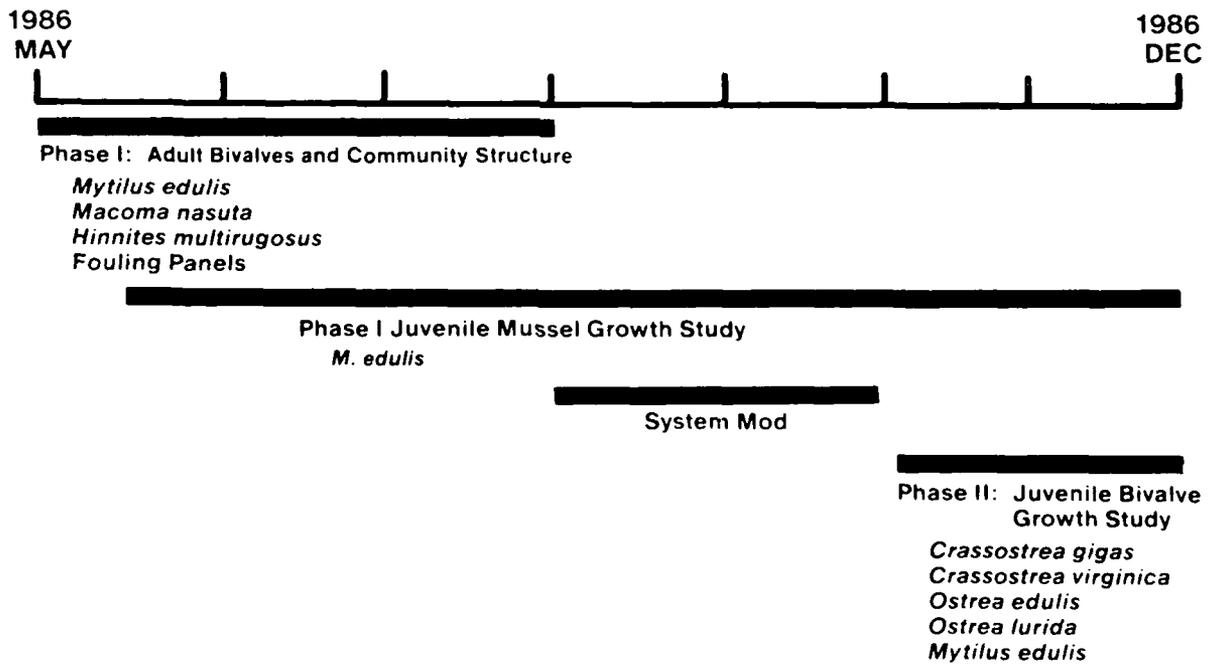

Figure 4. Time line for PETS experiments.

2. Receiving and Leachate Tanks. Fouling and sediment were also removed from the receiving and leachate tanks. The tanks were drained and the inside walls scrubbed with a nylon brush and rinsed with seawater. Fouling was always less in the leachate tank. However, sedimentation was similar in both.

3. Delivery Pipes. Hoses and pipes leading from the receiving and leachate tanks to the mixing/dilution units were disassembled and cleaned of accumulated sediment and fouling by brushing and rinsing. More sediment would tend to settle in the pipes traversing the ground, as they were very long and the lowest point in the seawater system. Even though the pipes were 1-1/2 and 2 inches in diameter, a small amount of fouling within them would significantly decrease the gravity-feed flow of the system and affect the delivery of required volumes.

4. Dilution/Mixing Bins. Due to the high volume of seawater passing through the dilution bins and their exposure to sunlight, algae grew profusely on the surfaces of these bins within a week. They were scrubbed lightly and rinsed with ambient seawater; the waste was drained through the overflow port.

5. Test Tanks. Test tanks were checked at least weekly for proper water flow and adequate aeration. During some periods of the test, filamentous algae would proliferate on the surface of the tanks. This was removed to avoid clogging the drain.

6. Lubrication and Adjustment. The electric motors and linkage used to rotate the dilution/mixing bins were lubricated weekly, and adjustments of the cam stop were made when necessary.

Other routine maintenance occurred throughout the tests which included checking the operational status of emergency generators and keeping the laboratory van functional.

Between Phases I and II, the entire system was cleaned thoroughly. All piping and hoses were opened, scrubbed, and cleaned. Receiving and leachate tanks were cleaned and recaulked where necessary. Individual test tanks were drained and scrubbed. Delivery pipes running from the dilution/mixing units to the test tanks were cleaned.

ORGANOTIN CHEMISTRY

Seawater Analysis

Seawater samples for TBT analysis were collected in 500-ml polycarbonate bottles from the test tanks, seawater intake, Shelter Island site, and leachate tank. Unless analyzed immediately, these samples were frozen and stored. TBT measurements were made by hydride derivatization and atomic absorption detection (Valkirs, et al., 1985).

Normally, collections were made from 100-percent leachate tanks twice per week and from the remaining tanks once per week. Organotin measurements were made approximately every 2 weeks on seawater from the bay and leachate tank. We presumed that the bay water would remain near nondetectable and the leachate tank

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was the same as the 100-percent leachate tanks. However, after obtaining widely fluctuating concentrations in the 100-percent tanks, samples were periodically collected from both the bay and leachate tank to help determine the source of variation.

Bioaccumulation

Mussel and clam tissues were frozen immediately after collection for TBT analysis. For each species, tissues of all replicates from each tank were pooled to obtain sufficient biomass for analysis. Tissue analyses were made on mussels collected from the Shelter Island, Pier Control, Tank Control, and 100-percent leachate exposures and on clams collected from the Tank Control and 100-percent exposures.

The tissues were thawed, homogenized, acidified with 6M HCl, and extracted twice with methylene chloride. The extracts were dried under a stream of argon and reconstituted in toluene. To remove any mono- or dibutyltins, the concentrated extracts were mixed with a solution of -5M NaCl in 3-percent aqueous NaOH, and the aqueous layers were discarded.

The TBT-containing toluene extracts were analyzed by gas furnace-atomic absorption spectrophotometry using graphite tubes fitted with L'vov platforms. A matrix modifier of 0.1 M ammonium dichromate, isopropyl alcohol, and toluene (1:6:3, respectively) was added to the extracts to both enhance the tin signal and "level" the response to various molecular forms of organotins. Each sample was analyzed in triplicate with standard additions of TBTCl. Linear regression of the averages was used to determine the concentration of TBT in each tissue sample (Meyers-Schulte and Dooley, 1987).

PHYSICAL/CHEMICAL MONITORING

Routine Measurements

Water quality was measured approximately twice weekly throughout both phases of the PETS tests. Temperature ($^\circ$C), pH, conductivity (mv/cm), and dissolved oxygen (ml/l) were measured in each tank and at the seawater intake using a model U-7 Water Quality Checker (Horiba Instruments, Inc.). Salinity was calculated from conductivity and temperature data (Perkin and Lewis, 1980).

24-Hour Study

A study was performed to determine the daily fluctuations in physical/chemical parameters and organotin concentrations. The following parameters were monitored during 24 consecutive hours in the test tanks and at the seawater intake:

1. Temperature, pH, conductivity, and dissolved oxygen. Horiba Water Checker (half-hourly).

2. In-vivo fluorescence (relative units), 10-ml sample measured before and after the addition of 0.100 μl of 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU) using a Turner Model 10-000R fluorometer (hourly).

