INTERACTIONS BETWEEN TEMPERATURE AND TRIBUTYLTIN (TBT) TOXICITY TO THE ESTUARINE MYSID, MYSIDOPSIS BAHIA

A Thesis
Presented to
The Faculty of the School of Marine Science
The College of William and Mary

In Partial Fulfillment
of the Requirements for the Degree of
Master of Arts

---

by

Prentiss H. Balcom
1991

APPROVAL SHEET

This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Arts

Prentiss H. Balcom

Approved, May 1991

Morris H. Roberts, Jr., Ph.D.
Committee Chairman/Advisor

Robert J. Huggett, Ph.D.

Mark W. Luckenbach, Ph.D.

Peter Van Veld, Ph.D.

Lenwood W. Hall, Jr., M.S.
University of Maryland
Queenstown, MD

DEDICATION

For my mother, Doris Durkee Balcom,
for giving me direction and encouragement

TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

ACKNOWLEDGEMENTS

I want to thank my major professor, Mory Roberts, for his guidance during my graduate school "experience," for his suggestions during the planning stages of this project, and for carefully reviewing and helping to improve several drafts of this manuscript. Thanks go to Mark Luckenbach for his input through the course of this work and helpful discussions on statistical analysis. I also thank the remaining members of my thesis committee, Lenwood Hall, Bob Huggett and Peter Van Veld for their thoughts at various stages of this project, and for their comments on this manuscript. Mike Bender is acknowledged for his comments and suggestions during the initial stages of this work, and a note of thanks goes to Shirley Sterling for typing a draft of this manuscript.

I am indebted to Pete De Lisle, Dan Sved, Ruth Williams and Karen Sheppard for their assistance, ideas, and friendship during the course of this work. Special thanks go to Ellen Travelstead and Donna Westbrook their help in getting me started with the TBT analyses. The staff of the Dept. of Chemistry and Toxicology is acknowledged for their assistance at various overwelming periods.

Above all, thanks go to my wife, Nancy Chartier Balcom, for her assistance during all phases of this study, and for her support throughout my graduate school career. Without her, I am sure this work could not have been completed. I am also indebted to my family for their encouragement and understanding throughout the course of this work.

For financial support of my graduate research assistantship and this study, I thank my major professor and the Dept. of Chemistry and Toxicology. A grant from the VIMS Minor Research Grant program partially funded the chemical analyses. Special thanks are due my father, Dudley C. Balcom, for special "grants," given cheerfully and with encouragement, that helped to support me through my graduate school career.

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

The limiting effect of temperature on reproductive output and growth of Mysidopsis bahia is unknown. Mysid neonates (<24-h-old) were exposed to four temperatures (20, 24, 28 and 32°C) for 29 days under static-replacement test conditions. Proportional survival was low at each test temperature following 29 days, but was higher at 20°C (0.41) and 24°C (0.39) than at 28°C (0.05) and 32°C (0.0). The first brood appeared earliest in marsupia of females at 28°C (10 days), and the day of appearance was increased at both higher and lower temperatures. Appearance of the first brood was significantly delayed ($p < 0.01$) until day 20 at 20°C. Mysid mean dry weights were not significantly different among temperature treatments, and few offspring were produced at any test temperature. The low survival, slow growth, and low offspring production seem to have resulted from the test methods and feeding regime employed. Life-cycle toxicity tests should be conducted under flow-through conditions to obtain better survival.

Aquatic animals living at temperatures near their tolerance limits may respond to lower levels of pollution than they would at optimal temperatures. Few studies in the literature have examined the effect of temperature on toxicity of organotin compounds, or the effect of temperature on the toxicity of any pollutant to M. bahia through a complete life cycle. This 28-day flow-through test incorporated five TBT concentrations (40, 74, 141, 273, 539 ng/L) plus controls at four temperatures (20, 23, 26, 29°C). Both temperature and the interaction of TBT with temperature affected survival. A 28 day LC50 of 472 ng TBT/L (287 and 1516 ng TBT/L, 95% fiducial limits) was calculated at 20°C. At 29°C, proportional survival at 539 ng TBT/L (0.40) was significantly reduced ($P < 0.05$) compared to the control (0.78) after 19 days, and on day 28, survival was significantly reduced ($P < 0.01$) at 273 ng TBT/L (0.13) compared to the control (0.45).

Temperature had an affect on the day of appearance of the first brood, day of first brood release, total number of offspring produced, and the number of offspring per female reproductive day (FRD), while TBT and the interaction of TBT with temperature affected only offspring production. The number of offspring per female reproductive (FRD) day was significantly reduced ($P < 0.01$), compared to the control, at 273 ng TBT/L following 28 days of exposure at 29°C. For controls, female mean dry weight was significantly greater ($P < 0.05$) at 23°C (1010 ug) than at the extremes in temperature (740 ug), but TBT concentration did not have a significant affect on weight. In general, mysids were more sensitive to TBT at extremes in temperature, particularly at elevated summer-like temperatures. Full life-cycle toxicity tests should be conducted for substances like TBT which are of high ecological risk at environmentally realistic concentrations.

INTERACTIONS BETWEEN TEMPERATURE AND TRIBUTYLTIN (TBT) TOXICITY TO THE ESTUARINE MYSID, MYSIDOPSIS BAHIA

I. GENERAL INTRODUCTION

Thermal Tolerance and Biology

A number of continually varying environmental factors (abiotic and biotic) modify the physiological performance of estuarine invertebrates. As an estuarine organism, Mysidopsis bahia must be able to functionally adapt to a host of dynamically changing environmental variables which are characteristic of the harsh estuarine environment (Vernberg & Vernberg, 1972; Vernberg & Vernberg, 1981). Temperature is one of the dominant physiochemical parameters in the environment, and is an important modifying factor to the physiological and ecological properties, as well as the distribution, of estuarine species (Alderice, 1972; Kinne, 1970; Prosser, 1973).

Tolerance limits for environmental factors such as temperature are controlled genetically, and to a lesser extent by acclimation, and within these limits conditions exist for optimal physiological performance (Cairns et al., 1975a, 1975b). An animal's thermal tolerance zone is the range of temperatures that can be tolerated more or less indefinitely. Temperatures above or below the zone of tolerance are considered to be in the zone of resistance. Some stages in the life cycles of estuarine invertebrates require specific, and often restrictive, temperature regimes. The tolerance zone is determined by the stage of the organism's development, its age, physiological condition, stage in the life history, etc., and juvenile stages often have a narrower thermal tolerance zone than adult stages (Cairns et al., 1975b).

Mysids are commonly known as opossum shrimp because of their shrimp-like

appearance and direct development of the young in a brood sac (marsupium). Most mysids are epibenthic, swimming in the water layer immediately above the sediment (Lussier et al., 1986). M. bahia is a subtropical estuarine species, found in shallow ponds near the substratum (Nimmo et al., 1977). Nimmo et al. state that this species occurs naturally in the Gulf of Mexico with other species of the genus Mysidopsis. Mysidaceans are dominantly carnivorous filter feeders, creating a feeding current while swimming. M. bahia is apparently omnivorous, but feeds readily on Artemis nauplii (Lussier et al., 1986).

M. bahia carries its embryos in the marsupium within which larval development takes place. It completes a life cycle (embryo to embryo) in about 25 days (Gentile et al., 1983). Gentile et al. state that juveniles released from the marsupia of sexually mature females are sexually differentiated in 12-14 days. Ova then appear in the oviduct of females within 24 hours and are released into the brood sac within 5 days. After about seven days embryos emerge from the marsupia as post-larvae with most of the adult features (Lussier et al., 1986). New eggs are released into the marsupia within 24 hours of the release of the juveniles (Gentile et al., 1983). Newly released juveniles are planktonic for the first 24 hours, after which they are epibenthic and orient positively toward a current for feeding (Nimmo et al., 1977).

In general, the range of temperatures over which a species survives is wider than the range over which the species grows and reproduces (Prosser, 1973). Temperature is known to exert a significant effect on time to sexual maturity (appearance of marsupium) in the estuarine mysid, M. bahia (McKenney, 1987), but its limiting effects on reproductive output and growth are presently unknown. With continued use of M. bahia as a test organism in aquatic toxicity tests, an increased understanding of the optimal temperature range for this species should

further its successful culture and enhance adequate assessment of the ecological hazards of various contaminants (McKenney, 1987).

TBT in the Environment

Tributyltin (TBT) is one of the most toxic chemicals ever introduced to natural waters. Of all organotin compounds, tributyltin, and triorganotins in general, are by far the most toxic to aquatic organisms (Maguire, 1987). Tributyltin-containing antifouling paints are highly effective for the control of fouling organisms on boat hulls; a major problem in estuarine and marine waters. The excellent fouling control activity of TBT is attributable to its toxicity to target organisms (fouling organisms) at low concentrations. Unfortunately, these same toxic properties make TBT harmful to nontarget organisms as well (Hall et al., 1988a).

Biological fouling can lead to serious economic consequences for industries such as electric power generation and shipping, and for recreational boating as well (Hall & Pinkney 1985). This has resulted in the widespread use of TBT-containing antifouling paints. These paints have been cited as the main source for input of TBT into aquatic environments (Maguire, 1987). Surveys of marina and boat owners conducted in recent years suggest that 50-75% of the recreational boats that use the Maryland waters of Chesapeake Bay are painted with TBT-containing paints (Hall et al., 1988b). The usage of TBT on commercial watercraft is believed to be even greater (Hall et al., 1988b). The Virginia waters of Chesapeake Bay are potentially affected by TBT paints introduced by recreational and commercial use, large shipbuilding operations, and a large Naval facility.

The highest concentrations of TBT in the U.S. marine environment have been found in yacht harbors and near vessel repair facilities. Seligman et al. (1989)

reported that investigators in the U.K., Canada, and the U.S. have documented concentrations of TBT between 50 ng TBT/L and "several" ug TBT/L in small boat harbors and high-use estuaries. However, concentrations were generally less than 20 ng/L and frequently not detectable in open, well-flushed regions of bays and estuaries. A similar pattern of TBT contamination exists in Chesapeake Bay. The highest concentrations of TBT reported from natural waters and sediments of Chesapeake Bay have been found in harbors, marinas, and areas associated with high boating activity (Hall et al. 1987, 1988c). TBT concentrations have generally been low or non-detectable in the more open waters of the Chesapeake Bay and its tributaries, as well as in the open ocean.

TBT contamination in some areas could pose a serious threat to estuarine biota. Peak environmental TBT levels may exceed concentrations which are acutely toxic to some sensitive invertebrates in the laboratory, and average concentrations may exceed chronic effect levels by an order of magnitude. Hall et al. (1987) reported 12-month average TBT concentrations ranging from 51-408 ng TBT/L in four marinas in the Maryland portion of Chesapeake Bay. A peak spring concentration of 998 ng TBT/L was reported form one marina. Westbrook et al. (1986) reported concentrations as high as 670 ng TBT/L in one marina along the Hampton River, Va. Average concentrations of TBT in three marinas ranged from 3-254 ng TBT/L at five stations during 50 weekly sampling periods (Huggett & Westbrook, 1987). These investigators reported a maximum TBT concentration of 1300 ng/L in the Hampton Roads marina. Aqueous concentrations of TBT were generally low in the Hampton Roads-James River-Elizabeth River system of the southern Chesapeake Bay, with the exception of one high value (920 ng TBT/L) at a station near a shipyard on the Elizabeth River.

Temperature Interaction with Toxicity

It is well known that estuarine animals are subjected to highly variable temperatures which may affect their survival, growth, and metabolic physiology. The temperature dependence of juvenile growth rate, time to maturity, and hardiness have been demonstrated in studies with an estuarine mysid, Neomysis americana (Thompson, 1984). Temperatures which favor maximum rates of growth and reproduction in N. americana seem to cause significant physiological stress and are very near levels that limit distribution. Marine and estuarine animals living near their distributional limits of temperature tolerance in estuaries and brackish water tend to be more susceptible to any stress, and can suffer at pollution levels considerably lower than those living under optimal environmental conditions (McLusky et al., 1986).

There is no single pattern for the effects of temperature on the toxicity of pollutants to aquatic organisms. Temperature change in a given direction may increase, decrease, or cause no change in toxicity, depending on the toxicant, the organism, and in many cases the investigator who has selected a particular procedure, exposure time, and response (Sprague, 1985). However, temperatures well within the ordinarily harmless range can greatly influence the tolerance of aquatic organisms for lethal agents. Important factors in the interaction of temperature with pollutants are the inate temperature tolerance (genetic nature) and the immediate prehistory or acclimation of the organism with respect to temperature (Cairns et al., 1975a; 1975b).

McLusky et al. (1986) reviewed information on the effects of temperature and salinity on the toxicity of heavy metals to estuarine invertebrates. The effect of temperature on the toxicity of arsenic, cadmium, chromium, copper, lead, mercury and zinc was considered. In general, increased temperatures resulted in increased

toxicity for all of the metals examined. In a few cases, the range of acute toxicity values for a single species exposed to different temperature and salinity regimes exceeded the entire range of acute toxicity values previously published for that pollutant with a variety of marine and freshwater animals (Bryant et al., 1984). The distinct effects of temperature on the acute toxicity of heavy metals suggest that the effect of temperature must also be taken into account in sub-lethal studies. Despite the considerable effort which has been expended on toxicity testing of harmful substances to aquatic organisms, relatively few studies have examined the effect of temperature (or salinity) on toxicity (Sprague,1985). Incorporating a range of temperatures in toxicity tests may be appropriate for evaluating the effect of heavy metals on estuarine or inshore marine communities since these communities may experience regular variations in environmental factors (McLusky et al., 1986).

A few studies have examined the effects of temperature in toxicity tests with mysids (Hargreaves et al., 1982; Thompson, 1984; McLusky & Hagerman, 1987; and Voyer & Modica, 1990). As mentioned above, the toxicity of heavy metals increases with an increase in temperature, and toxicity increased with increasing test temperature in each of these studies. Higher temperatures significantly increased the acute toxicity of naphthalene to N. americana (Hargreaves et al., 1982; Thompson, 1984). McLusky & Hagerman (1987) found that increased temperature reduced median lethal concentrations of metals to Praunus flexuosus. Using the same test species employed in the present study, M. bahia, Voyer & Modica (1990) found that median lethal concentrations for cadmium decreased with an increase in temperature.

In a recent review, McLusky et al. (1986) pointed out the paucity of information on the effects of temperature on the toxicity of both inorganic metals

and organometals (e.g. TBT) to estuarine invertebrates. Therefore, a study of the interaction effects of TBT and temperature would be informative. In the only published study found on the effects of environmental variables on the toxicity of an organotin compound, an increase in temperature sharply increased acute trimethyltin toxicity to zoea of the fiddler crab, Uca pugilator (Wright & Roosenberg, 1982). TBT loading has been reported to be higher during warm early summer months when boating activity increases in Chesapeake Bay and other areas (Hall et al., 1988a). The detrimental effects of TBT pollution may be pronounced when combined with elevated temperatures, normally in the harmless range. Although M. bahia is not a resident Chesapeake Bay inhabitant, Bay organisms of sensitivity similar to M. bahia, and with lower thermal tolerance limits, may be detrimentally affected at some times of the year.

Research Objectives and Justification

A thermal tolerance test was conducted to develop a 28-day static-replacement test design, and to verify the observations reported in the literature regarding temperature effects on survival and reproductive development in M. bahia. A static-replacement design might prove to be the most manageable approach for life-cycle toxicity tests. As recommended by American Society for Testing and Materials (ASTM) designation E 1191-87, this test provided preliminary information for later tests by determining, a) whether mysids survive, grow, and reproduce successfully under static-replacement conditions, b) whether food, water, handling procedures, mysid density in test chambers, etc., were acceptable, and c) the magnitudes of the within-chamber and between-chamber variances. The experimental design allowed for examination of the temperature effect on time to appearance of marsupia, time to appearance of first brood, day of first

brood release, survival, and mean dry weight of mysids alive at the end of the test.

An acute toxicity test was conducted at a single temperature to determine the 96-h LC50 of TBT to M. bahia. This test was used as a range finding test to assist in determining concentrations of TBT to be used in a life-cycle toxicity test. In addition to obtaining basic toxicological information, the acute test was conducted to develop a reliable test/dosing system for long-term testing of TBT.

Subsequently, a 28 day life-cycle toxicity test was conducted to study the individual and combined effects of TBT and temperature on M. bahia. The life-cycle toxicity test with M. bahia is a sensitive and versatile tool for use in environmental toxicology. It can be used to assess standard toxicological responses such as survival and growth, and a variety of sublethal reproductive responses as well (Gentile et al., 1983). The experimental design in the present study allowed for examination of effects on the following biological responses: survival, day of appearance of first brood, day of first brood release, offspring production, and the mean dry weights of mysids alive at the end of the test.

M. bahia was used as the test organism in these tests for several reasons. The use of mysids, and the genus Mysidopsis in particular, has become widely accepted in aquatic toxicology for acute and chronic (life cycle) toxicity tests. In general, Mysidopsis spp. have been shown to be as or more sensitive to toxic substances than other marine vertebrates and invertebrates tested (Nimmo & Hammaker, 1982), and acute and chronic toxicity data developed for this species are among the most comprehensive currently available for a marine invertebrate (Lussier et al., 1985). M. bahia is an excellent test species for life-cycle toxicity tests because of its short life cycle, ease of handling, excellent long-term survival, sensitivity to toxicants, and the uniqueness of its reproductive mode (Lussier et al., 1985). The later makes monitoring of reproductive processes achievable and quantifiable

under testing conditions. Use of this crustacean permits not only a comparative evaluation of differential toxicity among various life stages, but also an assessment of the impact of chronic exposures on ecologically important biological responses (McKenney, 1987). Increased mortality, retarded growth, and diminished reproductive success have direct effects on population production of the species (McKenney, 1985). These effects on productivity are important because mysids serve as an important link in the marine food chain between primary producers and fish (Markle & Grant, 1970; Smith et al., 1984).

II. THERMAL TOLERANCE TEST

INTRODUCTION

To our knowledge, the experiments of McKenney (1987) are the only published attempt at conducting life-cycle tests with mysids under static-replacement conditions. Tests were begun with neonates (<24-h post-release) from females maintained at 25±1°C and 24±2 °/$_{oo}$. Survival greater than 50% through 28 days of testing fell within the rather restricted range of 21-25°C and 17-24 °/$_{oo}$). Surprisingly, other than the work of McKenney (1987), little has been published on the thermal tolerance of M. bahia.