3. Turbidity (NPU units), 20-ml sample was measured using a Turner Designs Model 40-002 nephalometer (hourly).

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4. Seawater for organotin analysis. Samples collected from the leachate tank and seawater intake only. Samples were collected in 500-ml polycarbonate bottles and frozen until analyzed (hourly).

5. Suspended particulates (mg/l). Seawater samples were collected from each tank and the seawater intake every 6 hours, near the change of each tidal cycle. Samples were collected in 250-ml bottles and refrigerated until filtration through 0.22-micron Millipore filters.

PHASE I

Fouling Study

The effects of TBT on newly developing and established fouling communities were assessed by censusing Plexiglas panels (21.25 by 30.0 cm) for attached organisms (Henderson. 1985). Clean, unfouled panels (three replicate panels/tank) provided substrate for newly developing communities in PETS tanks. Prefouled panels represented established fouling communities in PETS tanks (three replicate panels/tank). To obtain established fouling communities, clean panels were suspended under the floating dock at the test site for 126 days before the study.

All fouling panels were suspended in test tanks -15 cm below the seawater surface. Every 2 weeks settlement on the panels was censused. A predetermined section (18.5 by 13.0 cm) of each previously unfouled panel was documented with an underwater camera (Nikonos II). Species identifications and counts were made from the projected slides. Thick tunicate growth on the prefouled panels precluded photographic analysis; thus, species counts were made directly on a predetermined 20-by 20-cm section of the panels. Panels were removed from the test tanks and placed horizontally in a small tub of clean seawater for photographs or direct species counts and replaced into test tanks within 15 minutes of removal. On days 31, 47, 67, and 110, the biomass of attached organisms was measured to the nearest 0.1 gm using a modified triple beam balance.

The density of mussels on the prefouled panels increased over the course of the experiment, but it was difficult to identify and monitor the growth of individuals. Therefore, mussel lengths were measured to the nearest 0.5 mm with vernier dial calipers at the conclusion of the test to distinguish between animals settling during the experiment and those that had settled during the prefouling period. Animals ≥25 mm in length were considered to have settled during the prefouling period. Mussels less than 5.0 mm were indicated as 5.0 mm due to the difficulty in measuring these animals while still attached to the panels. Mussels that migrated to the back of the panels were censused at this time.

Multiple one-way analyses of variance (ANOVA) (a = 0.05) were used to test for differences in abundance, biomass, and *M. edulis* settlement among controls and treatments at each sampling interval.

Adult Bivalve Tests

The Phase I adult bivalve tests were designed for endemic San Diego Bay species of recreational or commercial value that may be impacted by the use of

organotin AF coatings. Therefore, mussels (*Mytilus edulis*), clams (*Macoma nasuta*), and scallops (*Hinnites multirugosus*) were included in this test. Adult mussels and clams were monitored for TBT bioaccumulation and condition indices; gonad indices were measured in mussels only. Animals were collected from each tank (9 mussels and 10 clams), the Pier Control (9 mussels), and Shelter Island (8 mussels) every 2 weeks for these measurements.

Condition indices have been used to measure the relative health of bivalves (Drinkwaard, 1957; Baird, 1958; Westley, 1961; and Chew et al., 1965). The index described by Baird (1958) and Galtsoff (1964) was modified to the ratio of total soft tissues (g wet weight) to internal shell volume (ml). A wet weight method was necessary because tissues were used for index determinations and bioaccumulation. Dry weight methods would have affected the subsequent TBT analyses.

Gonad indices provide a measure of developing gametes of individuals (Bayne et al., 1985). The index used was the ratio of mantle (g wet weight) to total soft tissues (g wet weight) (Ouellette, 1978).

Two null hypotheses were tested by means of one-way ANOVAs (a = 0.05): (1) H_o = exposure to test levels of TBT has no effect on the condition index of mussels or clams, and (2) H_o = exposure to test levels of TBT has no effect on the gonad index of mussels. If the H_o was rejected, a Duncan's multiple range test determined at which test concentrations the differences occurred.

Animal Collection

Mussels (*Mytilus edulis*) from 40 to 60 mm long were collected from concrete pilings adjacent to North Island (figure 1) 52 days prior starting the study. All fouling was scraped from their shells. These mussels were held in plastic mesh bags and suspended from the pier at the test site for a 50-day acclimation period.

At the start of Phase I, 83 mussels were introduced to each tank. Animals were divided between two plastic mesh trays and suspended in the test tanks. The Pier Control mussels (n = 83) and those at Shelter Island (n = 56) were held in plastic mesh bags suspended -0.5 m below the surface.

Clams (*Macoma nasuta*) were collected from Tomales Bay, California, by a private collector and shipped to San Diego 28 days before starting the test. Upon arrival, they were counted and sorted into three size classes: small (20-30 mm), medium (30-50 mm), and large (50-60 mm). Ten clams (approximately three small, five medium, and two large) were placed in plastic tubs containing 1.5 liters of presieved sediment. The sediment was collected from an area immediately adjacent to the pier at the test site and was sieved through a 1-mm screen to remove endemic organisms. The tubs containing sediment and clams were held in a 3000-gal tank receiving unfiltered seawater at the NOSC Marine Sciences Laboratory until starting the test. At that time, 10 tubs were placed on the bottom of each test tank. One tub was removed from each tank for condition index determinations and bioaccumulation measurements.

Juvenile Mussels (Test I)

The effect of TBT on juvenile mussel growth was assessed by monitoring whole animal wet weights and lengths. Juvenile mussels (*M. edulis*) were collected from the supporting structure of the clean fouling panels suspended at the test site in January. The initial size range of test animals (n = 192) was 10 to 17 mm in length ($\bar{x}$ = 14.41 mm) and 0.124 to 0.563 mg in weight ($\bar{x}$ = 0.313 mg). One plastic ice cube tray, drilled with 1/4-inch holes on the sides and bottoms to allow water circulation, was suspended 10 cm below the surface in each tank. Lengths and wet weights were measured weekly for 29 weeks using vernier dial calipers and an electronic balance, respectively.

Statistical analyses were only performed on data for animals surviving the entire study to maintain a constant "n" for each replicate and to prevent biased comparisons if means were significantly influenced by deaths. Weekly mean and cumulative percent increases in weight were determined for each species at each treatment. These data were used for graphical representation. ANOVAs (a = 0.05) were performed on weight data at each sampling interval to test the null hypothesis, H_o = exposure to test levels of TBT has no effect on the growth of test organisms as measured by whole-animal wet weight. If the H_o was rejected, a Duncan's multiple-range test determined where the differences occurred.

System Modifications

The system was modified between Phases I and II to solve some of the problems encountered in Phase I. After draining the tanks, attached biota and accumulated sediment were removed. Flow rates were increased and diluters were adjusted to bring actual TBT concentrations closer to nominal. The total biomass/tank was markedly reduced at the beginning of Phase II.

PHASE II

Juvenile Bivalves

The Phase II study monitored growth in juvenile oysters and juvenile mussels (Test II). The oyster species used were *Crassostrea gigas, Crassostrea virginica, Ostrea edulis,* and *Ostrea lurida.* Oysters were selected because of concern over potential organotin-contamination problems in the culture industry. Although *O. lurida* is not commercially cultured, it was used because it is the only oyster found in San Diego Bay.