In his experiments, McKenney attempted to find the optima in temperature and salinity during the early reproductive phase of the life-cycle of M. bahia. He found that the time to sexual maturity (appearance of marsupium) was shortest at 28°C (11 days). Development to sexual maturity was slowed at both higher and lower temperatures. In contrast, survival through 28 days was maximized at about 23-24°C. McKenney concluded that when considering both survival capacity through a complete life cycle and time required for juvenile mysids to become reproductively mature, temperature-salinity conditions of 25°C and 20 °/$_{oo}$ appear to be optimal for this estuarine crustacean.

Information about the effect of temperature on reproductive development in M. bahia can also be derived from various toxicity tests carried out with this species. The reproductive parameter of time to first brood release has been measured in a number of life-cycle toxicity tests with M. bahia (McKenney, 1986; Lussier et al., 1985; Gentile et al., 1982a, 1983). The results from control

treatments in these experiments generally indicate a decrease in the number of days to first brood release with increasing temperature. Brood release occurred earliest at 25°C (19 days), later at 23-24°C (22 days), and latest at 21°C (21-26 days); slight variations were seen depending on the study examined.

MATERIALS AND METHODS

Production of Test Animals

Mysids in the laboratory brood stock (cultures established in 1979) were held in 37 L aquaria with undergravel filters and 1 um filtered York River (Gloucester Point, VA) water, and were fed Artemia sp. nauplii ad libitum daily. A room air temperature of 25-30°C and a light:dark (L:D) cycle of 14h:10h were maintained in the culture room at all times; water temperature in the laboratory culture tanks varied somewhat less than the room air temperature.

Newly released, uniform-aged (<24-h post-release) mysids were obtained from ovigerous females maintained in static cultures. A retention basket constructed of Nitex screen (1.0 mm mesh) was immersed in each of three 37 L brood tank containing 1 um filtered York River water (27-28°C). Several hundred mature mysids from the laboratory brood stock were added to each retention basket. In order to produce a given number of juveniles, approximately twice that number of mature mysids were required. A few hours after loading, animals small enough to escape the baskets were removed. Approximately 100,000 Artemia sp. nauplii (24-h-old) were added to approximately 30 L of water in each brood tank (De Lisle & Roberts, 1986). After 24 h the retention baskets were removed and neonates were carefully netted from each tank.

Thermal Tolerance Test

The 28 day thermal tolerance tests were conducted in incubators maintained at test temperatures with a 16h:8h L:D cycle. Test temperatures were as follows: 20, 24, 28, and 32°C. These temperatures were chosen based on the findings of McKenney (1987) which showed that 25°C was optimal when considering both survival and reproduction. Neonates were adjusted from brood release

temperatures (27-28°C) to test temperatures gradually over 48 h at a rate which did not exceed 3°C in any 12-h period. This method of initiating the test was necessary, since preliminary work indicated that cultures of mature mysids acclimated to the extreme test temperatures (20 and 32°C) over a period of several weeks would not release enough juveniles to start the tests.

Test chambers were 8-inch glass bowls containing 1 L of water. Tests were begun with 7 replicate bowls at each temperature and 10 neonates per bowl. Neonates were randomly placed in test bowls using a wide bore glass pipet, adding only a few at a time. Filtered (1 um) York River water was adjusted to the test salinity ($20°/_{oo}$) with high salinity water (Finney Creek, Wachapreague, VA). Carboys holding filtered water, replenished concurrently as required, were kept in the incubators at each test temperature, and were used to replace test water in the replicate bowls. In each incubator, light was provided by flourescent bulbs mounted vertically on one side of the incubator. Incubator illumination, measured with a Lycor LI-185A quantum radiometer/ photometer, varied by no more than 15% between incubators (630-750 Lux).

Observations on survival and reproductive development were made every other day when water was changed. The 20 and 24°C treatments were examined on one day and the 28 and 32°C treatments were examined together on the next day. The mysids in each replicate were carefully pipetted (wide-bore glass pipet) to clean bowls every fourth day (every other time water was changed), and the live mysids in each replicate were counted. The Kruskall-Wallis test and a nonparametric multiple comparison test (Zar, 1984), both calculated by hand, were used to test for differences in survival among the treatments. Excess food and debris were removed every other day before test water was renewed, and dead mysids were removed daily. Replicate bowls with 10 animals received 0.8 ml

of Artemia suspension per day, resulting in a density of about 3000 nauplii/bowl/day. As the test progressed and animals died replicates were fed in direct proportion to the number of mysids in the bowl in order to ensure comparable growth data and to avoid giving chambers with fewer mysids the advantage of extra food (ASTM Designation E 1191-87). All replicates received at least 150 nauplii/mysid/day (Lussier et al., 1986).

Temperature, salinity, dissolved oxygen, and pH were monitored in two randomly selected replicate bowls every other day in each treatment. Temperature was measured with thermometers marked in 1°C increments and estimated to tenths of a degree. All thermometers were standardized against a thermometer marked in 0.1°C increments (±0.5% accuracy, Fisher Scientific). A max-min thermometer was submerged in a container of water in each incubator, and maximum and minimum temperatures at each test temperature were recorded daily.

Reproduction Measurements

The developmental stages relating specifically to reproductive development and reproductive output were examined in detail. Mysids in replicate bowls were examined every other day for appearance of brood sacs and appearance of embryos (first brood) in brood sacs. Each bowl was examined daily for young (second generation), and when applicable, the day of brood release in each bowl recorded. Following the procedures of Lussier et al. (1986), on the final day of the test the live mysids in each bowl were examined under a dissecting microscope to determine sex, the number of immature animals, and presence or absence of eggs in the oviducts or brood sacs of females. Reproductive responses were examined for differences among treatments with the Kruskal-Wallis test and a

non-parametric multiple comparison test for use unequal sample sizes, both by hand calculation (Zar, 1984).

Growth Measurements

At the end of the experiment, live mysids in each treatment were processed for dry weight measurement. Females were separated from males plus immature animals in each replicate and the number in each group recorded. Mysids were rinsed with deionized water and the animals from each replicate bowl were placed on two tared weighing pans, one for each group (Lussier et al., 1986). The mysids were oven-dried at 60°C for 48 h. After cooling to a steady weight in a dessicator, the dry weight of each group of mysids was subsequently measured to the nearest microgram (ug) using a Mettler balance (BA25/BE22/ME22). To obtain the mean weight per individual mysid, the total weight of each group was divided by the number of animals in the group. Separate mean dry weights were calculated for females, males plus immatures, and total (females and males plus immatures) mysids in each replicate; comparison among treatments could then be made for each of these groups. Dry weight measurements were examined for differences between treatments using the Mann-Whitney test, calculated by hand (Zar, 1984).

RESULTS

Water Quality

For each treatment, the time-weighted mean temperatures differed from the desired test temperatures by less than 0.3°C (Table 1). The extreme values in the range of measured temperatures at each test temperature were attributable to short-term interruptions in incubator equilibrium temperature. The time-weighted average maximum and minimum temperatures were within 1.3°C of the desired test temperature at 20°C, 0.5°C at 24°C, 1.1°C at 28°C, and 1.4°C at 32°C (Table 1). These values were probably a good indication of the daily variation of temperature in each incubator.

Temperature measurements were made concurrently in all replicates at each treatment on days 5, 22, and 28 (none at 32°C on day 28). The temperatures of replicates at 20°C, 24°C, and 32°C were always within 1°C of each other, and usually within 0.5°C. Most of the replicates at 28°C were within 1°C of each other, but on each day one replicate increased the range of temperatures to 1.1-1.2°C.

Measured pH values were consistently lower than actual pH because of a problem with the pH probe. Values obtained with the Fisher Accumet meter (Orion combination electrode) used in this test (Table 1) were compared to pH values obtained using a Nestor mini-meter (model 55, Thomas combination electrode). Concurrent pH measurements using these meters revealed that values obtained using the Fisher meter were lower by 0.19-0.40 units one day and by 0.15-0.26 units on another day. On several different days, values obtained after calibration using the Fisher meter were approximately 0.25 units lower than values obtained after a second calibration. The Nestor meter was consistent between calibrations, revealing pH differences of only 0.05-0.1 units. If pH values

Table 1. Water quality measurements for thermal tolerance test. Time-weighted mean values at each temperature with range in parentheses.

Test Temp.	Measured Temp. (°C)	Max./Min. Temp. (°C)	Salinity (°/oo)	Dissolved Oxygen (mg/L)	pH
20°C	20.3 (19.4-21.2)	21.3/18.9 (20.4-22.3/17.9-20.0)	20 (19-20)	6.69 (6.20-7.30)	7.47 (7.26-7.69)
24°C	24.0 (22.5-24.8)	24.5/23.5 (23.9-25.1/23.0-24.0)	20 (19-21)	6.35 (5.60-6.80)	7.52 (7.33-7.81)
28°C	28.2 (26.9-29.3)	29.1/27.6 (28.2-30.9/26.5-28.5)	20 (20-21)	6.17 (5.50-6.55)	7.55 (7.36-7.70)
32°C	32.1 (31.0-33.6)	33.4/31.3 (31.7-34.8/29.9-32.2)	20 (20-21)	5.94 (4.90-6.50)	7.72 (7.46-7.91)

measured using the Fisher meter were corrected for the difference from the Nestor meter, mean pH values would be above the minimum of 7.70 recommended by ASTM (Designation E 1191-87).

The measured dissolved oxygen (D.O.) values remained above 60% of saturation (percent saturation calculated from measured D.O. values with a program that compensates for temperature and salinity) at all times at each test temperature, although dissolved oxygen concentration varied among treatments as a function of temperature (Table 1). The mean measured salinities at each test temperature were equal. The slight variability in ranges is attributable to adjustments to the test water made with high salinity water.

Survival

After 28 days, the mean proportional survival was 0.41 and 0.39 at 20 and 24°C, respectively, and 0.06 and 0.0 at 28 and 32°C, respectively (Table 2). Temperature had a significant affect on day 28 survival in these tests ($Chi^2=23.33$, d.f.=3, $P<0.01$, based on Kruskal-Wallis analyses). Survival at 20°C was significantly greater than at 28°C ($Q=2.76$, d.f.=4, $P<0.05$) and 32°C ($Q=3.76$, d.f.=4, $P<0.01$)(Zar, 1984). Survival at 24°C was not significantly different from that at 20°C. At 20 and 24°C there was a relatively constant rate of decrease in survival during the 28 day test (Figure 1), and survival remained above 0.50 until after day 21 at 24°C and until after day 25 at 20°C. In replicates at both 28 and 32°C the mortality rate increased markedly after 8 days (Figure 1). This increased rate of mortality persisted until day 24 when survival was approximately 0.10 at 28°C and 0.0 at 32°C.

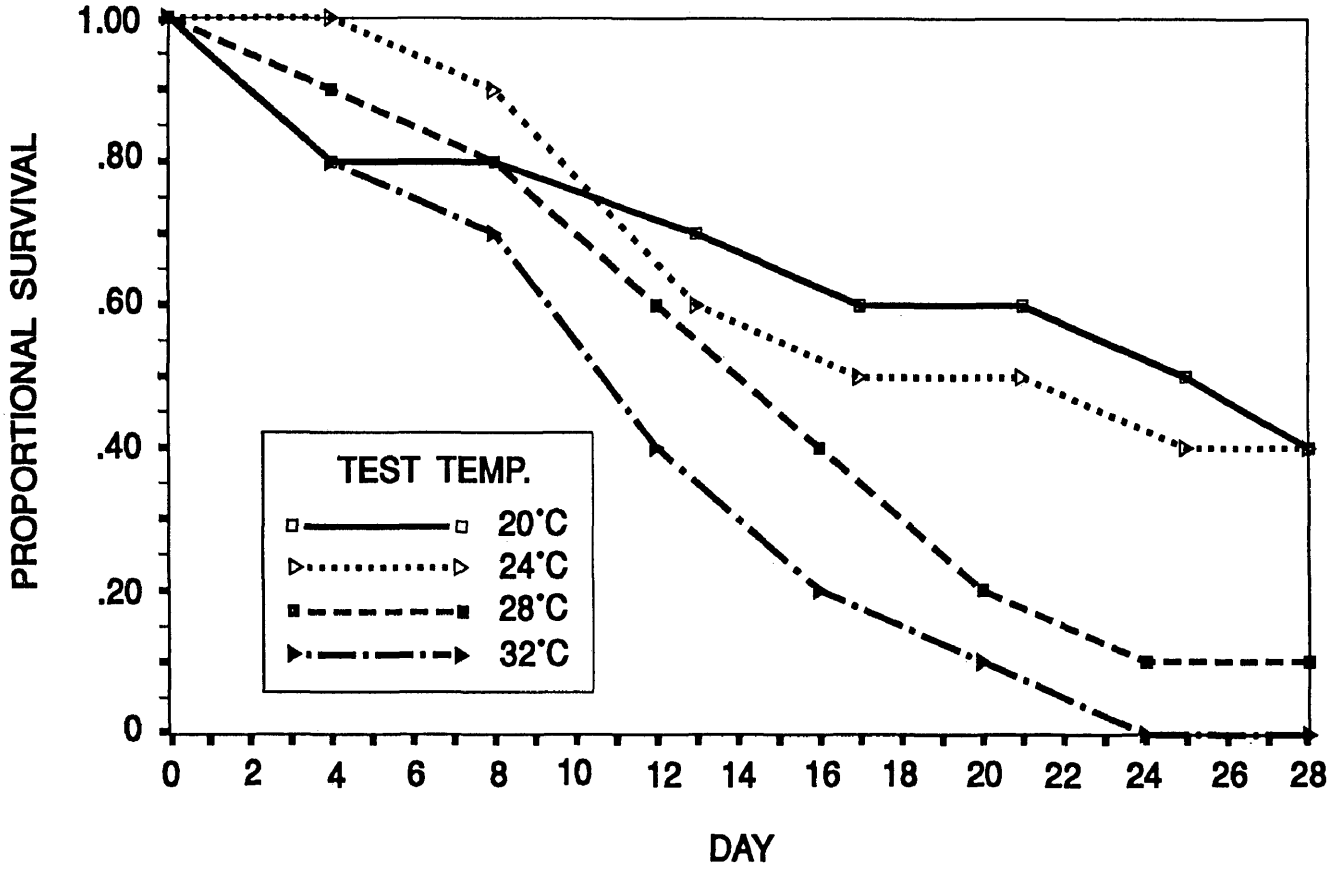
Reproductive Responses

The number of days until appearance of marsupia of females was least at

Table 2. Influence of temperature through 28 days on the development and reproduction of M. bahia. Each value represents the mean ± standard deviation with the number of replicates in parentheses.

	BIOLOGICAL RESPONSE						
Test temp. (°C)	Percent survival (day 28)	Appear. of marsupia (days)	Appear. of first brood (days)	First brood release (days)	Total no. young released	Female dry weight (ug)	Male dry weight (ug)
20	41 ± 20 (7)	16 ± 2 (7)	20 ± 3 (7)	26	5	587±166 (5)	518±166 (6)
24	39 ± 23 (7)	11 ± 2 (7)	18 ± 4 (7)	19	9	627±77 (6)	530±54 (4)
28	6 ± 5 (5)	9 ± 2 (6)	10 ± 2 (5)	15	15	357±115 (3)	----
32	0 ± 0	10 ± 2 (7)	14 ± 4 (4)	--	--	----	----

Figure 1. Mean survival through 28 days at each test temperature.


PROPORTIONAL SURVIVAL
1.00
.80
.60
.40
.20
0
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
DAY
TEST TEMP.
20°C
24°C
28°C
32°C

28°C (9 days, Table 2). Development was slower at the two lower test temperatures, and was also slightly slower at the highest test temperature (Table 2). Temperature had a significant affect on the number of days to appearance of marsupia ($Chi^2 = 18.08$, d.f.=3, $P < 0.01$). The number of days was significantly greater at 20°C than at 28°C ($Q = 3.88$, d.f.=4, $P < 0.01$) and 32°C ($Q = 3.33$, d.f.=4, $P < 0.01$). The day of appearance was not significantly different between 24°C and 20°C.

Temperature had a significant effect on time to appearance of the first brood in the marsupia of females ($Chi^2 = 13.37$, d.f.=3, $P < 0.01$). Embryos appeared first at 28°C after 10 days (Table 2). As was the case for the earlier developmental stage of appearance of marsupia, embryos appeared later in the marsupia of females at the two lower temperatures, and were also slower to appear at the highest test temperature (Table 2). Brood appearance was significantly slower at 20°C than at 28°C ($Q = 3.48$, d.f.=4, $P < 0.01$) but was not different from the other test temperatures.

Overall, there was little production of offspring in these tests. The data on production of young are summarized in Table 2, but only a few replicates released broods at any temperature. These data were not analyzed statistically because of the low sample sizes. Broods were released first at 28°C, and the day of release increased with decreasing temperature. A maximum number of young (15) were released at 28°C, and the number of young released decreased with decreasing temperature. Four juveniles at 28°C were dead when first observed. No juveniles were produced at 32°C.

Growth Measurements

Dry weight measurements were made on animals alive at the end of the test at 20 and 24°C, and for the few live animals (4) remaining at 28°C (Table 2). Small differences in mean dry weight were found between the 20° and 24°C treatments. Using the Mann-Whitney test, no significant differences were found between either females ($U=20$, $P>0.05$) or males plus immatures ($U=16$, $P>0.05$). A larger but still non-significant ($U=70.5$, $P>0.05$) difference in total (females and males plus immatures) mean dry weights was found for animals at the 20 and 24°C treatments. Mean dry weights were much lower at 28°C than at the other test temperatures, but the difference was not evaluated statistically because of the low sample size.

DISCUSSION

Survival

This experiment was conducted to evaluate the use of static renewal procedures for life cycle tests with M. bahia. However, survival in this test was low, indicating that the procedure is not appropriate. Survival at 20 and 24°C (0.41 and 0.39), the highest for this test, was similar to, but slightly lower than, that found by McKenney (1987) under static renewal conditions (these results presented earlier). According to ASTM designation E 1197-87, life-cycle toxicity tests with saltwater mysids should not be considered valid if more than 30% of the first generation control mysids die between the beginning and the end of the test. The survival obtained with the current methods is, therefore, not acceptable for toxicity tests.

The low survival after 28 days at all test temperatures indicates that the bowl change schedule (every 4 days) employed was inadequate. After 4 days of use, there was considerable bacterial or algal fouling in the bowls. The rapid mortality rate at 28 and 32°C indicates a problem with the test methods employed. More frequent water changes and transfers to clean bowls might improve the survival in tests lasting as long as 28 days, but this was not tested. Daily water and bowl changes would have involved considerably more labor and may have caused considerable stress to the test animals.

Most life cycle toxicity tests with mysids have been conducted under flow-through conditions, and have shown higher survival as compared to static replacement tests. McKenney (1986) found approximately 90% survival up to the time of first brood release in control treatments (25 ± 1°C, 20 ± 2°/$_{oo}$). In the experiments of Lussier et al. (1985), survival in control treatments was as high as 97% in an experiment which lasted 29 days (24 ± 1°C, 30 ± 2°/$_{oo}$) and survival was

generally above 70% in the controls of experiments lasting for a minimum of 35-days (22-23±1°C, 30 ± 2°/$_{oo}$). Gentile et al. (1982a) found 80 and 73% survival in control treatments of experiments lasting for 44 and 36 days (21±1°C, 30°/$_{oo}$), respectively. Finally, Gentile et al. (1983) found 80% survival in control treatments to day 35 (21±1°C, 30°/$_{oo}$). While flow-through tests are inherently more difficult to maintain than static replacement tests, especially with temperature as a test factor, they offer the advantage of considerably better survival. The toxicity tests discussed below were carried out under flow-through conditions in order to obtain adequate survival for life-cycle toxicity testing.

Reproductive Responses

Temperature had a significant effect on the time to appearance of marsupia in the present study. Although differences were found, it was often difficult to clearly recognize marsupia when they first appeared. Empty marsupia were not easy to recognize when mysids were swimming freely in the replicate bowls. Comparison of the results of the present study with the results of other studies reveals a somewhat confused picture of the effects of temperature. Based on the results of McKenney (1987), the number of days required to reach sexual maturity (appearance of marsupia) as a function of temperature in this study are predicted to be as follows: 20 days at 20°C, 14 days at 24°C, 10 days at 28°C, and 11.5 days at 32°C (20°/oo). The results of the present study agree with those of McKenney at the high temperatures, 28 and 32°C, but animals in the present study showed slightly faster development to sexual maturity at the two low temperatures, 20°C (16 days) and 24°C (11 days). Conversely, the 10-14 days required to reach sexual maturity at 20-21±1°C and 30°/$_{oo}$) in the control treatments of experiments by Gentile et al. (1982a, 1983) was shorter than that observed in either the

present study or that of McKenney (1987).

The first brood appeared first in the marsupia of females at 28°C (10 days), and brood appearance was slowed at both higher and lower temperatures. Two other experiments with M. bahia which included time to appearance of first brood as an endpoint have demonstrated slightly faster development at low temperatures as compared to the present test. Gentile et al. (1982a) reported appearance of the first brood (obtained by subtracting brood duration from time to first brood release) after 18 days and 14 days at 20±1°C in control chambers in separate experiments. Gentile et al. (1983) reported that the first brood appeared in 18 d at 21±1°C. The delay in appearance of the first brood at 20°C, as well as the low number of offspring produced, in the present test may reflect the feeding regime employed, bacterial contamination, or a combination of both of these factors. This point is discussed further below. Reproductive development may have been slower in the present study than that found in other tests because one expects less productive output from animals under nutritional and possibly lethal stress.

Feeding and Growth

McKenney (1987) investigated the effect of food density on growth and reproductive output in M. bahia. He reported that the availability of food during the reproductive phase of the life cycle influenced reproductive capacity and growth. The number of days to first brood release, number of young produced per female, and the total number of young released all seemed to be adversely affected by a reduced food supply. For maximum reproductive success with M. bahia, the threshold food concentration appeared to be a regime averaging approximately 2-3 Artemia nauplii/mL of test water.

As indicated previously, mysids in the present study were fed in direct

proportion to the number of animals in the test chamber, providing at least 150 nauplii/mysid as recommended by ASTM designation E 1191-87. The food density was initially above 2 nauplii/ml in all replicates, but once the number of animals was reduced to 7 in any replicate, the proportional feeding regime employed reduced the food concentration to less than the 2 nauplii/ml recommended by McKenney (1987). In the experiments of McKenney (1987), following 21 days of testing at 25°C, individual mysid weights averaged between 690 and 725 ug at feeding densities of more than 2 nauplii/ml. In the present experiments, at 24°C, individual male and female dry weights averaged 530 and 627 ug, respectively, after 28 days. These weights were as low as the mean weights (508-663 ug) found by McKenney at suboptimal food concentrations.

The feeding regime employed may also account for the delay in time to appearance of marsupia and time to appearance of the first brood at 20°C in the present study as compared to Gentile et al. (1982a, 1983). Food was being supplied at less than the optimal concentration as animal numbers decreased late in the experiments, and therefore animals probably grew and developed at a suboptimal rate. Overall, the results of the present study were confounded by the feeding regime employed. The delayed reproductive development, poor reproductive output, and low mysid weights are at least partially attributable to food limitation. The feeding rate of 150 nauplii/mysid/day does not seem to be acceptable for long-term flow-through tests with mysids, particularly when animal numbers are greatly decreased at the end of the test. Mysids should be fed proportionally as recommended in ASTM designation E 1191-87, but the minimum food density should be 2 nauplii/mL of test water.

Data Analysis and Experimental Design for Future Studies

Power calculations for a one-way ANOVA (Zar, 1984) were performed on the reproductive response and growth results of this study. The high variance in the data of the present study resulted in low power of hypothesis tests applied to these data. The only hypothetical situations which yielded satisfactory power (>70%) were those which incorporated 10 replicates (an unreasonably high number for later life cycle experiments) or greatly increased the minimum detectable difference (i.e. 50% of the mean for dry weights). Similarly, the predicted power of one-way ANOVA's applied to survival and reproductive results of McKenney (1986) indicate that power would be acceptable (>70%) only when the minimum detectable difference was greatly raised (i.e. 50% of the mean for survival data). In the two factor studies described in the next chapter, the number of groups was greater than the 4 employed in the present test. Since power decreases as the number of groups increases (Zar, 1984), the power of hypothesis tests applied to the results of those later tests is predicted to be even lower than that reported here.

Data from experiments dealing with reproductive output and growth typically have high inherent variability, and, as far as statistical power is concerned, the best way we have to compensate for this variability is to increase replication. In the two-factor study on the effect of temperature on the chronic toxicity of TBT discussed later, increasing replication in order to obtain adequate power in hypothesis tests was impractical. The large increase in replication required would be unmanageable with that experimental design. For these reasons, future studies should be designed for regression analyses similar to those used by McKenney (1986). With this statistical approach, the number of replicates and the agreement between replicates (variance) will only affect the confidence limits of

an estimated endpoint (Stephan & Rogers, 1985). These statistical tests can be used to fit an equation for the relationship between biological responses and both exposure concentrations and levels of an environmental factor. Regression analysis should be useful in future tests since it can be used to examine the relationship between individual factors and their interaction, and to provide a basis for interpolating to untested concentrations (Zar, 1984).

III. INTERACTION OF TEMPERATURE WITH TBT TOXICITY

INTRODUCTION

Acute Toxicity

Ranking species by sensitivity to either acute or chronic exposure to TBT produces a continuum of values extending from approximately 100 to less than 0.05 ug/L; most species are found clustered between 0.5 and 1 ug/L (Laughlin & Linden, 1987). Acute organotin toxicity (24- to 144-h LC50's) was found to range from 400 to 4000 ng TBT/L for several zooplankton species (Hall & Pinkney, 1985). Three species of mysids, including M. bahia, have 96-h LC50's which fall in the sub-parts-per-billion range. According to Hall & Pinkney (1985), acute morality data available at the time for macroinvertebrates showed a 96 h LC50 of 500 ng TBTO/L for juvenile mysids, Metamysidopsis elongata, at the low end of the range. In a static acute test with a TBT-containing leachate and mysid neonates (<24-h-post release), Holmesimysis costata (formerly Acanthomysis sculpta, Kathman et al., 1986), a 96-hr LC50 of 420 ng TBT/L was reported based on measured concentrations (Davidson et al., 1986).

The acute toxicity of TBT to Mysidopsis bahia is known only from one study in the literature (Goodman et al., 1988), and has also been investigated in unpublished work from this laboratory. The static replacement toxicity tests conducted in this laboratory indicate that the survival of neonates (<24-h-old) of M. bahia through 96 hours is severely affected at concentrations between 800 and 1600 ng/L (3% - 83% survival). In a separate 96-h test, the survival of 7-d-old mysids was affected at concentrations between 1450 and 1700 ng/L (55% - 90%

survival). In a paper published after the present project was initiated, Goodman et al. (1988) reported a 96-h LC50 for M. bahia (<24-h-old) of 1100 ng TBT/L (680-1400 ng/L, 95% C.I.).

Chronic Toxicity

The literature on TBT toxicity reveals reduced survival and growth, impaired reproductive output and developmental rate, imposex in some gastropods (the development of male sex organs on the female), shell thickening in some pelecypods, avoidance responses, and histological changes in various animals after sublethal exposure to organotin compounds. Hall & Pinkney (1985) reviewed much of the early toxicity data on TBT in a paper on the acute and sublethal effects of organotin compounds on aquatic biota. Maguire (1987) updated the current status of toxicity testing with TBT in his review of environmental effects of TBT compounds, and Hall (1988b) reviewed information regarding TBT toxicity studies with Chesapeake Bay biota.

Even though many organisms show tolerance towards organotin compounds administered in sublethal quantities, repeated doses of organotins, even at very low levels can eventually prove fatal (Saxena, 1987). Saxena states that a variety of biochemical and physiological alterations occur at TBT dose levels lower than the lethal dose, and effects become increasingly severe as the concentration increases. Tributylin has been shown to adversely affect the development of aquatic organisms from fertilized egg through various early life stages, and has a detrimental effect on reproductive functions in mature individuals (Rexrode 1987). However, few chronic studies have been undertaken with TBT, or organotin compounds in general, until quite recently. Hall & Pinkney (1985) noted the need to evaluate sublethal effects of long-term, low level exposures of organotins

to invertebrates and fish. Among other important areas, behavorial and physiological studies are needed to assess these effects.

Oysters, clams, and other bivalves are the most sensitive organisms, particularly during embryonic and larval periods, but other groups are also highly sensitive to TBT. Sensitivity to TBT varies among aquatic species, but various species of gastropods, bivalves and crustaceans have been found to be the most susceptible, with fish being somewhat less susceptible (Rexrode, 1987). The reproductive biology of the European dog-welk, Nucella lapillus, in both laboratory and field studies, may be adversely affected by TBT concentrations as low as 20 ng/L (Bryan et al., 1986). In the Baltic amphipod, Gammarus oceanicus, TBT compounds were slow acting toxicants, but sublethal effects were found at concentrations as low as 300 ng TBT/L (Laughlin et al., 1984). Lethal effects were observed in a copepod, Eurytemora affinis, exposed to concentrations less than 200 ng TBT/L (Hall et al., 1988a). Most notable, and relevant to the present study, sublethal reproductive effects were observed in a mysid, H. costata, at 190 ng TBT/L (Davison et al., 1986). Bushong et al. (1990) have reported that Acartia tonsa nauplii are very sensitive to TBT, at environmentally realistic concentrations, relative to other estuarine organisms. In two 6 day experiments, 23 and 24 ng TBT/L were found to significantly reduce survival relative to the controls (Bushong et al., 1990).

At a number of specific sites in Chesapeake Bay, TBT concentrations have been high enough to cause harmful effects in some aquatic organisms if exposure was continuous (Hall et al., 1987). While acutely toxic effects would be likely only with the more sensitive species of bivalves and zooplankton, potential environmental hazards could occur with various trophic groups after long-term exposures to sublethal concentrations (Hall et al., 1988a). Since TBT is a slow-

acting toxicant at sub-lethal levels, toxicity tests based on short term exposures have failed to indicate some effects on non-target marine organisms (Laughlin & Linden, 1987). Long-term toxicity exposures are essential to assess ecological threats posed to non-target organisms by TBT, and are necessary for the development of water quality criteria (Davidson et al., 1986). Both Hall et al. (1988a) and Rexrode (1987) contend that chronic TBT experiments (full larval or full life cycle exposures) with sensitive aquatic organisms must be conducted. Preferably, these tests should be conducted at environmentally realistic concentrations of TBT in order to provide useful information for writing valid regulations.

MATERIALS AND METHODS

Acute Test

A flow-through exposure system was used to deliver 1 um filtered York River dilution water heated to 27°C. The test incorporated three replicates at each of 5 TBT concentrations, a dilution water control, and a triethylene glycol solvent vehicle control. The TBT concentrations were chosen based on the results of static tests conducted in this laboratory, a preliminary flow-through acute test with M. bahia, and an LC50 value reported in the literature. The nominal concentrations are taken to be the stoichiometrically calculated values based on the stock solution concentrations and measured flow rates. The 5 nominal test concentrations were as follows: 5000, 3000, 1800, 1080, 648 ng TBT/L (dilution factor=0.6).

In a preliminary 96-h acute toxicity test, measured TBT concentrations were low on three days of the test on which samples were taken. Mean values were 641 ng TBT/L (21% of nominal) and 150 ng TBT/L (8% of nominal) at 3000 and 1800 ng TBT/L treatments, respectively. A single dead animal was observed in some replicates after 96 hours, but mortality was not correlated with measured exposure concentration. It was decided to operate the toxicant delivery system continuously for several weeks prior to initiating another test in order to fully saturate the test system and, thus, increase the measured TBT concentrations. The high survival, low percent recovery of TBT from the test system, and the LC50 value reported by Goodman et al. (1988) indicated that a test nominal concentration of 5000 ng/L should be incorporated into the definitive acute test design.

A primary stock solution of tri-n-butyltin chloride (TBTCl, Aldrich) was prepared by dissolving 7.443 mg TBT/ml in acetone. A range of TBT stock

concentrations was prepared by adding an appropriate amount of the primary stock solution to a known volume of triethylene glycol (TEG). Acetone was added to the TEG of the solvent vehicle control stock at a concentration equal to that in the highest TBT stock solution (0.002%). Stock solutions were stored at 4° C and were warmed to room temperature and mixed well before replentishing stock containers which supplied the test system.

The experiment was started by placing neonates (<24-h-old) in exposure chambers after test solutions had been flowing long enough for the concentrations of TBT to have reached steady state. In order to obtain an estimate of equilibration time in the test system, a 16 day equilibration period was allowed prior to the start of this test. These results are discussed below. Neonates were obtained for testing as described in the thermal tolerance test. Water quality measurements (means) in the brood tanks were as follows: temperature 27-28°C; dissolved oxygen 7.1 ppm; salinity 22°/$_{oo}$ (same as culture tanks). Individual mysids were randomly assigned five at a time to replicate test chambers at each TBT test concentration. Three replicates were employed at each test concentration plus controls, and ten mysids were exposed in each. Replicate glass exposure chambers (12x12x15 cm) were randomly assigned to test chamber locations on the water table. Within each exposure chamber, mysids were confined to retention baskets consisting of a cylinder of Nitex screen (202 um), 15 cm high, attached with silicone adhesive to a glass petri dish (10 cm). Exposure chambers were flushed regularly by a tidal flush mechanism which caused the water level to rise and fall.

Dilution water was aerated vigorously in a primary head tank heated (27°C) by an electric immersion heater. Heated dilution water was delivered to a flow-splitting head tank which delivered dilution water at a known rate of flow (range of means = 153-162 ml/min) to glass toxicant mixing boxes (10x12x12 cm). TBT

stock solutions were delivered by peristaltic pump at a known flow rate (range of means= 0.049-0.052 ml/min) to each of five mixing boxes. All treatments except the dilution water control received the same solvent vehicle concentration, 0.32 ml TEG/L. Here stock solutions mixed with inflowing diluent water to produce the desired test concentrations. The test solution in each mixing box was split by means of calibrated pipet tips, and delivered to the three replicate exposure chambers at a constant rate of 50 ml/min. All exposure chambers were located on a water table under conditions of even lighting (approximately 500 Lux) and a 14:10 L:D cycle. During the tests, mysids were fed Artemia nauplii daily in direct proportion to the number of individuals in each retention chamber, but always above a minimum density of 2 nauplii/ml of test water. Since water volume in exposure chambers averaged 600 ml, all replicates were initially fed 1 mL of Artemia feeding suspension daily using an Eppendorf pipet.

Observations on the number of dead and affected animals were made in each replicate at 3, 6, 12, and 24 hours, and twice a day thereafter until the end of the tests. Observations were made by eye under a bright light. Retention baskets were carefully removed from exposure chambers, the water was allowed to drain down to the level of the petri dish, and baskets were replaced as soon as observations were made. Dead animals were removed from the chambers when observed. At the end of the test, the weights of mysids alive in control replicates were measured according to procedures described in the thermal tolerance test. Three or four animals were added to each replicate weigh pan, and since all animals were still immature, they were not separated by sex before weighing.

Temperature, salinity, dissolved oxygen and pH were measured daily in one randomly chosen replicate at each test concentration. Water quality measurements were made concurrently in all replicates at the beginning and end

of the test. Maximum and minimum temperatures were measured daily in a separate exposure chamber receiving dilution water using a max-min thermometer. A randomly chosen replicate at each test concentration was sampled once daily for chemical analysis. Test water was siphoned through pretreated silicone tubing into one liter polycarbonate containers. Duplicate exposure chambers at each test concentration were sampled for chemical analysis once near the beginning of the test. This provided an estimate of the difference in TBT concentration between replicate test chambers.