All oysters except *C. virginica* were obtained from a professional rearing facility in Eureka, California. *C. virginica* were obtained from the Harbor Branch Oceanographic Institution, Inc., Ft. Pierce, Florida. Oysters obtained from Eureka, California, were in excellent condition. All animals were alive upon arrival at which time they were distributed in holding trays and maintained in control tanks until starting the test. There was significant mortality in *C. virginica* during shipment from Florida. Approximately 40 percent were dead upon arrival. Another 30 to 35 percent died within the following 24 hours. No further dead *C. virginica* were found. Because of the large number of dead, the number per replicate was reduced to 15 for all tank treatments and 11 for the Pier Control. In addition, all available animals

were used which included some relatively small (<150 mg) and large (>1000 mg) individuals. *C. virginica* from the extremes of the size range were selected for the Pier Controls to minimize the standard deviation in tank replicates.

The initial weights of oysters were *C. gigas* - 150 to 300 mg ($\bar{x}$ = 211 mg), *C. virginica* - 96 to 1256 mg ($\bar{x}$ = 296 mg), *O. edulis* - 140 to 280 mg ($\bar{x}$ = 199 mg), and *O. lurida* - 100 to 300 mg ($\bar{x}$ = 189 mg).

Phase II juvenile mussels were collected from the rubber tire bumpers secured to Coronado Bay Bridge piling No. 18 approximately 1 km from the test site (figure 1). There were insufficient numbers of juveniles available at the test site for Phase II. Initial lengths were 10.10 to 15.00 mm ($\bar{x}$ = 12.61 mm), and weights were 0.142 to 0.553 mg ($\bar{x}$ = 0.287 mg).

All five species were held in each tank and at the Pier Control in plastic mesh trays. Each tray contained 18 individuals of a given species, except *C. virginica*, with 15 individuals per tray. Weekly measurements of oyster and mussel wet weights were made using an analytical balance. Before weighing, the shells were blotted of excess water and any material on the shell was removed. Byssal threads protruding from the mussels were carefully snipped off. Mussel lengths were measured using vernier dial calipers.

Statistical procedures for the Phase II juvenile bivalve data were the same as those for the Phase I juvenile mussel data.

RESULTS

TBT CONCENTRATIONS AND WATER QUALITY

Overall mean TBT concentrations were 0.193, 0.077, and 0.065 μg/l for the nominal 0.200, 0.050, and 0.020 μg/l treatments, respectively (figure 5, table 1). Mean TBT concentrations in Phase I were 0.204, 0.092, and 0.079 μg/l for the respective nominals. Mean TBT concentrations in Phase II were 0.157, 0.051, and 0.038 μg/l TBT for the respective nominals. Mean TBT concentrations for the Phase I juvenile mussel study were 0.197, 0.080, and 0.067 μg/l.

TBT concentrations in the leachate and 100-percent treatment tanks fluctuated markedly during the experiment. There was also a high degree of variability among replicates for a given treatment. TBT concentrations in the 10- and 25-percent treatment tanks showed nearly 50 percent less variability than in the 100-percent treatment tanks. However, there was very little separation between TBT concentrations in the 10- and 25-percent dilutions. Instead of differing by a factor of 2.5, these dilutions only differed by a factor of 1.2.

Overall mean TBT concentration in seawater at the intake and in control tanks was 0.009 μg/l; Phase I and Phase II averaged 0.006 and 0.010 μg/l TBT, respectively (table 1). The mean TBT concentration measured at the Shelter Island site was 0.452 μg/l ($\pm$0.247).

All physical parameters measured were reasonably constant except temperature. The minimum, maximum, and mean values are presented in table 2. Results of the 24-hour study are presented in table 3.
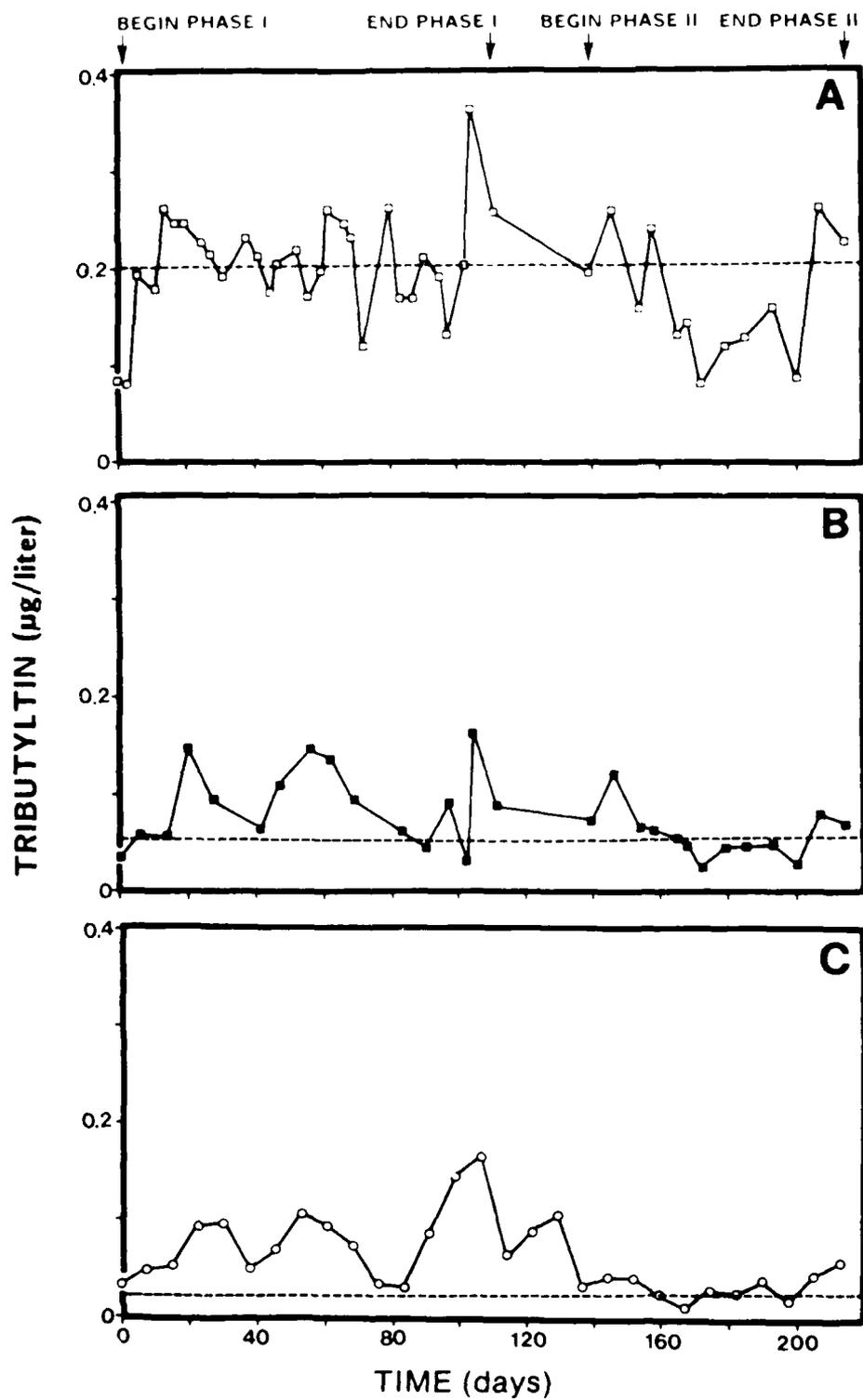
Figure 5. TBT leachate treatments: A – 100%, B – 25%, C – 10%
(———) measured TBT concentration, (------) nominal TBT concentration.