28 Day Test

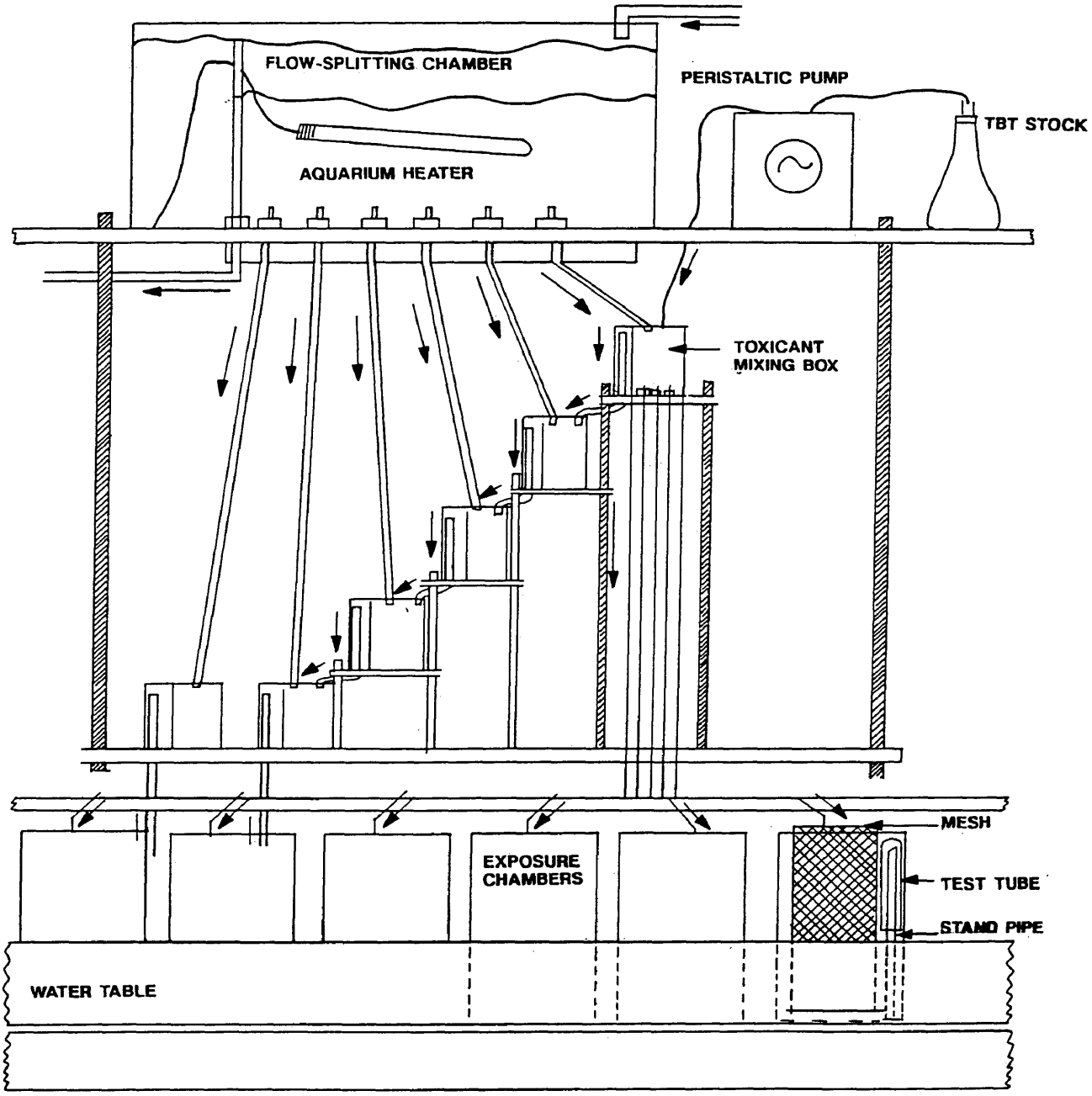
For the life-cycle test, a flow-through exposure system delivered 1 um filtered York River water at four test temperatures. Test temperatures were chosen based on the results of the thermal tolerance test to produce a range of responses in survival and reproductive developement. These temperatures were 20, 23, 26, and 29°C. Inflowing dilution water was heated to the lowest test temperature (20°C) with an electric immersion heater as it passed through a PVC heating chamber. As the experiment progressed and the ambient water temperature rose above 20°C, the inflowing dilution water was diverted to a heat exchanger/chiller. The water was chilled to less than 20°C and then delivered to the heating chamber. The 20°C water entered a primary head tank at the top of the dosing system where it was aerated vigorously. Siphons from this head tank delivered water to four flow-splitting chambers; one for each test temperature (Figure 2). The three remaining test temperatures (23, 26, and 29°C) were produced in separate flow-splitting chambers with submersible electric aquarium heaters. The flow-splitting chamber at 23°C was 50Lx16Wx13H cm and 38 L aquaria (50Lx26Wx30H cm) were used at 26 and 29°C to accommodate the number of

heaters needed to produce these temperatures.

The toxicant delivery system used in this test differed from that used for the acute test. Each of the four flow-splitting chambers delivered test water to six toxicant mixing boxes (Figure 2), one for each of five TBT concentrations and a solvent vehicle control. A total of 24 mixing boxes were used to produce the 5 TBT concentrations plus control at each temperature. The toxicant mixing boxes were on elevated platforms, with the high TBT concentration in the highest position, and mixing boxes at successively lower concentrations at progressively lower elevations. Dilution water was delivered to the highest mixing box (highest test concentration) at each temperature at 497 $\pm$ 19 ml/min (mean $\pm$ standard deviation). Dilution water was delivered to the remaining TBT mixing boxes at each temperature at 252 $\pm$ 10 ml/min (mean $\pm$ standard deviation), and to the solvent vehicle control mixing boxes at each temperature at 310 $\pm$ 8 ml/min (mean $\pm$ standard deviation). Since solvent vehicle control mixing boxes delivered water to the max/min thermometer chambers, they received an additional 50 ml/min of dilution water. The mean flow rates were calculated from delivery rates to all mixing boxes measured several times during the test.

The toxicant mixing boxes formed a gravity driven serial dilution apparatus. Overflow standpipes formed a conduit to transfer test water between the mixing boxes (Figure 2). The 500 ml of dilution water entering the uppermost mixing box at each test temperature was split. Approximately 250 ml/min of the test water was delivered to the replicates at the highest concentration (50 ml/minute to each of five replicates), while approximately 250 ml/min overflowed to the next mixing box. In each of the remaining TBT mixing boxes in the dilution series, excluding the solvent control, water from the preceeding box (250 ml/min overflow) mixed with incoming dilution water (250 ml/min) to produce the next

Figure 2. Diagram of serial diluter apparatus for 28 day test.


FLOW-SPLITTING CHAMBER
PERISTALTIC PUMP
TBT STOCK
AQUARIUM HEATER
TOXICANT
MIXING BOX
MESH
EXPOSURE
CHAMBERS
TEST TUBE
STAND PIPE
WATER TABLE

test concentration in the dilution series (0.5 dilution factor).

The TBT treatment water produced in each mixing box was divided into outflow streams by calibrated, plastic pipet tips, each delivering test water at 50 ml/min to one of five replicate exposure chambers. The Nitex retention baskets and glass exposure chambers employed were the same as those described for the acute test. The experiment was a stratified random design of temperature and TBT concentration, since all exposure chambers at each temperature were grouped together on the water table and constituted one of the four strata. The five replicates at each TBT concentration plus controls were randomly assigned to a test chamber location within each strata. This test design was necessary to avoid temperature regulation problems associated with a completely randomized design.

Test material concentrations were chosen as follows. First, the highest concentration of test material was set equal to the lowest concentration that caused adverse affects on survival in the acute test (ASTM designation E 1191-87). Since no test concentrations produced partial mortality in the acute toxicity tests, the arithmetic mean of the dose that caused 100% mortality (1487 ng TBT/L) and the dose that caused no mortality (391 ng TBT/L) was used as the highest test concentration. The mean of these two doses was 939 ng/L. A second estimate was based on acute:chronic ratios (ACR's). Since no chronic toxicity information exists for M. bahia and TBT, an ACR of 3.0 for TBT with a mysid, Holmesimysis costata, of comparable sensitivity (Davidson et al., 1986) was used. Although it would have been more appropriate to use the geometric mean (LC50) for the calculation (Stephan, 1977), results from the acute test (arithmetic mean of 939 ng TBT/L) with M. bahia were divided by the ACR to yield an estimate of the maximum acceptable toxicant concentration (MATC) for the chronic test

(ASTM designation E 1191-87). Test concentrations were chosen to bracket this estimate, and this method produced an estimate of the highest test concentration (935 ng TBT/L) similar to that produced by the arithmetic mean method.

The nominal test concentrations employed in this test were as follows: 939, 470, 235, 117, and 59 ng TBT/L. The stock solution delivered to the dilution system was prepared by adding 6.28 ml of 7443 mg/L TBTCl primary stock (in acetone) to 4000 ml triethylene glycol. The resulting 11.7 mg/L stock solution was delivered by peristaltic pump to the first mixing box (highest test concentration) in the dilution series at each temperature at 0.04 $\pm$ 0.01 ml/min (mean $\pm$ standard deviation). The TEG stock for the solvent vehicle control, prepared as described for the acute tests, was delivered to the control mixing box at each temperature at 0.04 $\pm$ 0.01 ml/min (mean $\pm$ standard deviation). The solvent vehicle concentration was less than 0.1 ml/L of dilution water at each test concentration. The stock solution supplying the dosing system was replenished every third day during the test. On days 7 and 23 of the test, new stock solution for delivery to the dilution system was prepared as described above. The stock was stored at 4°C and was warmed to room temperature and mixed well before use.

Juveniles were produced for testing as described in the thermal tolerance test with a few modifications. Juveniles were <60-h post-release at the start of the test. This lengthy brood release period was necessary in order to obtain enough juveniles for all replicate test chambers; 960 neonates in 120 replicates. All brood tanks were fed rotifers (Brachionus sp.) liberally during the brood release period but no Artemia. The salinity of the brood tanks was adjusted to the salinity of the mysid cultures (20 $^{\circ}/_{oo}$) with artificial sea salt brine solution before animals were added to brood tanks. The brood tank temperatures were generally 26.0-27.0°C during the 60-h brood release period but may have been slightly higher for short

periods since the maximum recorded laboratory air temperature was 29.2°C during this period. Neonates were held in an incubator at 25 ± 0.5°C and 18° /$_{oo}$ S (York River ambient salinity) prior to starting the tests.

The test was initiated by placing animals in retention baskets after TBT concentrations had reached steady-state. Based on TBT concentrations measured during the equilibration period prior to the acute test, one week was deemed appropriate for equilibration. Eight juvenile mysids were randomly assigned four at a time to replicates at each TBT-temperature treatment. After juveniles had been placed in the test system, adjustments from brood release temperatures to test temperatures did not exceed 3°C in a 12-h period.

Once daily, mysids were fed live brine shrimp nauplii. As recommended by McKenney (1987), the density of Artemia was greater than 2 nauplii/ml of test solution at all mysid densities. Counts of Artemia feeding suspensions indicated that there were 2,720 ± 760 nauplii/ml (mean ± standard deviation, N=11). For the first eight days of the test, mysids in all replicates were fed a mixture of 1 ml Artemia (24-h-old) suspension and 0.5 ml of rotifer (Brachionus sp.) suspension as a supplement; the various volumes were produced with an Eppendorf pipet. For the remainder of the test, animals were fed only Artemia. Replicates received between 0.57 and 1 ml of Artemia suspension once daily, depending on mysid density, for a concentration of approximately 3-4 nauplii/ml. Dead brine shrimp and other debris were removed from exposure baskets when daily observations were made.

The primary data collected in these life cycle tests were, a) survival of first generation mysids; b) time required to reach certain reproductive and developmental stages; c) the number of live young produced; and d) mean dry weights of all first-generation mysids alive in each test chamber at the end of the

test. Live mysids in each exposure chamber were counted every other day to account for deaths as a result of treatment exposure. The criteria for determination of death were immobility (absence of movement of respiratory and feeding appendages), opaque white coloration, and a lack of reaction to gentle prodding. Dead mysids were counted and removed when observed. Observations on stage of development and reproductive output were also made every other day. These observations included the number of days to appearance of first brood in the marsupia of females, days to first brood release in each replicate, and the number of live young released in each replicate. For each replicate, the day of appearance of the first brood was the first day on which any female in the replicate reached this developmental stage.

To make observations, retention baskets were carefully removed from exposure chambers, and water was allowed to drain down to the level of the petri dish. Baskets were placed on a light table, six at a time, for observations. All baskets were returned to the appropriate replicate exposure chamber before the next set was removed for observation. Both first and second generation mysids were observed during the test for signs of abnormal development and aberrant behavior. On the final day of the test, live second generation mysids in each chamber were examined under a dissecting microscope to determine sex, number of immature animals, presence or absence of eggs in the oviducts or brood sacs of females, and any obvious morphological abnormalities were noted. The live second generation mysids in each replicate were weighed as a measure of growth according to the procedures described in the thermal tolerance test.

Throughout the test, maximum and minimum temperatures were measured daily with max/min thermometers placed in a 2 L flow-through container at each test temperature. Thermometers used to make temperature measurements were

standardized against a thermometer marked in 0.1°C increments (0.05% accuracy, Fisher Scientific). On a daily basis, temperature (estimated to tenths of a degree) and dissolved oxygen were measured in two randomly selected exposure chambers at each TBT-temperature treatment plus controls; pH was measured in one randomly chosen replicate daily. Temperature was measured concurrently in all exposure chambers on a weekly basis. Flow rates from the toxicant mixing boxes to all exposure chambers were checked several times during the experiment using a graduated cylinder. Salinity was measured daily in a few randomly selected exposure chambers.

The soundness of the diluter and toxicant delivery system was checked daily, and all pipet tips were cleaned with monofilament and/or pipe cleaners at least every other day in order to maintain the desired flow rates. Whenever there was an interruption of flow in the toxicant delivery system, corrective action was taken, and water quality measurements were made in at least some of the affected replicates.

On day one of the test, water samples were taken from a randomly chosen replicate at nominal concentrations of 59, 235, 939 ng/L, and a control, at each test temperature. Thereafter, water samples were taken for TBT analyses once a week throughout the test from randomly chosen replicates at the same nominal concentrations. This sampling scheme was chosen with the assumption that the variability of the toxicant delivery system was kept at a minimum through cleaning and daily checks of the system. During sampling, test water was siphoned through a preconditioned glass and silicone siphon tube into a 1 liter polycarbonate container. Immediately after collection, samples were acidified to below pH 2 with concentrated HCl. On the last two sampling days (days 22 and 28), glass solvent bottles were used to collect water for TBT analyses. Previous TBT

analyses had indicated minimal adsorption to either polycarbonate or glass following extraction with hexane.

Analytical Methods

Water samples were analyzed for tributyltin using the methods of Unger et al. (1986). This method for analyzing butyltins relies on derivatization by Grignard reaction to produce tetraalkyl tin compounds. Each water sample (pH<2) was added to a separatory funnel and spiked with tripentyltin chloride (Pe_3SnCl) in ethanol (internal standard) at the anticipated measured concentration. The sample volume for extraction was 500 ml for the acute test (a 1 liter sample was shaken and half was used for analysis) and 1000 ml for the 28 day test. Each sample was extracted with three 40 ml aliquots 0.2% tropolone in n-hexane and the combined extract was reduced to 10 ml by rotoevaporation.

The extracts were transferred to 50 ml centrifuge tubes, the lower aqueous layer removed, and the extracts were frozen to assist with water removal. The extract was derivatized with excess n-hexyl magnesium bromide (0.5 ml of 2.0 M) for 30 minutes, during which time it was agitated every five minutes. The tube was acidified with concentrated HCl, shaken and vented three times, and allowed to sit for 30 minutes. The lower aqueous layer was discarded, and the derivative-containing organic phase was reduced to 1 ml under dry N_2. The extract was transferred to a chromatographic column containing 20 g of activated Florisil (100-200 mesh) with 2 g of anhydrous Na_2SO_4 added to the top. The sample was eluted with 300 ml hexane which was then rotoevaporated and reduced under dry N_2 to 0.1-0.55 ml. Blanks consisting of deionized water and 2 ml of concentrated HCl were run concurrently with each set of sample extractions; blanks were extracted and put through the same procedure as were the samples.

The hexyl-organotins resulting from derivatization were quantified by gas chromatography (Varian) with flame photometric detection (GC/FPD). The column for most of the sample injections was a 20 m glass capillary (0.32 mm I.D.) coated with 1 um SE-52, and immobilized with dicumyl peroxide; the carrier gas was helium. A few of the injections were made to a 30 m fused silica capillary (0.32 mm I.D.) coated with 1 um DB5. The detector and injection port temperatures were 280°C, and the column oven temperature was programmed from 135° to 300°C at 10°C/min. Detector output was collected on an HP laboratory automation system. Butyltin peak areas were integrated and quantified by response relative to that of the internal standard. The integrator reported TBT and DBT concentrations as the cations, and TBTCl and DBTCl concentrations were calculated by adjusting the molecular weight from ion to chloride.

Unger (1988) addressed questions concerning the accuracy and precision of the analytical methods employed. Unger added TBT to replicate samples of York River water at a concentration of 7.9 ng TBT^+/L (N=9). The mean ($\pm$ standard deviation) measured concentration was 7.4$\pm$0.66 ng TBT^+/L; a mean recovery of 94%. The coefficient of variation for these analyses was 0.09, indicating the reliability of the methods employed. Unger stated that the high percent recovery indicated that the detection limits of the flame photometric methods were less than 10 ng TBT/L.

Data Analysis

Survival results are reported as proportional survival, where 1.0 is equal to 100% survival. Proportional mortality data from the acute toxicity test were used to calculate a 96-h median lethal concentration (LC50) and its 95% confidence limits. The binomial test described by Stephan (1977) was the statistical method

employed. For the 28 day test, proportional mortality data at each test temperature was used to determine a 28 day LC50. Data was subjected to probit analysis using the SAS statistical package (SAS Institute Inc., 1985).

Both regression analysis and ANOVA (SPSSx, 1986) were used to analyze data from the 28 day interaction experiment. Stepwise multiple regression (SPSSx, 1986; Sokal & Rohlf, 1981) was used to examine biological effects (i.e. survival, time to reach various developmental stages, reproductive output) in response to exposure to TBT-temperature treatments. Data were regressed on the quadratic function of temperature and TBT:

$$Y = b_o x_o + b_1 C + b_{11} C^2 + b_2 T + b_{22} T^2 + b_{12}(C*T)$$

where Y = the estimated biological response, $b_0 x_0$ = the intercept, C and T = the linear effect of TBT concentration and temperature, C^2 and T^2 = the quadratic effect of TBT concentration and temperature, C*T = the linear by linear interaction between TBT concentration and temperature, and b_0, b_1, etc. = the regression coefficients. Terms were added to the stepwise regression model one at a time, starting with the term explaining most of the variance (SPSSx, 1986). Terms which did not contribute significantly to the regression model were not included in the final predictive equation. Residuals were checked to see that normality and homoscedasticity assumptions had been met (SPSSx, 1986; Sokal & Rohlf, 1981), and data were transformed when necessary (Sokal & Rohlf, 1981).

One-way ANOVA was used to examine survival, growth, stage of development, and reproductive output data as a function of either TBT concentration or temperature (SPSSx, 1986). These analyses were followed by either the Student-Newman-Keuls or Dunnett's multiple comparison procedures when significant

differences were found (Zar, 1984). The predicted power of hypothesis tests was shown to be low when applied to data of the thermal tolerance test, and, therefore, it was concluded that use of hypothesis tests should be avoided. However, the power of parametric tests was acceptable and homoscedasticity assumptions were met when hypothesis tests were applied to data from the present test. Data were transformed prior to analysis when required (Sokal & Rohlf, 1981). A nonparametric one-way ANOVA (Kruskal-Wallis) was tested as an alternative to parametric ANOVA whenever homogeneity of variance tests indicated that the data were not homoscedastic (Zar, 1984). When significant differences were found, these tests were followed by an appropriate nonparametric multiple comparison procedure (Zar, 1984).