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Table 1. Mean measured TBT concentrations (μg/1) by treatment, tank, and phase.

TREATMENT		Tank	Overall (16 May - 16 Dec 1986)		Phase I Juvenile Mussels (3 Jun - 16 Dec 1986)		Phase I (16 May - 4 Sep 1986)		Phase II (21 Oct - 16 Dec 1986)	
			$\bar{X}$ (±S. D.)	$\bar{\bar{X}}$ (±S. D.)	$\bar{X}$ (±S. D.)	$\bar{\bar{X}}$ (±S. D.)	$\bar{X}$ (±S. D.)	$\bar{\bar{X}}$ (±S. D.)	$\bar{X}$ (±S. D.)	$\bar{\bar{X}}$ (±S. D.)
Leachates	100%	Tank 1	0.181 (±0.074)	0.193 (±0.071)	0.184 (±0.073)	0.197 (±0.070)	0.189 (±0.077)	0.204 (±0.070)	0.161 (±0.068)	0.157 (±0.066)
		Tank 11	0.203 (±0.074)		0.209 (±0.076)		0.212 (±0.074)		0.166 (±0.076)	
		Tank 12	0.194 (±0.059)		0.199 (±0.056)		0.212 (±0.055)		0.143 (±0.049)	
	25%	Tank 3	0.073 (±0.040)	0.077 (±0.040)	0.076 (±0.041)	0.080 (±0.040)	0.086 (±0.046)	0.092 (±0.044)	0.050 (±0.016)	0.051 (±0.019)
		Tank 8	0.074 (±0.040)		0.078 (±0.041)		0.096 (±0.045)		0.048 (±0.015)	
		Tank 10	0.082 (±0.038)		0.085 (±0.039)		0.098 (±0.041)		0.056 (±0.023)	
	10%	Tank 4	0.065 (±0.036)	0.065 (±0.039)	0.068 (±0.037)	0.067 (±0.040)	0.076 (±0.036)	0.079 (±0.041)	0.038 (±0.012)	0.038 (±0.015)
		Tank 6	0.066 (±0.042)		0.068 (±0.045)		0.079 (±0.048)		0.041 (±0.017)	
		Tank 7	0.063 (±0.038)		0.064 (±0.040)		0.083 (±0.040)		0.035 (±0.014)	
Control		Tank 2	0.007 (±0.008)	0.009 (±0.007)	0.008 (±0.008)	0.008 (±0.008)	•	0.006 (±0.006)	0.011 (±0.008)	0.010 (±0.007)
		Tank 5	0.006 (±0.007)		0.007 (±0.008)		•		0.010 (±0.007)	
		Tank 9	0.008 (±0.009)		0.009 (±0.008)		•		0.010 (±0.005)	

* = Samples pooled from Tanks 2, 5 and 9 to yield one result.

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Table 2. Physical/chemical measurements for Phase I
(17 May - 3 Sep 1986) and Phase II (16 Oct - 16 Dec 1986).

	Phase I		**Phase II**	
	Mean	Min - Max	Mean	Min - Max
T (°C)	23.1	19.5 - 25.9	18.6	15.0 - 21.7
Sal (ppt)	36.4	29.6 - 37.6	35.7	35.1 - 36.0
D. O. (ml/l)	7.3	5.1 - 9.8	7.6	6.1 - 10.1
pH	7.6	5.9 - 8.3	7.7	7.1 - 7.9

Table 3. Physical/chemical measurements taken in PETS
tanks and at the seawater intake during the 24-hour study
(11 - 12 Dec 1986).

	PETS Tanks		**Seawater Intake**	
	Mean	Min - Max	Mean	Min - Max
T (°C)	15.0	13.5 - 16.9	15.4	15.0 - 16.0
Sal (ppt)	35.6	35.2 - 36.5	35.6	35.4 - 35.8
D. O. (ml/l)	7.1	6.3 - 8.2	7.1	6.5 - 8.0
pH	7.7	6.8 - 7.9	7.8	7.7 - 7.9

PHASE I

Fouling Study

Prefouled Panels. A list of species observed on the prefouled panels is presented in table 4. In summary, at T_o these panels were primarily covered with the solitary tunicates. Present in fewer densities were mussels, arthropods, and sponges. After placement in the test tanks, the tunicates began to deteriorate and slough off, and by day 60 the panels were nearly 75 percent unfouled. The newly available substrate was slowly recolonized by sponges, anemones, tunicates, worms, mussels, and arthropods. Statistical analyses of the data indicate no significant difference between controls and treatments in species abundance of attached organisms.

The biomass measurements were similar over time for all conditions. There was no statistically significant difference in biomass between controls and treatments.

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Table 4. Animal species observed on the prefouled panels.
(f = front panel; m = middle panel; b = back panel)

The majority of juvenile mussels settled on the prefouled panels while they were held in test tanks. There was no statistically significant effect on settlement due to organotin exposure. However, actual differences may have been masked by tank effects as the 100-percent treatments did have considerably less settlement than the other treatments.

Tanks 7 to 12, located on the west side, experienced heavier mussel settlement than those on the east side. This difference was statistically significant (a = 0.05). The mussels found on panels in tank 12 (100-percent leachate) were much smaller than those in the other five tanks on the west side. The overall mean TBT concentration for tank 12 was similar to the other 100-percent leachate tanks; however, there were times when the TBT concentration in tank 12 was higher than in the other replicates. Although not confirmed by statistics, these data suggest that organotin concentrations greater than 0.2 μg/l may interfere with the settlement of juvenile *Mytilus edulis*.

Unfouled Panels. A list of species observed on the unfouled panels is presented in table 5. In summary, after 95 days relatively little fouling had taken place with approximately 20 percent of the panels colonized by tube worms, tube-building amphipods, bryzoans, and limpets. Variability in settling between replicate tanks was high. Statistical analyses of the data indicate no significant difference between controls and treatments in species abundance of attached organisms. The method used to estimate biomass was not sensitive enough to measure the sparse settlement on these panels.

Bioaccumulation

Mussels and clams accumulated increasing amounts of TBT in their tissues for 60 days. From day 60 to 110, body burdens appeared to stabilize and approach a threshold (figure 6). Control mussels and clams maintained body burdens at 0.42- and 0.22-μg TBT/g tissue, respectively, over the 110 days.

Mussels accumulated more TBT than clams. The amount of TBT measured in mussel tissues was proportional to the exposure concentration. The average maximum body burden for mussels held at Shelter Island ($\bar{x}$ TBT = 0.452 μg/l) was 10.38-μg TBT/g tissue. Mussels in PETS tanks with mean TBT concentrations of 0.204- and 0.079-μg/l had average body burdens of 5.40- and 2.96-μg TBT/g tissue, respectively. The average for clams at the 0.204-μg/l TBT exposure was 2.13-μg TBT/g tissue. For the same three treatments, mussel bioconcentration factors (BCFs), to the nearest hundred, were 23,000, 26,500, and 37,500. The BCF for clams was 10,400. BCFs for control mussels and clams were 70,000 and 36,700, respectively.