In order to normalize reproductive output data, the number of offspring per female reproductive day (FRD) was calculated according to the procedures presented by Gentile et al. (1982a, 1983). In each replicate, the cumulative number of young released was divided by the product of the number of mature females in the replicate and the number of reproductive days available to the replicate. The number of reproductive days were those between the day of first brood release and the end of the test (ASTM designation E 1191-87), or the day on which no more mature females were found in the replicate. Calculating the number of offspring per FRD provides an index that corrects for the number of mature females per replicate and the number of days those females were alive in each replicate.

RESULTS

Acute Test

Water Quality

The mean measured temperatures in all treatments were close to the test temperature of 27°C (Table 3). Average maximum and minimum temperatures were within 1.1°C of the test temperature (Table 3). The range in the maximum and minimum values is very likely due to the diurnal variation in temperature of the inflowing York River water.

In general, water quality was similar among treatments (Table 3). Within TBT and control treatments, concurrent water quality measurements at 0- and 96-h indicated that variability among replicates was small. The mean pH values are within acceptable limits for toxicity testing with M. bahia. The mean pH for each treatment was close to 7.7, which is the lower end of the optimal range in pH recommended by ASTM (designation E 1191-87). The mean dissolved oxygen concentrations remained above 80% of saturation in all treatments during the test (percent saturation was calculated from measured D.O. values with a program that compensates for temperature and salinity). However, dissolved oxygen was lower for TBT treatments than for the control treatments and may indicate some response of the mysids to TBT exposure. The mean flow rates to replicates at each treatment varied by no more than 4 ml/min.

TBT Concentrations

The mean measured TBT concentrations were generally only a small percentage of the nominal concentration for each treatment (13-30% of nominal), and TBT concentrations did not increase significantly from the beginning to the end of the test (Table 4). Samples for the 648 ng TBT/L nominal

Table 3. Water quality measurements for 96-h acute test. Time-weighted mean values with range in parentheses.

Nominal Conc. (ng/L)	Temperature (°C)	Diss. Oxygen (mg/L)	pH	Flow Rate (ml/min)
Control	27.3 (26.6-28.0)	6.67 (6.45-6.90)	7.76 (7.69-7.81)	47 (45-52)
Solvent Control	27.0 (26.7-27.2)	6.49 (6.35-6.60)	7.71 (7.65-7.79)	48 (45-51)
648	27.1 (26.8-27.4)	6.26 (5.80-6.60)	7.67 (7.62-7.73)	49 (44-52)
1080	27.2 (26.8-27.8)	6.17 (5.95-6.40)	7.66 (7.61-7.71)	49 (42-53)
1800	27.5 (27.0-28.0)	6.14 (5.95-6.40)	7.65 (7.59-7.72)	45 (40-49)
3000	27.5 (26.9-28.2)	6.15 (5.90-6.55)	7.64 (7.61-7.68)	47 (40-52)
5000	27.2 (26.8-28.0)	6.16 (5.95-6.40)	7.65 (7.61-7.70)	47 (38-52)

Maximum/Minimum Temperature (°C): 28.1/25.9 (27.5-28.6/25.3-26.4)

Salinity ($^{o}/_{oo}$): 22 (22-23)

Table 4. Measured TBT concentrations (ng/L) for 96-h acute test. ND means not detectable.

Nomimal Conc. (ng/L)	Internal Standard Conc. (ng/L)	Hours 0-h	24-h	48-h	72-h	96-h	Mean	SD	CV
Control	47	ND		ND		ND	ND	---	---
Solvent Control	47	ND		ND		ND	ND	---	---
1080	553	237 232	173	148	121	234	191	50	0.26
1800	948	212 236	227	245	203	267	232	23	0.10
3000	1500	381 407	275	310	430	543	391	96	0.24
5000	2530	823 1016	1101	3402	1055	1578	1496	967	0.65

concentration were not analyzed due to the low percent recovery in the test, lack of biological effects, and additional cost of the analyses. Despite this, measured TBT concentrations in duplicate exposure chambers at each treatment (day 0) differed by only 2-14%. While this estimate of the variability in TBT concentration between replicates is based on a limited number of samples, it does not point to major discrepancies. Repeat GC injections varied by 2-11% for a few samples which were injected twice, indicating an acceptable precision for the GC/FPD analyses. Except at the 5000 ng TBT/L nominal concentration, the coefficient of variation for the measured TBT concentrations was acceptable (<0.30). The high measured concentration at 48 hours for the 5000 ng TBT/L treatment resulted in an unusually large CV for this treatment (Table 4).

The average dibutyltin (DBT) concentrations for the 3000 and 5000 ng TBT/L treatments were 79 and 126 ng DBT/L, respectively. These DBT concentrations are a small percentage of the measured TBT concentrations for these treatments ($<20\%$), indicating that there was no excessive degradation of TBT to DBT either during the tests or during sample storage.

Water was sampled from one replicate at the 3000 ng/L treatment on three separate days during the 16 day equilibration period prior to the acute test. These analyses indicated that measured TBT concentrations were as high after eight days (335 ng TBT/L) as they were after fourteen days (300 ng TBT/L). Therefore, an eight day equilibration period would be suitable for future tests. The low percent recovery of TBT from the test system was a problem which would have to be addressed before further tests were conducted.

Mortality

Proportional mortality was 0.03, 0.03, 0.0, and 0.97 at measured exposure

concentrations of 190, 232, 391, and 1487 ng TBT/L, respectively. The high proportional mortality at the highest exposure concentration may be closely correlated with a period during the test when the measured TBT concentration increased to approximately 3,400 ng TBT/L (Table 4). Results from control treatments were pooled for statistical analysis as there was no mortality in either the dilution water control or the solvent vehicle control. An approximate LC50 of 816 ng TBT/L was obtained by nonlinear interpolation between measured concentrations of 391 and 1497 ng TBT/L (Stephan, 1977). The binomial test indicates that 391 and 1487 ng TBT/L can be used as statistically sound, conservative 95% confidence limits because the actual confidence level associated with these limits is greater than 95% (Stephan, 1977).

Dry weight measurements of animals from the dilution water control replicates yielded a mean dry weight of 128 ug per animal (N=9), with a standard deviation of 18 ug and a range of 97-152 ug per animal.

28 Day Test

Water Quality

The time-weighted mean measured temperatures were within 0.3°C of each test temperature (Table 5). Although the range of measured temperatures at each test temperature included values that were more than 1°C from that test temperature, overall, temperatures did not vary unacceptably. The standard deviation reported for each time-weighted mean measured temperature is much smaller than the range (Table 5). In general, extremes in measured temperatures at all test temperatures were infrequent, were usually short in duration, and were not indicative of all replicates on a given day at a given temperature. The summary statistics for measured temperatures (Table 5) do not include days 0

Table 5. Water quality measurements for 28 day test.' Time-weighted means with the range in parentheses. The mean ± standard deviation are reported for temperature measurements.

Test Temp. (°C)	Temp. (°C)	Maximum Temp. (°C)	Minimum Temp. (°C)	Dissolved Oxygen (mg/l)	pH	Flow Rates (ml/min)
20°C	20.3 ± 0.6 (19.0-21.5)	21.4 ± 0.7 (20.5-23.0)	19.2 ± 1.0 (17.1-21.0)	6.40 (5.10-7.60)	7.76 (7.46-8.01)	51 (38-60)
23°C	22.9 ± 0.5 (21.8-23.8)	23.8 ± 0.5 (22.7-24.6)	21.7 ± 0.8 (20.0-23.0)	6.11 (5.05-7.10)	7.75 (7.48-7.98)	49 (40-58)
26°C	26.0 ± 0.6 (24.5-27.2)	26.9 ± 0.5 (26.0-27.8)	24.7 ± 1.1 (22.8-26.4)	5.72 (4.50-6.50)	7.73 (7.47-7.98)	50 (38-60)
29°C	28.8 ± 0.7 (27.2-30.2)	29.8 ± 0.6 (28.4-30.6)	27.7 ± 1.0 (25.1-29.0)	5.35 (4.00-6.30)	7.73 (7.48-7.95)	51 (44-60)

Mean Salinity: 17 °/oo (15-18 °/oo)

through 3 of the test when temperatures were still being adjusted and test temperatures had not yet been established.

At each test temperature, the time-weighted mean maximum and minimum values were within 1.4°C of the test temperature (Table 5). Although the range of both the maximum and minimum values at each test temperature had some extreme values, the standard deviation of the maximum and minimum temperatures was between 0.5 and 1.1°C at each test temperature. Other than diurnal temperature variability in the inflowing York River diluent water, only interruptions in the toxicant delivery system produced temperature variation in the test system. The soundness of the test system (heaters, water levels) was monitored continuously by electrical alarm switches, and therefore, interruptions were known to be infrequent. However, the submersible heaters were not efficient enough to completely compensate for changes in ambient water temperature. The extreme values in the range of measured maximum and minimum values were probably related to extremes in the diurnal temperature cycle of the York River.

On days 9, 16, and 28 of the test, temperature was measured concurrently in all replicates at each test temperature. The mean measured temperatures were close to the desired test temperatures on each day that concurrent measurements were taken. The variability between replicates was low (Table 6). Replicates at 20°C did not differ by more than 0.8°C on any of the three days. Replicates at 23°C were within 1°C of each other, except on day 9, when one replicate increased the range to 1.1°C. Replicates at 26 and 29°C were generally within 1°C of each other. However, on day 9 at 26°C and day 16 at 29°C, a few replicates increased the range to 1.2°C at both temperatures. The only major inconsistency was on day 9 at 29°C when a few replicates increased the range of

Table 6. Concurrent temperature measurements (mean ± standard deviation) in all replicates during 28 day test with range in parentheses.

Test Temp. (°C)	Day 9	Day 16	Day 28
20°C	20.1 ± 0.2 (19.8 - 20.4)	19.4 ± 0.1 (19.0 - 19.5)	20.5 ± 0.2 (20.4 - 21.2)
23°C	23.2 ± 0.3 (22.5 - 23.6)	22.3 ± 0.2 (21.6 - 22.5)	22.6 ± 0.1 (22.5 - 22.8)
26°C	26.4 ± 0.4 (25.8 - 27.0)	25.5 ± 0.2 (25.2 - 26.0)	25.0 ± 0.2 (24.6 - 25.2)
29°C	29.3 ± 0.6 (27.6 - 30.4)	28.7 ± 0.4 (28.2 - 29.4)	29.0 ± 0.3 (28.5 - 29.5)

temperatures to 2.8°C.

The time-weighted mean pH values were within the optimum range recommended by ASTM (designation E 1191-87) and were similar between test temperatures (Table 5). However, the range of pH values was roughly 0.5 at each test temperature and fell below 7.70, the low end of the optimal range. The significance of this variability was probably minimal as the low pH values were not persistent on successive days of the test, and were uniform across TBT treatments and test temperatures. Although the range of pH includes values below 7.5 at each temperature, at 20°C, the mean pH fell below 7.6 on only three days during the test. Similarly, the mean pH was below 7.6 on only two days at 23°C, and on only one day at 26°C and 29°C.

The time-weighted mean dissolved oxygen concentration decreased with increasing test temperature, but the mean dissolved oxygen levels at each test temperature were well above 60% of saturation (Table 5) (percent saturation was calculated from measured dissolved oxygen values with a program that compensates for temperature and salinity). Some of the measured dissolved oxygen concentrations in replicates at 29°C fell below 60% saturation, including the 4.00 mg/L value at the lower limit of the range of measured values. In a few isolated instances, very low dissolved oxygen levels such as those at 29°C were related to obstructions in the water delivery lines. This occurred no more than two or three times at any test temperature, and only one replicate was affected at a time. Because these low dissolved oxygen concentrations were attributable to a short-term problem which was corrected as soon as it was observed, the extreme values are not reported in Table 5.

The means of the measured flow rates to replicate exposure chambers at each test temperature were very close to the calibrated value of 50 ml/min (Table 5). However, the range of flow rates at each temperature included values which

varied from the mean by more than the maximum of 10% recommended by ASTM (designation E 1191-87). This occurred infrequently, and the standard deviation was only 3-4 ml/min at each temperature. The variability in flow rates was kept to a minimum by daily checks of the diluter system and by cleaning the pipet tips frequently.

Extreme Water Quality Measurements

Extreme values of water quality parameters were not persistent in any replicate exposure chamber, and were generally related to specific problems which were corrected when they were identified. On any given day during the test, and over the course of the experiment, the variability in water quality parameters among TBT treatments at a given temperature was small compared to the differences between test temperatures.

On day 18 of the test, chilled water delivery to the test system was initiated because the ambient water temperature had risen above 20°C. The increased ambient temperature accounted for a general rise in temperature of replicates at 20°C after 18 days, and the high mean maximum temperature (21.4°C) at this test temperature (Table 5).

A major interruption in the toxicant delivery system occurred on day 11 when the water delivery system failed during the night. Since water delivery was stopped by an electronic safety valve, animals were not exposed to severe changes in temperature. Water delivery to replicates at each test temperature was reestablished within an hour. After eight hours, the temperatures in the max./min. thermometer chambers were 18, 19.5, 22, and 25.5°C at test temperatures of 20, 23, 26, and 29°C, respectively. Test temperatures were not reestablished for 12 hours due to the time required for the temperatures to

stabilize in the flow-splitting chambers where water was exchanged rapidly. The remaining water quality parameters were normal at all test temperatures following this interruption.

While not all the extreme water quality values reported in Table 5 can be related to a specific interruption in the water delivery system, blocked toxicant delivery lines were observed on days 13, 15, 18, 19, 27, and 28. Only one replicate was usually affected, usually resulting in extreme temperature and dissolved oxygen values. Problems with toxicant delivery were corrected when observed, and, therefore, test animals in affected replicates were exposed to extreme conditions for less than 24-hrs in all cases.

TBT Concentrations

TBT concentrations were measured at each test temperature on days 1, 8, 15, 22, and 28 (Table 7). The mean measured concentrations were 63, 63, and 57% of nominal at the 59, 235, and 939 ng TBT/L treatments, respectively. At each nominal concentration, the mean measured concentration at each test temperature was close to the internal standard concentration (the predicted measured concentration). Coefficients of variation (CV) were acceptable, generally <0.30 for measured TBT concentrations at each treatment. However, at the 59 ng TBT/L treatment, the 0.42 CV at 20°C was abnormally high. This was apparently the result of low measured concentrations on days 15 and 22 for this treatment. This variability may represent the limitations of the peristaltic pump to deliver the TBT stock solutions at a consistent rate. The mean measured TBT concentration was above 5 ng/L for solvent vehicle control treatments at 20 and 26°C. At both temperatures, this seems to be the result of unusually high measured concentrations on day 15 (Table 7), and is not

Table 7. Measured TBT concentrations (ng/L) for 28 day test.
ND means not detectable.

Nominal Conc. (ng/L)	Internal Standard Conc. (ng/L)	Test Temp. (°C)	Day 1	8	15	22	28	Mean	SD	CV
Solvent Control	21	20	12	ND	20	ND	ND	6	9	----
		23	8	ND	ND	ND	ND	2	4	----
		26	8	8	27	ND	ND	9	11	----
		20	ND	ND	17	ND	ND	3	8	----
59	35	20	39	26	15	13	26	24	10	0.42
		23	42	56	38	34	46	43	8	0.19
		26	62	33	65	31	56	49	16	0.33
		29	45	32	32	19	33	32	9	0.28
235	141	20	153	161	92	132	232	154	51	0.33
		23	159	141	155	207	162	165	25	0.15
		26	138	105	185	126	143	139	29	0.21
		29	116	111	156	130	152	133	20	0.15
939	561	20	574	500	597	536	380	517	85	0.16
		23	488	620	454	587	538	537	68	0.13
		26	616	421	456	636	665	559	112	0.20
		29	429	517	530	590	619	537	74	0.14

indicative of uniformly high TBT levels in control replicates at these test temperatures. The most likely explanation is accidental contamination from other replicates while making observations.

During a preliminary chemical equilibration test with the toxicant delivery system, replicate exposure chambers were sampled after 7 days at each of two TBT concentrations to assess the consistency between replicates, and the soundness of the dosing system. Mean measured concentrations were 62% and 61% of the nominal concentrations of 235 and 470 ng TBT/L. The measured concentrations of replicates differed by only 4% at 235 ng TBT/L, and by only 6% at 470 ng TBT/L. A number of the samples listed in Table 7 were injected twice for GC analysis. The reported concentrations of repeat injections differed by a mean of 7% (range 4-11%, N=6), indicating an acceptable precision with regard to the GC/FPD analyses.

The mean DBT concentrations for the control, 59, 235, and 939 ng TBT/L nominal concentrations were 2, 10, 27, and 67 ng DBT/L, respectively. While measured DBT increased with increasing TBT concentration, these results show that the relative DBT concentration decreased with increasing TBT concentration. The measured DBT concentration did not seem to change significantly over time (28 days) at any TBT treatment, and was not influenced by test temperature.

Mean predicted TBT concentrations for each TBT treatment were estimated from regressions of nominal vs. measured concentrations (Table 8), and were used for subsequent statistical analysis. Regressions were performed separately for each sampling day using the measured concentrations for all TBT treatments and test temperatures on that day (Table 7). The power of each of these regressions was greater than 0.99 (Zar, 1984). Predicted TBT concentrations were calculated from the regression equations for each sampling day (Table 8). The mean

Table 8. Predicted (Y) TBT concentrations (ng/L) for 28 day test from regressions of nominal vs. measured concentrations. B = intercept, A = regression coefficient.

Nominal Conc. (ng/L)	Day 1	8	15	22	28	Mean	SD
0	11	3	15	0	12	8	6
59	43	35	45	33	46	40	6
117	76	67	77	70	80	74	5
235	141	131	139	144	149	141	7
470	269	259	263	292	284	273	14
939	527	514	511	588	556	539	33
B =	11.23	2.87	14.62	-4.26	12.31		
A =	0.552	0.547	0.531	0.634	0.582		
r =	0.968	0.966	0.968	0.98	0.931		

predicted concentrations for the 59, 235 and 939 ng TBT/L nominal concentrations were very close to the mean measured concentrations reported in Table 7. The mean predicted concentration for the solvent vehicle controls is 8 ng TBT/L, which is higher than 3 of 4 mean measured concentrations for the controls (Table 7). This predicted solvent control TBT concentration is an artifact of the regression methods employed, and is not indicative of unacceptably high TBT concentrations in control treatments.