Bivalve Indices

Mussel condition and gonad indices decreased over time for all controls and treatments (figure 7). Pier Control mussels had consistently higher indices than Tank Control mussels. Condition indices in the 0.204-μg/l TBT treatment were significantly lower than Tank Controls on days 31, 47, and 80. Gonad indices for the same treatments were significantly lower on days 47 and 95.

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Table 5 Animal species observed on the unfouled panels.

Conc	0			10			25			100		
Tank	2	5	9	4	6	7	3	8	10	1	11	12

Species

17 June, day 32

Species	2	5	9	4	6	7	3	8	10	1	11	12
Filamentous b.a.*	+	+	+	+	+	+	+	+	+	+	+	+
Enteromorpha						+					+	
Spirorbis		+	+	+	+	+	+	+	+	+	+	+
Ericthonius			+			+			+			+
Crucibulum spinosum								+				
Colonial tunicate 1			+	+				+				+
Colonial tunicate 2		+										

2 July, day 47

Species	2	5	9	4	6	7	3	8	10	1	11	12
Filamentous b.a.	+	+	+	+	+	+	+	+	+	+	+	+
Spirorbis	+	+	+	+	+	+	+	+	+	+	+	+
Ericthonius				+		+		+	+			+
Crucibulum spinosum												
Invertebrate egg mass	+					+		+				+
Diplosoma												
Tunicate		+										
Bryozoan							+				+	

18 August, day 95

Species	2	5	9	4	6	7	3	8	10	1	11	12
Filamentous b.a.	+	+	+	+	+	+	+	+	+	+	+	+
Spirorbis	+	+	+	+	+	+	+	+	+	+	+	+
Ericthonius												
Crucibulum spinosum								+		+		+
Invertebrate egg mass		+						+				

*Filamentous b.a. is an unidentified brown algal mat.

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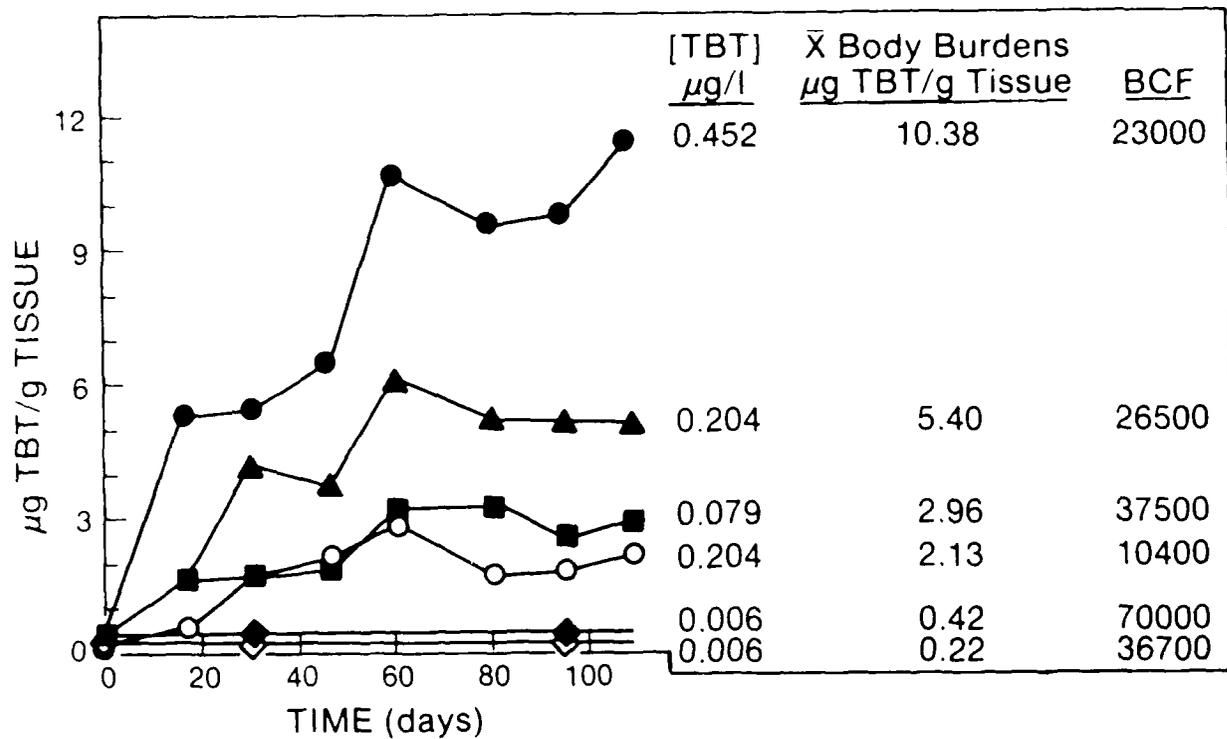
Figure 6 TBT bioaccumulation (μg TBT/g tissue). (●) Shelter Island mussels; (▲) 100% leachate mussels; (■) 10% leachate mussels; ( ) 100% leachate clams; (♦) control mussels; ( ) control clams.

The data table within the figure reads:

[TBT] μg/l	X̄ Body Burdens μg TBT/g Tissue	BCF
0.452	10.38	23000
0.204	5.40	26500
0.079	2.96	37500
0.204	2.13	10400
0.006	0.42	70000
0.006	0.22	36700

Figure 7 TBT effects on adult mussel condition index (A) and gonad index (B). ( ) Pier control, (●) Tank Control; (▲) Shelter Island; ( ) 100% leachate, (■) 25% leachate; ( ) 10% leachate.

None of the measurements indicated that TBT affected clams. Clams from all test tanks had high mortalities and highly variable condition indices. The data show no dose-dependency, but rather decreasing condition with time for all animals. No significant differences were found.

PHASE II

Juvenile Bivalve Growth Study - Oysters

Cumulative percent increases in weights for all species of oysters are presented in figure 8. Except for *O. lurida*, growth of the Pier Controls was significantly greater than growth in any Tank Control or treatment. There was no significant effect of TBT exposure on oyster growth when treatments were compared to Tank Controls.

Crassostrea gigas. Survival remained above 80 percent for controls and all treatments. Weight increases for animals in test tanks were all similar. Tank Controls achieved a 75.2-percent cumulative percent over 56 days, while Pier Controls increased 261.5 percent. A parameter observed but not quantified was shell thickening by *C. gigas* in all replicates, including the Pier Controls. This was not expected in controls since shell thickening has been attributed to much higher concentrations of TBT (Thain and Waldock, 1986).

Crassostrea virginica. Survival was very high for *C. virginica* under all conditions, with the Tank Controls the lowest at 88.9 percent. Weight increases for animals in test tanks were all similar. Tank Controls achieved the lowest cumulative increase of 53.4 percent after 56 days. Pier Controls increased 282 percent after the same 56-day period.

Ostrea edulis. Survival was 100 percent for all conditions through 35 days, after which it declined until the end of the test. Final survival rates ranged between 83.3 and 90.7 percent. Tank Controls achieved cumulative increase of 325.6 percent after 56 days. A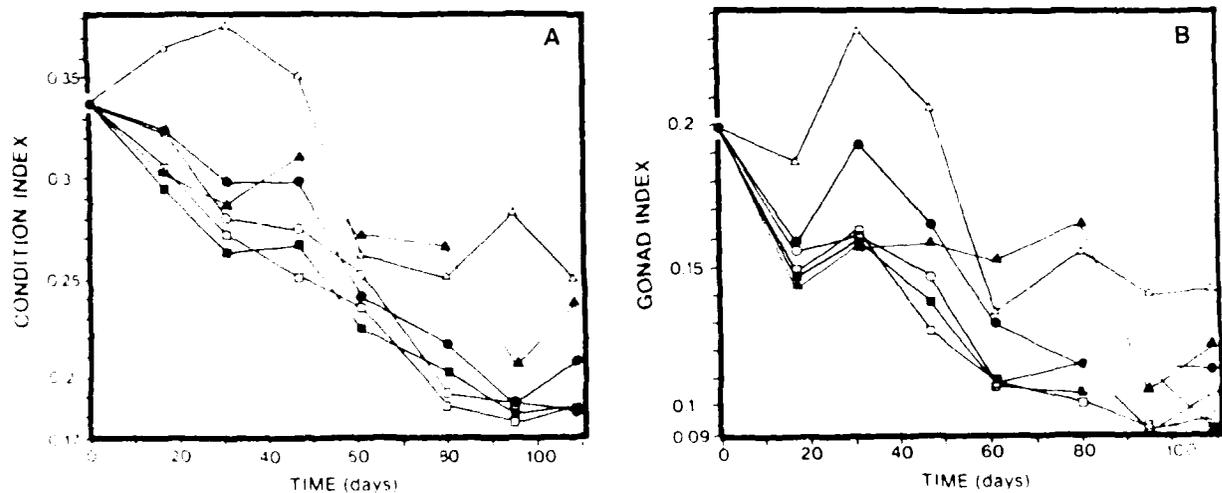ll tank treatments were statistically similar to the Tank Controls even though the 25-percent leachate treatments were consistently lower in weight increases for the entire test period. Pier controls increased 478.5 percent in weight after 56 days.

Ostrea lurida. Survival of *O. lurida* slowly decreased over the entire test with final survival ranging between 72.2 and 94.4 percent. Weight increases for animals in test tanks and Pier Controls were all similar. The greatest cumulative percent increase of 234.5 percent was for oysters in the 100-percent treatment. The Tank and Pier Controls were similar with cumulative increases of 204.4 and 208.0 percent, respectively.

Juvenile *Mytilus edulis* Studies - Phases I and II

Cumulative percent increases in weights and lengths for the Phases I and II juvenile mussels are presented in figure 9. In Phase I after 196 days, the Controls increased in weight by 450 percent; mussels at the 100-percent leachate treatment increased in weight by 250 percent. Weight increases for animals in both the 10 and
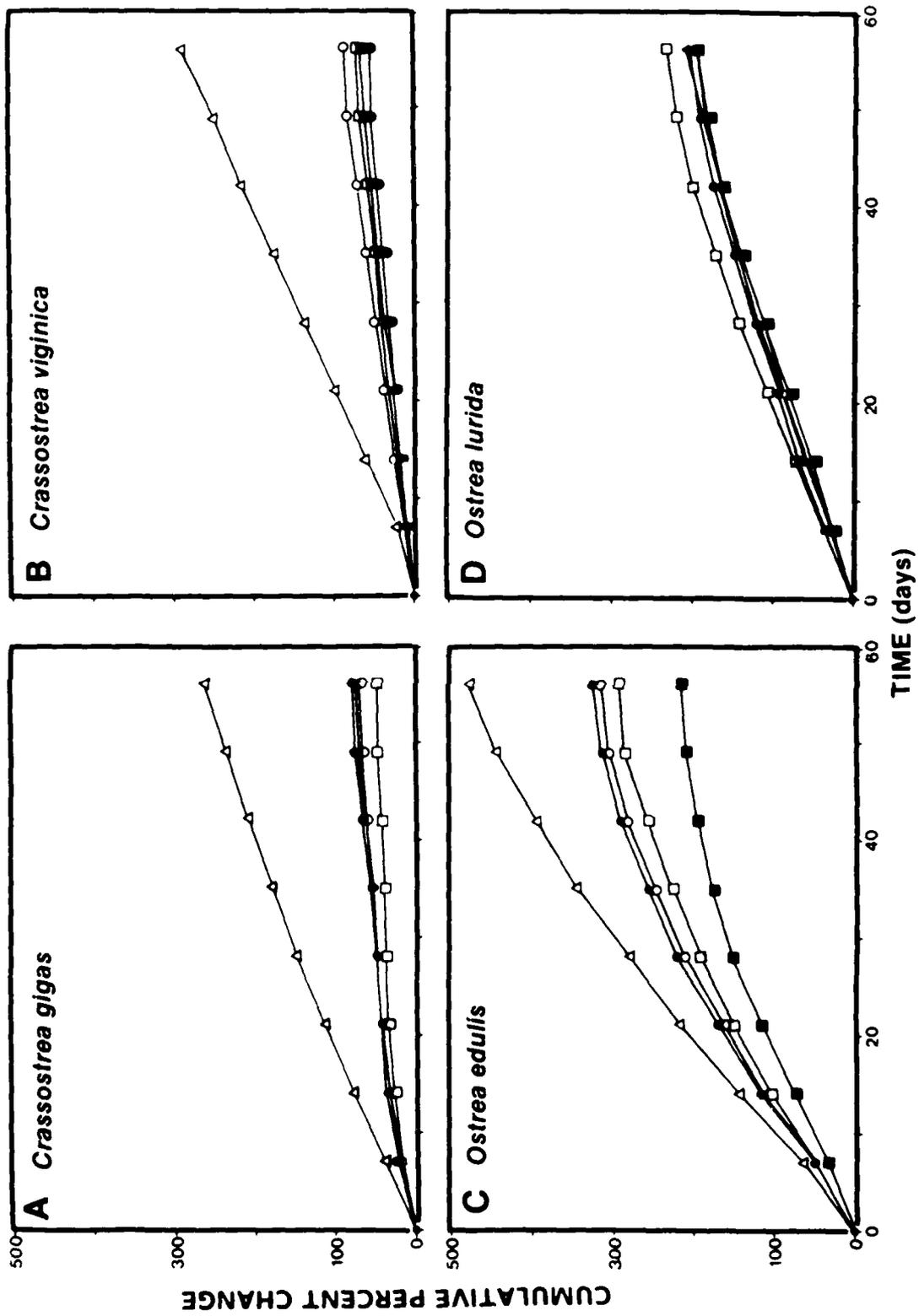
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Figure 8. TBT effects on juvenile oyster growth. (▽) Pier Control; (●) Tank Control; (□) 100% leachate; (■) 25% leachate; ( ) 10% leachate.
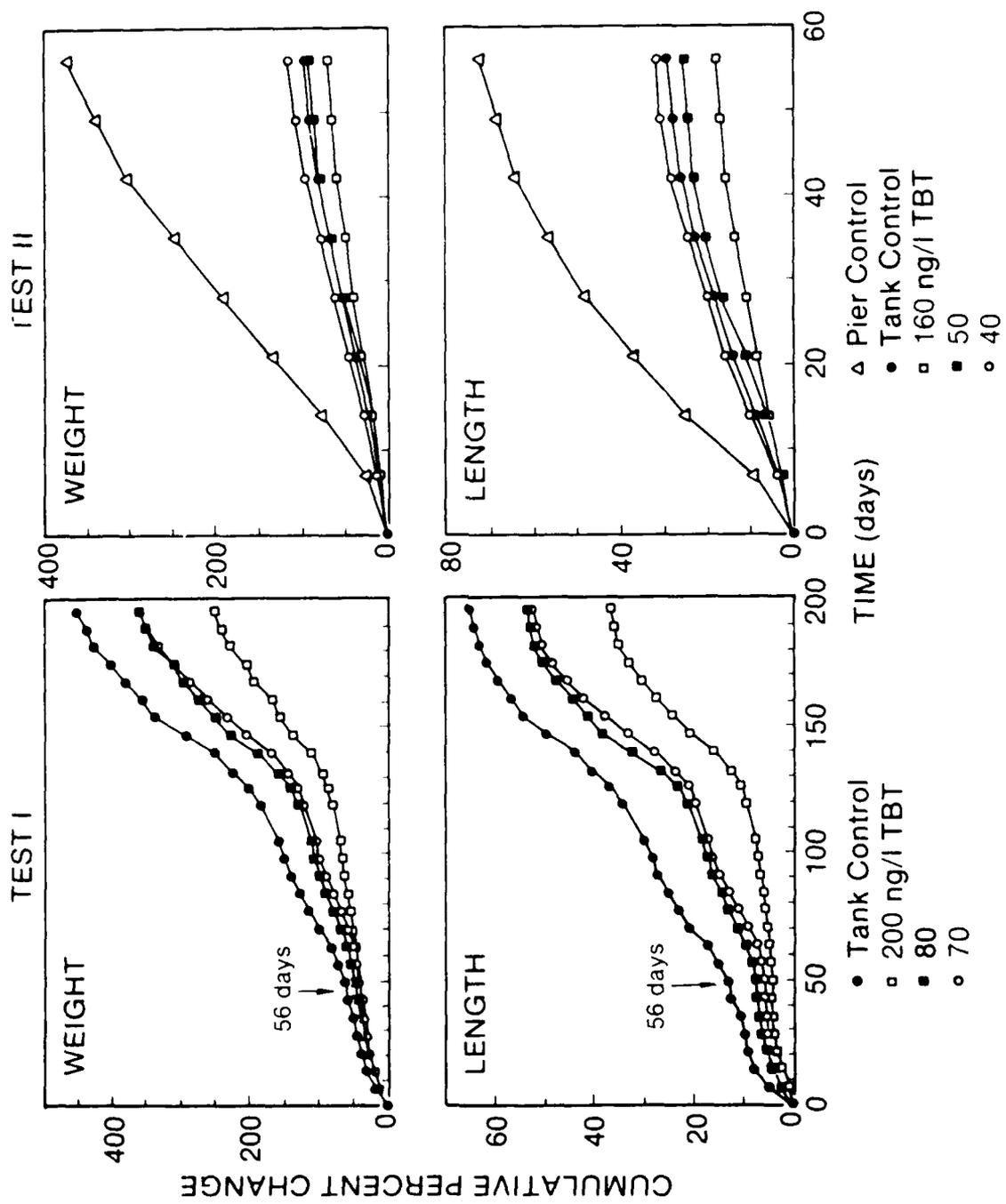
20