Survival

The day 28 proportional survival data (Figure 3, Table 9) were subjected to step-wise multiple regression analysis to test for significant trends in the data with respect to TBT concentration and temperature. Proportional survival data from day 28 were transformed using the arcsine of the square root prior to the analysis. Overall, the r^2 value for the multiple regression was small, indicating that the trends in the data were not adequately described by any combination of the independent variables. However, the C*T interaction term was significant, and was the most predictive component, followed closely by the quadratic effect of temperature (T^2) (r^2= 0.235, F=17.94, P=.000). Due to the poor fit of the model to the data, a multiple regression equation is not presented. Examination of residuals indicated that the data did not violate homoscedasticity or normality assumptions of multiple regression.

The effect of temperature and TBT on day 28 proportional survival is displayed in Figure 3 and Table 9. In general, survival was higher at the two lowest temperatures (20 and 23°C) than at the two higher test temperatures (26 and 29°C). At 20 and 29°C, the downward slope of the lines with increasing TBT concentration indicate that there was an effect of TBT on survival. Significant

Figure 3. Effect of TBT concentration on day 28 proportional survival at each test temperature. (n= 5, see Table 9 for errors associated with data points)

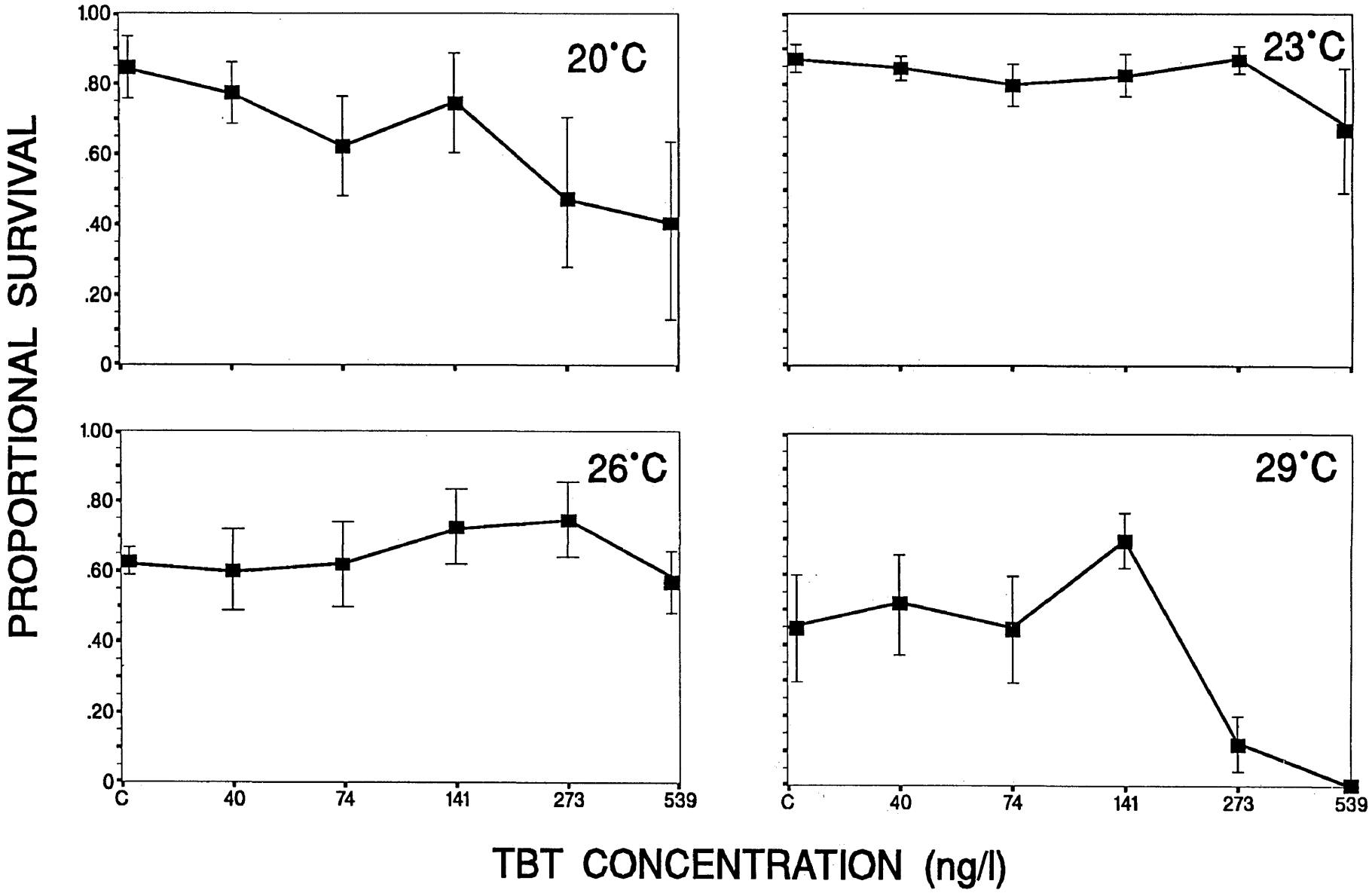

PROPORTIONAL SURVIVAL
1.00
.80
.60
.40
.20
0
20°C
23°C
26°C
29°C
C
40
74
141
273
539
TBT CONCENTRATION (ng/l)

Table 9. Day 28 mean proportional survival as a function of TBT concentration at each test temperature. Standard error in parentheses.

Temperature (°C)	TBT Concentration (ng/L)					
	Solvent Control	40	74	141	273	539
20	0.85 (0.09)	0.78 (0.09)	0.63 (0.13)	0.75 (0.13)	0.48 (0.20)	0.40 (0.24)
23	0.88 (0.04)	0.85 (0.03)	0.80 (0.06)	0.83 (0.06)	0.88 (0.04)	0.68 (0.17)
26	0.63 (0.04)	0.60 (0.11)	0.63 (0.12)	0.73 (0.10)	0.75 (0.11)	0.58 (0.09)
29	0.45 (0.14)	0.53 (0.13)	0.45 (0.14)	0.70 (0.08)	0.13 (0.08)	0.00 (0)

regressions were found at both 20°C (r^2=0.160) and 29°C (r^2=0.395) using data transformed by the arcsine of the square root, although coefficients of determination were low. As stated above, multiple regression analysis indicated that the interaction of TBT concentration and temperature had a significant effect on survival. This is seen graphically (Figure 3) at 20 and 29°C by the pattern of higher survival in the control and low TBT concentrations and the progressively lower survival at high TBT concentrations. In contrast, TBT concentration had little effect on proportional survival at either 23 or 26°C. The statistically determined interaction effect is supported by the fact that the most significant TBT effects were seen at the extremes of temperature.

The significant quadratic effect of temperature on day 28 survival can also be seen in Figure 3. Survival was highest at 23°C at all TBT treatments, and decreased at both higher and lower temperatures. In particular, survival was decreased at the higher TBT treatments at 20 and 29°C as was mentioned previously. An unexplained peak in survival of 0.70 was seen at 141 ng TBT/L (Figure 3), but survival then decreased sharply to 0.0 at 539 ng TBT/L. Additionally, a small peak in survival is seen at the 141 and 273 ng TBT/L treatments at 26°C (Figure 3). These peaks may be indicative of an effect of TBT on detrimental microbial populations which caused a general reduction in control survival at these temperatures. The TBT levels were not high enough to interfere with mysid survival, but may have had an effect on the more sensitive microbial populations present.

Probit analysis was used to calculate 28 day median lethal concentrations (LC50's) for proportional mortality data derived from proportional survival data at each test temperature. The effect of TBT was examined separately at each test temperature. At 20°C, the 28 day LC50 was 472 ng TBT/L, with upper and lower

95% fiducial limits of 287 and 1516 ng TBT/L. The data at 23, 26, and 29°C did not meet the assumptions of the probit method, so estimates of the LC50 were not calculated at these temperatures. No TBT treatment produced mean proportional mortality greater than 0.50 at either 23° or 26°C. However, proportional mortality at 539 ng TBT/L was 0.32 and 0.42 at 23 and 26°C, respectively. These relatively low mortality rates at the highest TBT concentration indicate that the LC50's at these temperatures lie above 539 ng TBT/L. Proportional mortality was not strictly concentration dependent at 29°C, and, therefore, the data are not suitable for probit analysis. However, there was no survival in the 539 ng TBT/L treatment at 29°C. Based on a comparison of proportional mortality, the LC50 at 29°C is predicted to be lower than the calculated LC50 at 20°C.

Daily proportional survival through the 28 day test was examined for mysids at at each TBT treatment and each temperature. At 20°C, there was a pronounced TBT effect at 273 and 539 ng/L (Figure 4). A sharp decrease in survival began on about day 10 in these treatments and leveled off after 19 days. The control and the remaining TBT treatments displayed high survival (>0.80) until after day 25. Statistical analysis of proportional survival data from each day of the test indicated no significant differences among TBT treatment groups at 20°C (Table 10). There were, however, nearly significant differences on days 19, 21, and 23. Despite the low power of the ANOVA's(<0.40), homogeneity of variance tests were not significant and confirmed that these data did not violate assumptions of parametric ANOVA. Nonparametric analysis of variance (Kruskal-Wallis) confirmed the lack of significant treatment affects.

There were no significant differences in proportional survival between TBT

Figure 4. Daily proportional survival for each TBT exposure concentration at 20 and 23°C (n= 5).

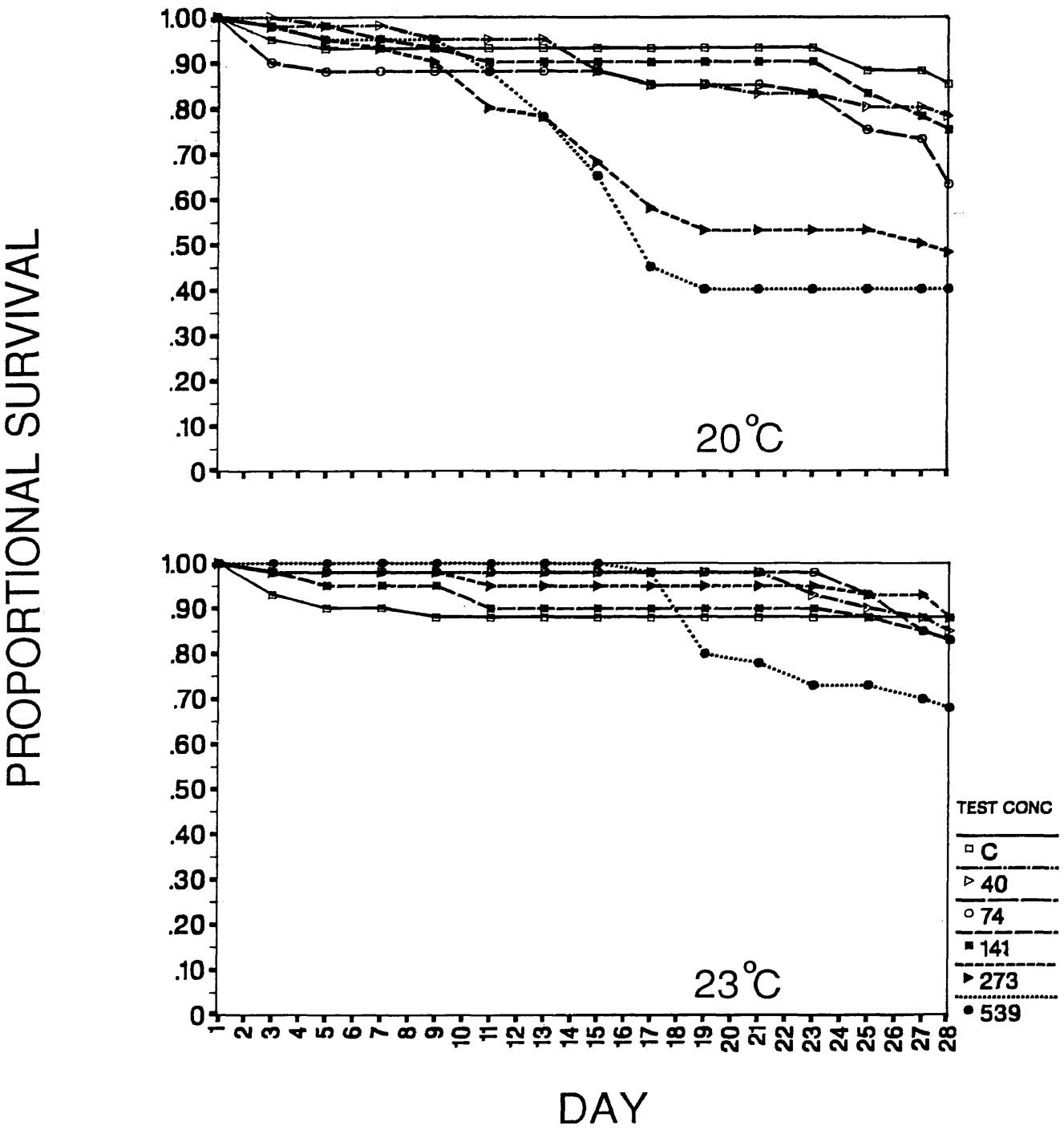

PROPORTIONAL SURVIVAL
1.00
.90
.80
.70
.60
.50
.40
.30
.20
.10
0
20°C
23°C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
DAY
TEST CONC
C
40
74
141
273
539

Table 10. Summary of one-way ANOVA's on proportional survival data at each test temperature for the 28 day test. For multiple comparisons, any two mean TBT concentrations not underscored by the same bar are significantly different (P<0.05). Two underscore lines indicate the 0.01 significance level.

Day	Trt.	Temp.	TBT Conc.				Multiple Comparisons
19	20°C	---	NS				
	23°C	---	NS				
	26°C	---	NS				
	29°C	---	*	F=2.656	P=0.048	DF=5	141 C 40 74 273 539[b]
	Controls	NS	---				
21	20°C	---	NS				
	23°C	---	NS				
	26°C	---	NS				
	29°C	---	*	F=3.693	P=0.013	DF=5	141 C 40 74 273 539[b]
	Controls	NS	---				
23	20°C	---	NS				
	23°C	---	NS				
	26°C	---	NS				
	29°C	---	*	F=3.708	P=0.013	DF=5	141 40 C 74 273 539[b]
	Controls	*	---	F=5.161	P=0.011	DF=3	20 23 29 26[a]
25	20°C	---	NS				
	23°C	---	NS				
	26°C	---	NS				
	29°C	---	**	F=5.126	P=0.002	DF=5	141 40 C 74 273 539[b]
	Controls	*	---	F=3.867	P=0.030	DF=3	20 23 26 29[a]

(continued)

Table 10. (continued)

Day	Trt.	Temp.	TBT Conc.				Multiple Comparisons
27	20°C	---	NS				
	23°C	---	NS				
	26°C	---	NS				
	29°C	---	*	F=3.819	P=0.011	DF=5	141 40 74 C 273 539[b]
	Controls	*	---	F=3.97	P=0.027	DF=3	20 23 26 29[a]
28	20°C	---	NS				
	23°C	---	NS				
	26°C	---	NS				
	29°C	---	**	F=6.621	P=0.001	DF=5	141 40 74 C 273 539[b]
	Controls	*	---	F=4.857	P=0.014	DF=3	20 23 26 29[a]

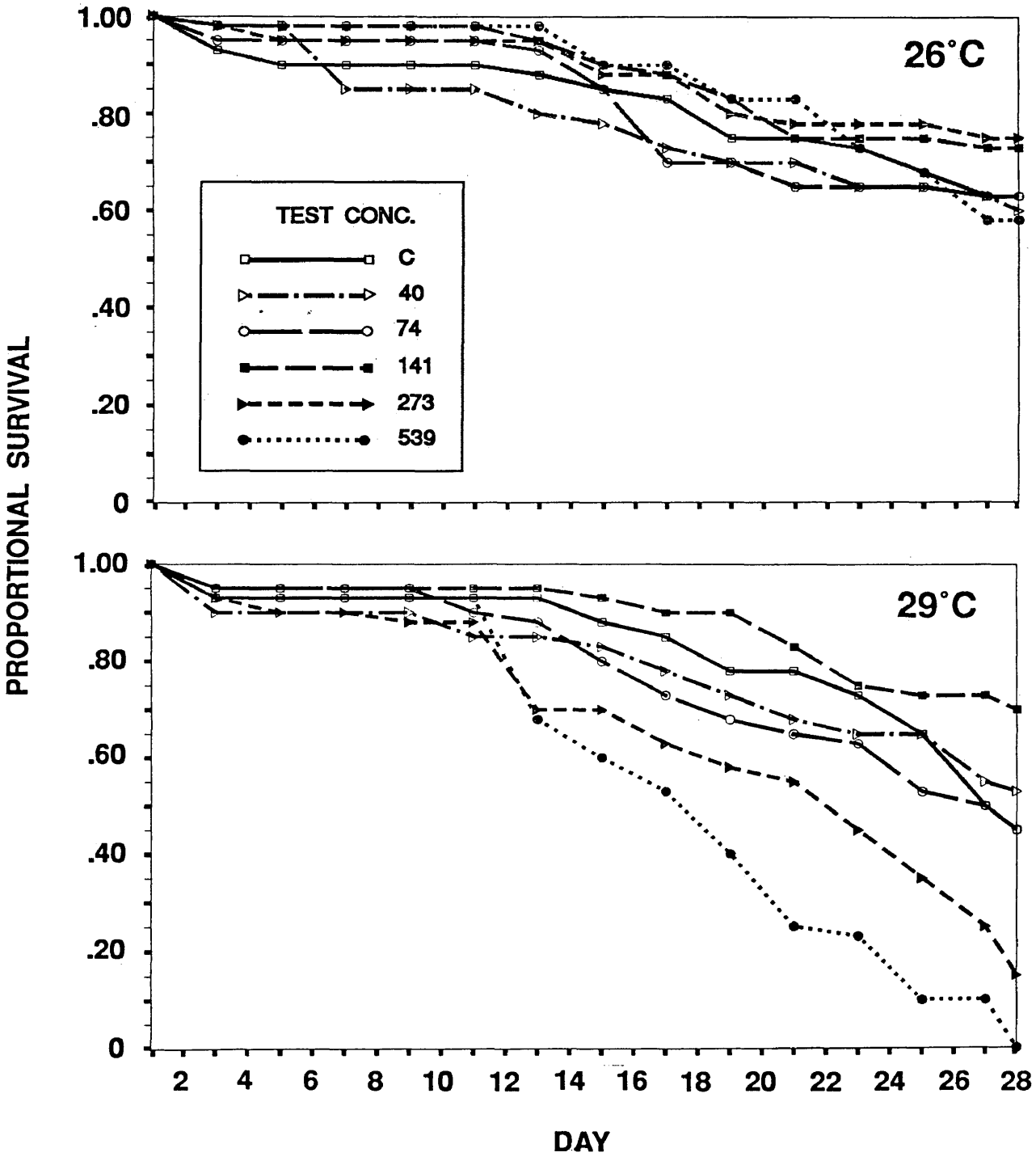
[a]Student-Newman-Keuls test
[b]Dunnett's Test

treatments at 23 and 26°C throughout the 28 day test (Table 10). Throughout the test, proportional survival at 23°C was above 0.80 at all TBT treatments except 539 ng TBT/L (Figure 4). At 539 ng TBT/L, survival fell to 0.80 by day 19 and was reduced to 0.68 by day 28. This is similar to the decrease in survival seen at 539 ng TBT/L and 20°C, but the effect was more pronounced at the lower temperature. Proportional survival at 26°C was nearly the same at all TBT treatments throughout the test (Figure 5). At all TBT concentrations, survival decreased at a nearly steady rate after day 13, and ranged between 0.60 and 0.75 by day 28.