Figure 9. TBT effects on juvenile mussel growth as measured by weights and lengths in Tests I and II, respectively.

25 percent leachate treatments were 355 percent. The ANOVA demonstrated there were significant differences in weights among Controls and treatments. The Duncan's New Multiple Range Test showed the following: $C \neq (0.067 = 0.080) \neq 0.197$ μg/l TBT.

In Phase II the Tank Control mussels increased in weight by 99 percent, while those at the 100-percent leachate treatment increased by 71 percent. The ANOVA demonstrated there were no significant differences in growth among Tank Controls and treatments after 56 days of exposure.

There were no significant mortalities at any concentration tested in either study. Only two animals died at the 100-percent leachate treatment after 196 days of exposure in the first study; only one animal died at the same treatment in the second study.

Records were also kept of byssal thread production when weekly measurements were made since previous experiments have shown this is also an indicator of stress. Byssal thread production decreased to a minimum by week 7 of the first study when over half of the mussels at the 100-percent leachate treatment did not produce byssal threads. The following week byssal thread production increased and, by the end of the experiment, there were no significant observable differences in byssal threads among controls and treatments. There were no differences in byssal thread production in Phase II.

In the Phase II study, there was a significant difference in growth between the Pier Control mussels and all tank-held mussels. Animals suspended at the pier increased in weight by 378 percent.

DISCUSSION

The prototype site-specific microcosm system evaluated in San Diego Bay is probably more environmentally realistic than most laboratory tests, even though conditions within our system did not duplicate the surrounding bay waters. Statistical analyses of the data indicate that within our exposure range there were no significant effects attributable to TBT on fouling communities (species abundance and biomass), mussel and clam condition index, mussel gonad index, or oyster growth. However, high variability within and among replicates in TBT concentrations, temperature, and available light may have masked actual TBT effects. In many cases statistical results indicated tank effects were high enough to severely reduce our ability to discriminate concentration effects. For these reasons the biological results of the TBT studies must be interpreted with caution. The only portion of this test which showed TBT effects was the juvenile mussel growth study, but the impact may have been overestimated as the animals were probably under stress, as suggested by growth differences between Tank Controls and Pier Controls (Salazar and Salazar, 1987).

The paucity of settlement on all fouling panels suggests that not all larvae drawn in from the bay successfully passed through the system to the test tanks, or that they were quickly filtered from suspension by the animals in the tanks. However, the presence of "fouling" within the seawater distribution lines and on tank walls and test containers indicates that some larvae were able to settle. The bivalves that settled, *O. lurida, M. edulis,* and *Musculista senhousia,* did so at concentrations

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shown to be highly toxic in laboratory studies (Beaumont and Budd, 1984). This suggests that wild larvae may not be as sensitive as laboratory-reared individuals or that laboratory studies are not accurate indicators of environmental toxicity. Further, TBT, the molluscicide developed to kill freshwater snails for schistosomiasis control, had no apparent effect on the marine snail *Navanax inermiss*, which settled, grew, and laid eggs in all tanks. The effects of TBT on the survival and development of these eggs were not monitored.

In general, bivalve condition and gonad indices decreased over the entire test period. We cannot be sure whether this decrease is part of the natural cycle for mussels in San Diego Bay or can be attributed to stress in the test tanks. The decrease in condition index between May and September is in general agreement with that observed by others (Bayne and Thompson, 1970; Dare and Edwards, 1975; and Lutz et al., 1980). Condition indices for the Pier Controls declined similarly after an initial 30-day increase. However, Pier Control condition indices were always greater than those for tank-held animals. Although calculated gonad indices also decreased over time, there were always some individuals that appeared to have mature gametes at each sampling period. No apparent differences in gamete development were observed at the TBT concentrations tested.