Survival at 29°C had begun to decrease following 11 days of exposure at 74, 273 and 539 ng TBT/L, and after 13 days at the remaining TBT treatments (Figure 5). Survival continued to decrease at all TBT treatments until the end of the test. After 28 days, proportional survival ranged between 0 and 0.45 for the control, 74, 273, and 539 ng TBT/L treatments. As mentioned previously, 28 day survival was not concentration dependent, and was above 0.50 at both 40 and 141 ng TBT/L. These unexpected results are not explained by anomalies in either measured TBT concentrations or in water quality. Significant differences in survival among TBT treatments at 29°C were detected on days 19, 21, 23, 25, 27, and 28 (Table 10). Dunnett's tests indicate that survival in the 539 ng TBT/L treatment was significantly different from that in the control on days 19 through 28, and survival in the 273 treatment was different on day 28. The power of these one-way ANOVA's ranged from 0.48 (day 19) to 0.97 (day 28). Homogeneity of variance tests indicated that the variance was homocedastic and suitable for ANOVA.

For each day of the test, ANOVA's were used to test for a significant effect of temperature on control survival. These analyses reveal that temperature had a

Figure 5: Daily proportional survival for each TBT exposure concentration at 26 and 29°C (n=5).


26°C
29°C
TEST CONC.
C
40
74
141
273
539
PROPORTIONAL SURVIVAL
1.00
.80
.60
.40
.20
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
DAY

significant effect on control survival after day 23 (Table 10). Multiple comparisons indicated that survival was higher at 20°C than at the other three test temperatures on days 23, 25, and 27. On day 28, survival was higher at both 20 and 23°C. The power of the ANOVA's ranged from 0.60 to 0.76, and homogeneity of variance tests indicated that the variance was homoscedastic and suitable for the ANOVA's.

Reproduction

Appearance of first brood:

There were no significant TBT effects on time to appearance of embryos at any test temperature. Examination of the effect of temperature across all TBT concentrations reveals that embryos appeared later at 20°C than at the other test temperatures (Figure 6, Table 11). Temperature had a significant effect on the development of mysids in control treatments (Table 12). The number of days to appearance of embryos decreased with increasing temperature. The mean day for appearance of embryos in controls at 20°C (18 days) was significantly different from the mean day at 23°C (15 days), and the mean day of appearance at both of these temperatures was significantly different from controls at 26°C (12 days) and 29°C (12 days). The differences in mean day of appearance at 23, 26 and 29°C decreased with increasing TBT concentration. Homogeneity of variance tests indicated that the variance of data from the controls is slightly heteroscedastic. The power of the ANOVA is high (approximately 1.0), however, so the results of this parametric test are reported. These findings on time to appearance of first brood are consistent with data from the thermal tolerance test presented earlier (Table 2). In that teat, brood appearance required 20 $\pm$ 3 days (mean $\pm$ standard deviation) at 20°C, 18 $\pm$ 4 days at 24°C, and 10 $\pm$ 2 days at 28°C. These

Figure 6. Mean number of days to appearance of first brood as a function of TBT concentration at each test temperature. (see Table 11 for errors associated with data points)

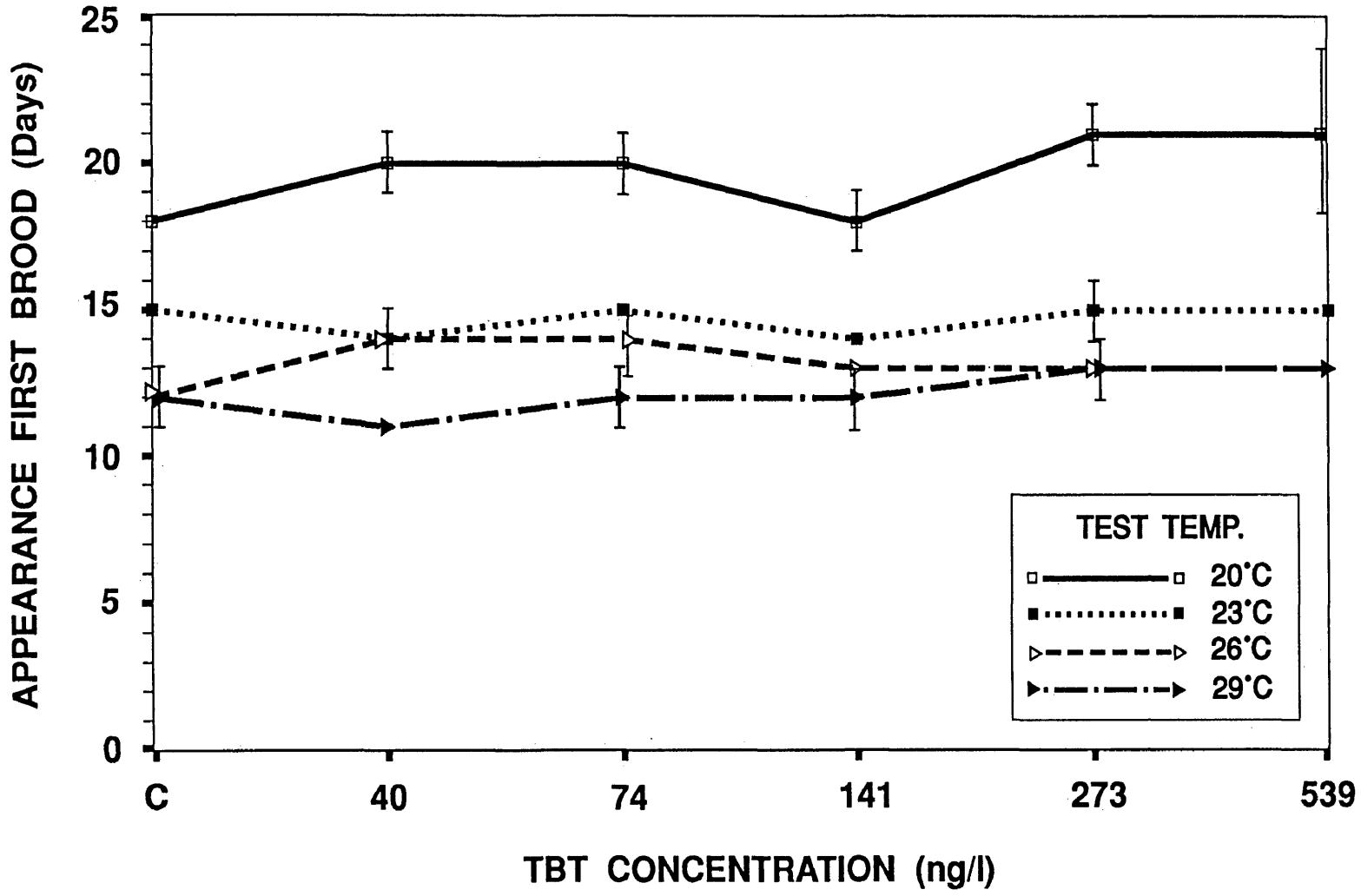

APPEARANCE FIRST BROOD (Days)
25
20
15
10
5
0
C
40
74
141
273
539
TBT CONCENTRATION (ng/l)
TEST TEMP.
20°C
23°C
26°C
29°C

Table 11. Reproductive Responses as a function of TBT concentration at each test temperature. Each value is the mean with standard error in parentheses.

Temp.	TBT conc.	Appearance of first brood (days)	First brood release (days)	Total no. offspring	No. offspring per FRD
20°C	C	18 (0)	-----	0 (0)	0.0 (0.0)
	40	20 (1)	-----	0 (0)	0.02 (0.02)
	74	20 (1)	-----	0 (0)	0.07 (0.07)
	141	18 (1)	-----	0 (0)	0.0 (0.0)
	273	21 (1)	-----	0 (0)	0.0 (0.0)
	539	21 (3)	-----	0 (0)	0.0 (0.0)
23°C	C	15 (0)	24 (3)	1 (1)	0.05 (0.03)
	40	14 (1)	22 (2)	3 (1)	0.14 (0.09)
	74	15 (0)	22 (3)	1 (1)	0.06 (0.03)
	141	14 (0)	20 (2)	1 (1)	0.05 (0.0)
	273	15 (1)	25 (2)	3 (2)	0.12 (0.10)
	539	15 (0)	----	0 (0)	0.01 (0.01)
26°C	C	12 (0)	15 (1)	19 (6)	0.56 (0.14)
	40	14 (0)	18 (2)	30 (9)	0.86 (0.18)
	74	14 (1)	18 (3)	5 (2)	0.33 (0.18)
	141	13 (0)	16 (2)	36 (4)	0.78 (0.10)
	273	13 (0)	17 (1)	21 (10)	0.50 (0.16)
	539	13 (0)	17 (2)	19 (5)	0.54 (0.13)
29°C	C	12 (1)	16 (2)	28 (7)	0.80 (0.12)
	40	11 (0)	13 (0)	45 (7)	0.87 (0.24)
	74	12 (1)	14 (1)	35 (7)	0.64 (0.08)
	141	12 (1)	14 (1)	38 (7)	0.69 (0.13)
	273	13 (1)	14 (1)	12 (5)	0.37 (0.09)
	539	13 (0)	15 (2)	3 (3)	0.14 (0.06)

Table 12. Summary of one-way ANOVA's of reproductive responses at each test temperature following 28 days of testing. See legend of Table 10 for further explanation.

Response	Trt.	Temp.	TBT Conc.				Multiple Comparisons
Appearance of first brood	20°C	---	NS				
	23°C	---	NS				
	26°C	---	NS				
	29°C	---	NS				
	Controls	**	---	F=33.92	P=.000	DF=3	20 23 26 29[a]
First brood release	20°C	---	NS				
	23°C	---	NS				
	26°C	---	NS				
	29°C	---	NS				
	Controls	*	---	F=6.670	P=.017	DF=2	23 29 26[a]
Total offspring released	20°C	---	NS				
	23°C	---	NS				
	26°C	---	NS				
	29°C	---	NS				
	Controls	**	---	F=32.43	P=.000	DF=3	29 26 23 20[a]
No. offspring per FRD	20°C	---	NS				
	23°C	---	NS				
	26°C	---	NS				
	29°C	---	**	F=5.093	P=.003	DF=5	40 C 141 74 273 539[b]
	Controls	**	---	F=24.43	P=.000	DF=3	29 26 23 20[a]

[a]Student-Newman-Keuls test
[b]Dunnett's Test

standard deviation ranges overlap the standard deviation ranges at similar temperatures in the present test.

First brood release:

The data at 20°C for day of first brood release were excluded because few replicates released broods at this test temperature. There were no significant TBT effects at any test temperature. The first brood was released latest at 23°C at all TBT concentrations (Figure 7, Table 11). Temperature had a significant effect on day of first brood release (Table 12) when controls alone were examined. Brood release was significantly more delayed at 23°C than at either 26 or 29°C. The power of this ANOVA was high (0.89), and homogeneity of variance tests indicated that the variance was homogeneous among controls.

Total offspring production:

Offspring production was much higher at 26 and 29°C than at 23°C, and there was no offspring production at 20°C (Figure 8). An interactive effect of TBT concentration and temperature is apparent when one examines total offspring production. Total offspring production was greater at 29°C than at 26°C for all TBT treatments below 141 ng TBT/L, including the control (Figure 8, Table 11). At 273 and 539 ng TBT/L, however, offspring production at 29°C was less than that at 26°C.

Total offspring production data were log transformed prior to statistical analysis. Stepwise multiple regression analysis indicated that temperature (T) was the most predictive component, and that the interaction term (C*T) was also significant. However, these independent variables accounted for only a small amount of variability in total offspring production (r^2=0.452, F=28.491, P=.000).

Figure 7. Mean day of first brood release as a function of TBT concentration at each test temperature. (see Table 11 for errors associated with data points)

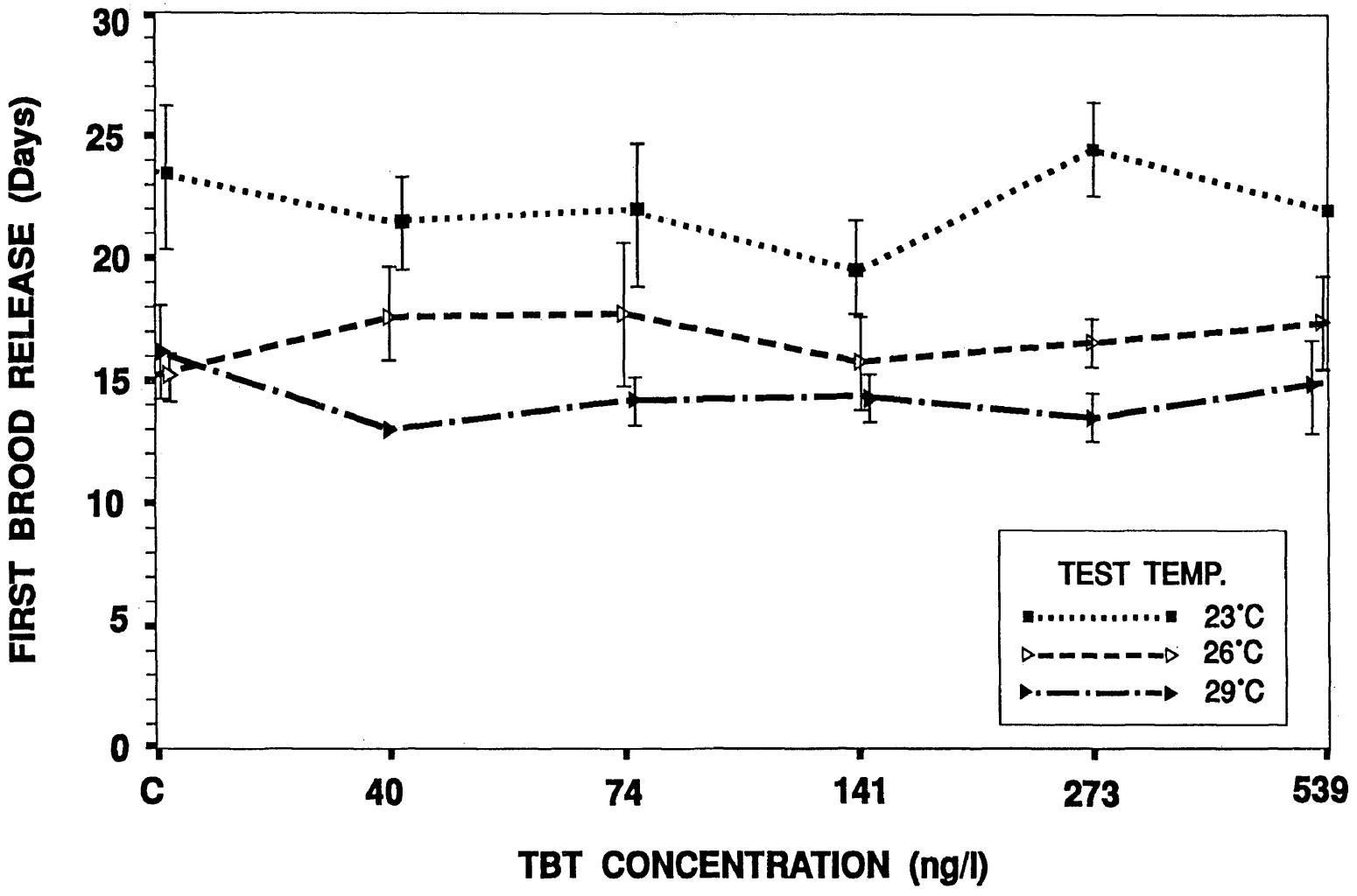
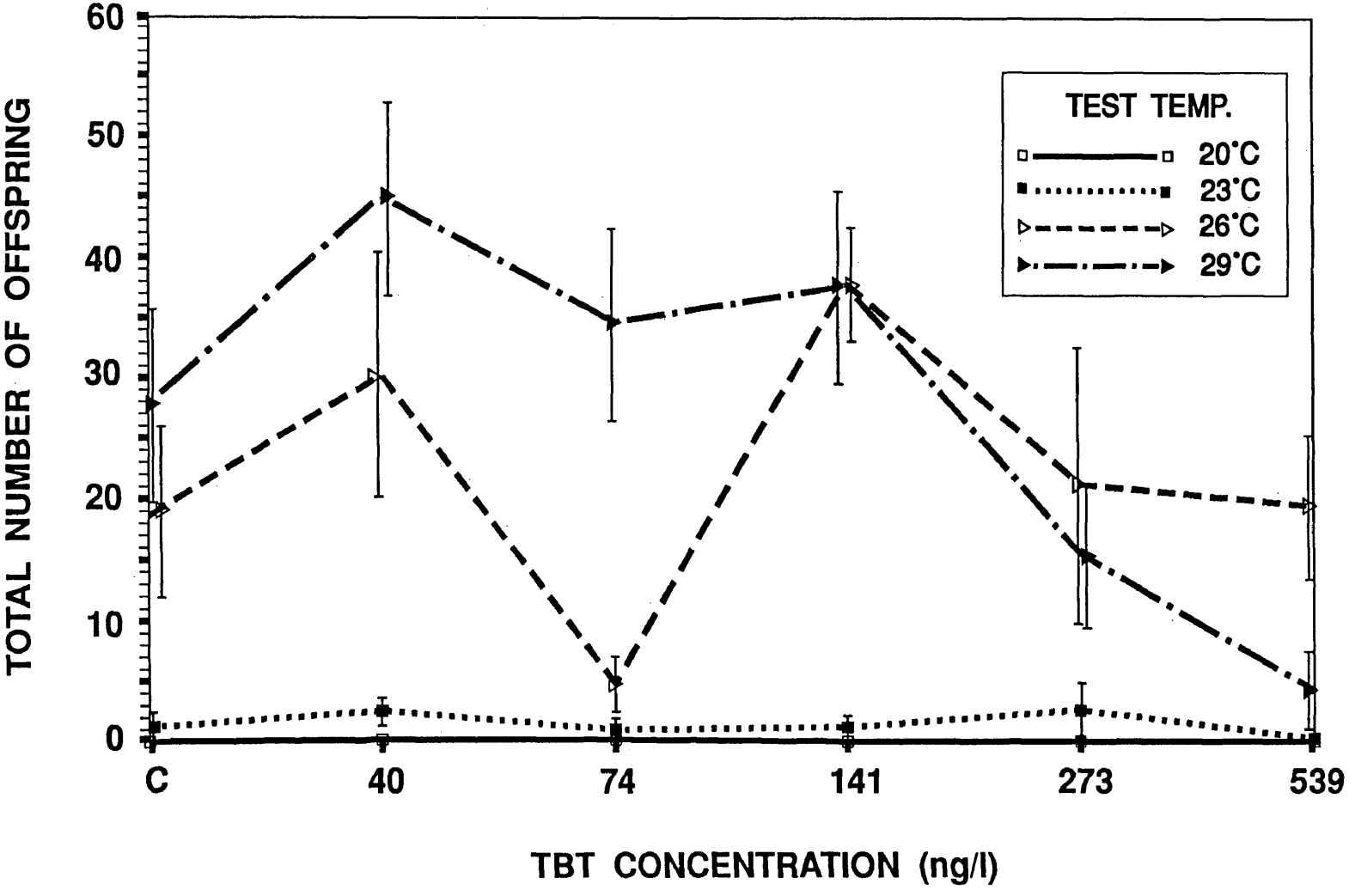

FIRST BROOD RELEASE (Days)
30
25
20
15
10
5
0
C
40
74
141
273
539
TBT CONCENTRATION (ng/l)
TEST TEMP.
23°C
26°C
29°C

Figure 8. Mean total number of offspring as a function of TBT concentration at each test temperature. (untransformed data; see Table 11 for errors associated with data points)


TOTAL NUMBER OF OFFSPRING
60
50
40
30
20
10
0
C
40
74
141
273
539
TBT CONCENTRATION (ng/l)
TEST TEMP.
20°C
23°C
26°C
29°C

Due to the poor fit of the regression model to the data, a predictive regression equation is not presented. Examination of residuals indicated that homoscedasticity and normality assumptions of the model had been met. Although total offspring production dropped dramatically at the highest TBT treatment at 29°C as compared to the control, this non-standardized (discussed below) offspring production data produced no significant differences from the control. The high variance associated with this data, even after transformation, did not allow significant differences to be found with standard hypothesis test methods.