We thought that the Phase II juvenile bivalve growth study would be more realistic and informative than Phase I because we improved the flow rate and stability of TBT concentrations and reduced total biomass for Phase II. We also thought that the Phase I animals were under nutritive stress, and, therefore, we reduced biomass in Phase II experiments to eliminate this problem. However, bivalve growth indicated that system modifications and improvements were insufficient to provide growth conditions equivalent to surrounding bay waters.

For all oyster species except *O. lurida*, Pier Control animals grew considerably faster than Tank Controls. The difference was most pronounced in *C. virginica*, which grew five times faster than tank-held animals. *M. edulis* grew four times faster in the bay than in test tanks (Salazar and Salazar, 1987). These data strongly suggest that all of our test animals, including Tank Controls, were stressed by the test system.

For *O. edulis*, control growth was similar to that reported by Thain and Waldock (1986). However, in their study juvenile (3 mm) *O. edulis* growth was markedly reduced at 0.060-μg/l TBT after 20 days. In contrast, we found no statistically significant reductions in juvenile (-10 mm) *O. edulis* growth at concentrations as high as 0.157-μg/l TBT after 21 or even 56 days. Thain and Waldock show no reductions in growth of larger (5 g) *O. edulis* exposed to 0.24-μg/l TBT for 45 days, while growth in *C. gigas* (2.5 g) under similar conditions was significantly reduced. Our study showed that juvenile (-15 mm) *C. gigas* were not affected by 0.15-μg/l TBT after 56 days of exposure.

There are several possible explanations for these differences in results. Thain and Waldock suggest that the sensitivity of juvenile bivalves is size-dependent. Since our juvenile oysters were larger, they might be expected to be more resistant to TBT. If their laboratory test animals were under more stress, greater sensitivity might be expected. Also, differences in TBT effects could be attributed to differences in bioavailability between the laboratory and microcosm (Salazar, 1986). Although we found no statistically significant differences in growth attributable to TBT for any

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oyster species, tank variability may have precluded detecting such differences. Therefore, this test may not have been sensitive enough to detect differences at these low concentrations.

TBT tissue values show that both mussels and clams accumulated significant amounts of TBT from bay and experimental environments and approached constant TBT tissue burdens after 60 days of exposure. This could be attributed to either approaching a steady-state condition or metabolic decreases associated with st·ess from containment and TBT. The PETS data suggest that TBT bioaccumulation in *M. edulis* did not reach steady state until 60 days at 0.070-μg/l TBT and that BCFs decreased with increasing exposure concentration.

Others have found a similar inverse relationship with TBT concentration and BCFs for *O. edulis* and *C. gigas* (Waldock et al., 1983) and *C. virginica* (Dooley, unpublished). Laughlin et al. (1986) suggest that their BCFs of -5,000 are about an order of magnitude above what can be predicted from model compounds and octanol water partitioning coefficients. BCFs calculated for animals held at Shelter Island ($\bar{x}$ = 0.452 μg/l TBT) and in test tanks ($\bar{x}$ = 0.204 μg/l TBT) were about 25,000. These values are nearly five times higher than measured values of Laughlin et al. and nearly 50 times higher than predicted. However, they indicate that laboratory-determined values are not reliable measures of the environmental bioconcentration process. Since bioavailability may be correlated with suspended particulates, which were higher in Shelter Island, we expected differences in BCFs between Shelter Island mussels and those in our tanks. However, bioaccumulation was similar for both groups of mussels. Bioavailability may have been similar at Shelter Island because only a small portion of TBT was associated with particulates in Shelter Island as suggested by Valkirs et al. (1986). The environmental significance of BCFs remains unclear.

In theory, the benefit of a microcosm system is that it combines the advantages of controlled laboratory dosing conditions with realistic field conditions. This permits meaningful environmental studies over extended periods. The main improvement over the Hawaii prototype was removing the leachate panels from individual tanks and using a primary leachate tank, dilution, and distribution system. This field-dosing system is very similar to our laboratory system (Valkirs et al., 1985). There is similar variability in both systems that is characteristic of the TBT leaching properties. We believe this is the best available system for long-term, flowthrough tests. Although not truly portable, the PETS design facilitates deployment in a small area at almost any location.

Unfortunately, several problems need to be solved before this prototype can be used to obtain conclusive biological results for reliably predicting the environmental impact of TBT. Since the test tanks were fed by gravity flow, the height of the receiving tank and size of delivery lines determined maximum available flow rate. The major problems in maintaining adequate flow rates throughout the test were sedimentation and fouling in the delivery lines, thus effectively reducing pipe size. *Without adequate flow rates, achieving and maintaining nominal TBT concentrations was difficult.* In addition, the design of the dilutor system precluded a separation of the lower two concentrations, as it could not consistently produce the volumes of control and leachate water for the required ratios. Improvements made between Phases I and II resulted in concentrations closer to nominal for all but the highest concentration. At the highest test concentration, TBT values varied by almost a

factor of two over the entire test period and during the 24-hour sampling period. This variability was also observed in a 66-day flowthrough laboratory test (Valkirs et al., 1985) and is probably characteristic of the TBT leachate system. Even more variability was observed in the field, where TBT concentrations near marinas fluctuated by more than a factor of 20 between tidal cycles (Clavell et al., 1986). How this type of variation affects the biota is not clear.

We found that the arrangement of tanks affected the conditions within tanks. Our tanks were placed in two rows of six tanks each, with the rows approximately 1 meter apart. This resulted in significant differences in temperature among the four end tanks and the inside eight. Even though we provided a 70-percent sunscreen, some tanks still received more light than others. This influenced the density of algae on the surface and sides of the tanks. Davis et al. (1977) have suggested a circular distribution of tanks to help eliminate some of these problems.

Numerous authors have stressed the need for field validation of microcosm experiments (Perez et al., 1977; Harte et al., 1980; Heath, 1980; Santschi, 1982; White and Champ, 1983; Donaghay, 1984; Oviatt, 1984; and Santschi, 1985); few have actually done so (Perez et al., 1977; Oviatt, 1984; Oviatt et al., 1984; and Santschi et al., 1984). Considering the marked differences between our Pier and Tank Controls, we feel that field controls are absolutely necessary in the validation of site-specific bioassays. Most studies have not used this approach.

Our suggestions for improving this particular system are as follows: (1) Increase the flow rate to provide seawater containing its complete particulate load and to reduce tank effects; (2) Configure the test system and use shading to minimize effects of atmospheric conditions; and (3) Always include a field control to verify that the measured biological parameters are really tracking natural variation.

The results of this microcosm study are helpful in assessing the fate and effect of TBT from organotin AF coatings. Under site-specific microcosm conditions, juvenile mussel growth rates were shown to be affected by TBT stress (Salazar and Salazar, 1987). The bioaccumulation study confirmed that TBT is accumulated by mussels and clams. The degree of accumulation is directly proportional to TBT concentration, although BCFs are inversely proportional to TBT concentration. Results from the other portions of this study were less tangible and less useful for assessing TBT effects. The absence of measurable biological effects associated with TBT exposure could be interpreted to mean that there would be no effects in nature or the measurements were too insensitive given the variability of the test system. Systems such as PETS can be useful in environmental management only if the investigator knows of their limitations and prudently applies the results.

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