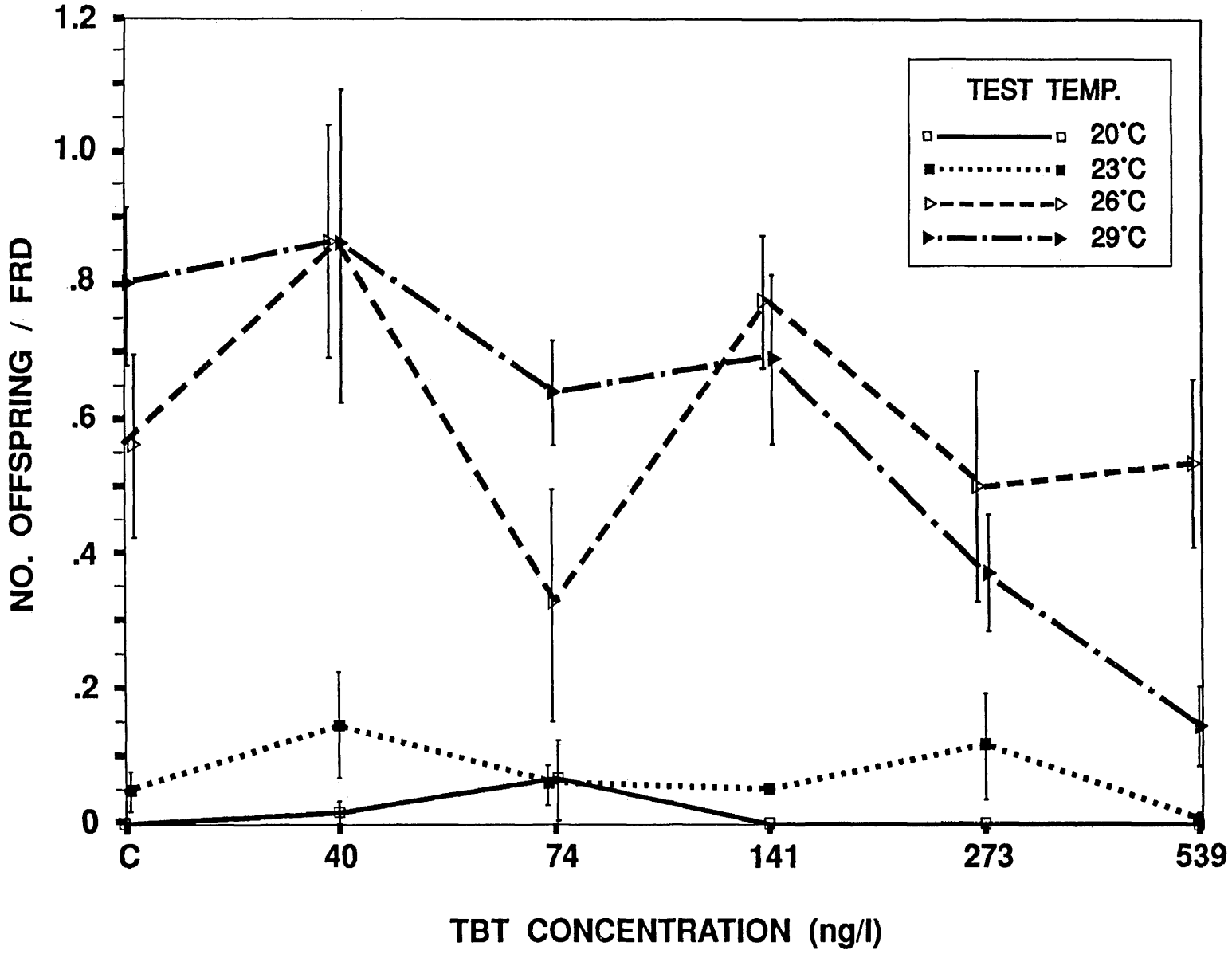
The effect of temperature was pronounced at all TBT concentrations (Figure 8). When control treatments alone were examined, mean total offspring production at 26 and 29°C were equal, and were significantly greater than production at 23°C (Table 12). The power of the ANOVA was approximately 1.0, and homogeneity of variance tests indicated that variance was homogeneous among the control groups.

Number of offspring per female reproductive day (FRD):

A standardized parameter called the number of offspring per female reproductive day (FRD) was calculated from the total offspring production data. The female reproductive days (FRD) for each replicate are the number of females in the replicate multiplied by the number of reproductive days available to the replicate (see p. 48). The total number of young released in each replicate was then divided by this product. Calculating the number of offspring per FRD provides an index that corrects for varying numbers of mature females among replicates and the number of days those females were alive.

The number of offspring per FRD data set (Figure 9, Table 11) was subjected to stepwise multiple regression analysis. These data were log transformed prior to

Figure 9. Mean number of offspring per female reproductive day (FRD) as a function of TBT concentration at each test temperature. (untransformed data; see Table 11 for errors associated with data points).


1.2
1.0
.8
.6
.4
.2
0
NO. OFFSPRING / FRD
TEST TEMP.
20°C
23°C
26°C
29°C
C
40
74
141
273
539
TBT CONCENTRATION (ng/l)

analysis. The linear effect of temperature was the most predictive independent variable, but the interaction term (C*T) also contributed significantly to the model (Table 13). The predictive equation produced is as follows:

$$Y = -0.62598 + 0.03064\ T - 0.00004\ C*T + 0.00079\ C$$

Although the regression model did not fully describe the variance in the data ($r^2=0.56$), as indicated by the moderately acceptable multiple regression coefficient, some important trends were highlighted. Additionally, the offspring per FRD response was predicted better than was the total number of offspring response discussed earlier. Examination of residuals indicated that homoscedasticity and normality assumptions of the model had been met.

The temperature term was included first in the regression model (Table 13). The important affect of temperature is indicated by the fact that the number of offspring per FRD was higher across all TBT treatments at 26 and 29°C than at 23°C (Figure 9). Offspring production in controls at the two high test temperatures were not different from each other, but had significantly higher than offspring production than controls at the two low temperatures (Table 12). The number of offspring per FRD was 0.067 for the 74 ng/L TBT treatment at 20°C (Table 11), even though total offspring production was zero across all treatments at 20°C (Figure 8). This is simply a function of the offspring per FRD calculation and the resulting scale (0.0 - 1.0) reported, and does not represent an error in the data presentation.

The interaction of TBT and temperature also contributed significantly to the regression model (Table 13). This interaction between factors can be examined graphically. At the control, 40 and 74 ng TBT/L treatments at 29°C, the number of offspring per FRD was higher than at any other test temperature (Figure 9).

Table 13. Results of step-wise multiple regression on log transformed offspring per FRD during 28 day test. Independent variables which were not significant are not included in the table. MS_{reg}(df)= 0.2774 (3), MS_{resid}(df)= 0.0060 (108)

Step	Variable Entered	R	F	Sig. F
1	T	0.7056	109.031	0.000
2	C*T	0.7353	64.153	0.000
3	C	0.7495	46.150	0.000

At the 141, 273, and 539 ng TBT/L treatments at 29°C, however, the number of offspring per FRD had dropped below that for corresponding TBT treatments at 26°C. The linear effect of TBT concentration contributed significantly, though only slightly, to the regression model (Table 13). The effect of TBT concentration on number of offspring per FRD at 29°C is seen by the downward sloping line in Figure 9. Following 28 days of testing, the mean numbers of offspring per FRD at 273 and 539 ng TBT/L were significantly different from the control at 29°C (Table 12). Offspring production was highly variable at 26°C, and there was no significant effect of TBT concentration. Homogeneity of variance tests indicate that the variance among groups was homogeneous, and that the assumptions of ANOVA had been met. The power of the ANOVA's was high; 0.80 at 29°C and 1.0 for the control comparisons.

The number of offspring per FRD was the most sensitive sublethal response examined. The 273 ng TBT/L treatment was significantly different from the control at 29°C, but there was no statistically significant effect at 141 ng TBT/L. Therefore, the 141 ng TBT/L treatment can be labeled as the No Observable Effect Concentration (NOEC).

Growth

Temperature had a significant influence on female mean dry weight. Female dry weight was highest at 23°C (Figure 10, Table 14). A non-parametric ANOVA (Kruskal-Wallis) indicated that female mean dry weight was significantly different between controls at each test temperature ($Chi^2=8.34$, d.f.= 3, $P<0.05$). Multiple comparisons showed that the mean weight of the control at 23°C (1,010 ug) was significantly different from the mean weight of the controls at 20°C (740 ug, $Q=2.14$, d.f.=4, $P\leq0.05$) and 29°C (740 ug, $Q=2.24$, d.f.=4, $P\leq0.05$)

Figure 10. Mean dry weight (ug) of females as a function of TBT concentration at each test temperature following 28 days of testing. (see Table 14 for errors associated with data points)

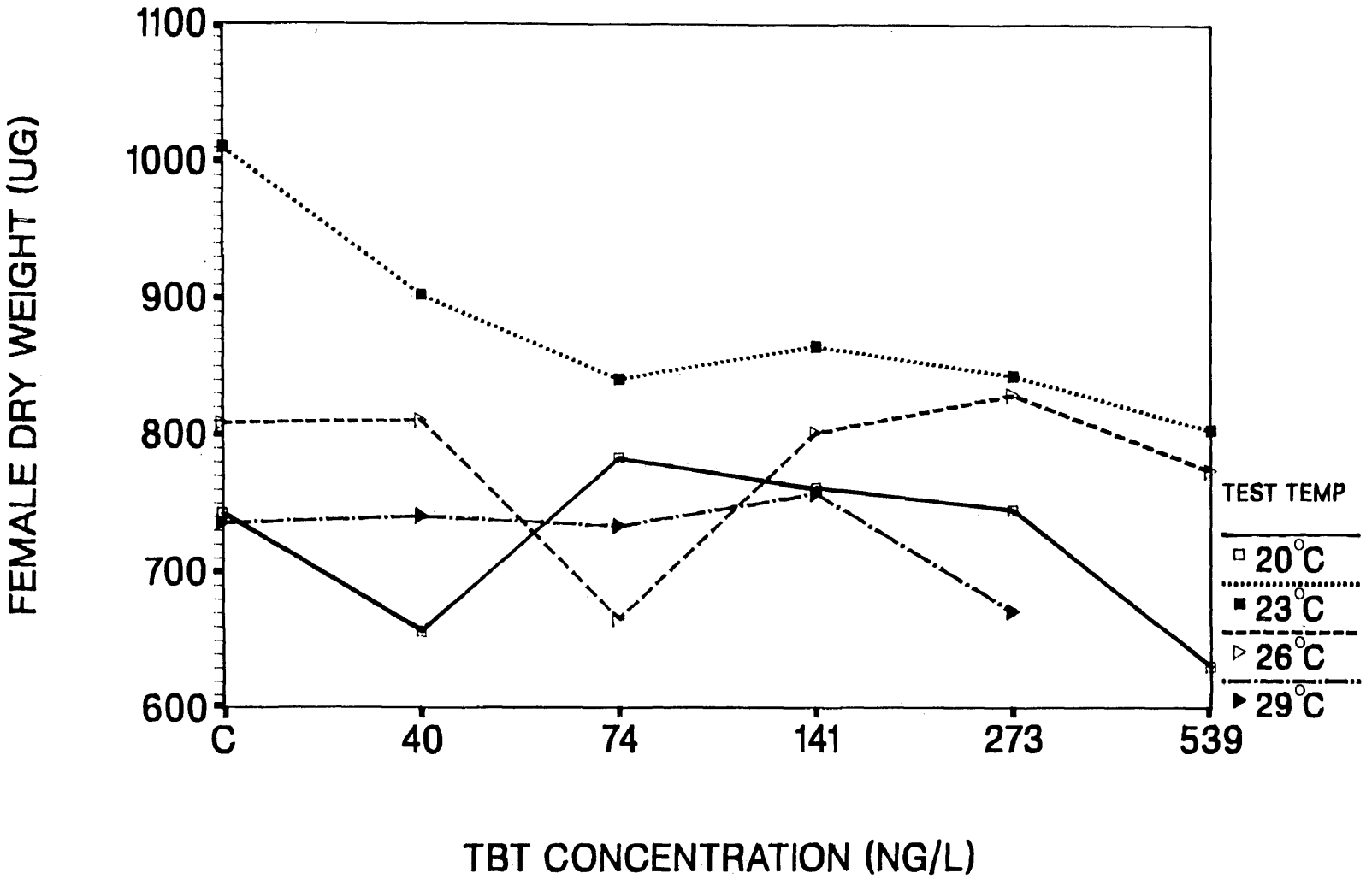

FEMALE DRY WEIGHT (UG)
1100
1000
900
800
700
600
C
40
74
141
273
539
TBT CONCENTRATION (NG/L)
TEST TEMP
20°C
23°C
26°C
29°C

Table 14. Mean dry weights (ug) of mysids following 28 days of testing as a function of TBT concentration at each test temperature. (n= number of replicates)

Temp.	TBT Conc. (ng/L)	Males			Females			Total (Males+Females)		
		Mean	SD	n	Mean	SD	n	Mean	SD	n
20°C	C	630	89	5	740	68	5	680	96	10
	40	620	67	4	660	91	5	640	78	9
	74	630	56	5	780	81	5	710	104	10
	141	700	61	5	760	79	5	730	73	10
	273	660	87	3	740	42	3	700	75	6
	539	560	50	2	630	85	2	590	71	4
23°C	C	730	31	5	1,010	285	5	870	240	10
	40	720	68	5	900	73	5	810	118	10
	74	670	78	5	840	77	5	760	114	10
	141	740	71	5	860	121	5	800	113	10
	273	670	51	5	840	60	5	750	107	10
	539	680	57	4	800	90	4	740	96	8
26°C	C	720	49	5	810	78	5	760	78	10
	40	680	76	4	810	91	5	750	104	9
	74	700	93	5	670	21	2	690	78	7
	141	720	80	4	800	119	5	760	107	9
	273	720	37	5	830	83	5	780	82	10
	539	740	41	4	770	137	4	760	95	8
29°C	C	620	40	3	740	108	4	680	102	7
	40	660	22	4	740	107	5	700	88	9
	74	680	87	3	730	30	4	710	61	7
	141	660	82	5	760	97	5	710	100	10
	273	640	14	2	670	99	2	660	60	4
	539	---	--		---	--	-			

(Zar, 1984). The mean dry weight of females in controls at 26°C (810 ug) was not significantly different from that for controls at other temperatures. In general, temperature had the same effect on male and total dry weight as it did on female mean dry weight (Table 14). However, the effect was less distinct and there were no significant differences between controls at each temperature. Female mean dry weights were higher than male mean dry weights at each test temperature.

TBT had little effect on dry weights at any test temperature. A significant TBT effect was found in only one instance. As is clearly seen in Figure 10, female mean dry weight at 74 ng TBT/L was significantly different from the control at 26°C ($F=2.77$, $P=0.045$, $df=5$). No explanation for the large decrease in weight was found, but this was one of the treatments in which there were only two replicates with live animals at the end of the test. There were no obvious effects of TBT on either survival or reproduction at this TBT-temperature treatment, and, therefore, toxicant concentration is not correlated with the low weights. Additionally, the low mean dry weight for this treatment are not correlated with obvious anomalies in water quality. There were no significant TBT effects on either male or total mean dry weights.

A Sartorius balance (model 1712 MP8) with precision of 0.01 mg (10 ug) and an internal calibration was used to make dry weight measurements. For the two days on which weight measurements were made, measured weights of 1 and 2 mg standards varied by no more than 0.02 mg (20 ug) from the true weights. Between the days on which weight measurements were taken, the measured weight of each standard varied by no more than 0.03 mg (30 ug). Several sets of duplicate weight measurements were made in order to address the precision of these methods. Duplicate measures of empty weigh pan weights, and duplicate weight measurements of empty pans before and after drying did not differ by

more than duplicate weighings of the standard weights. However, repeated animal total dry weight measurements (randomly chosen among treatments and sexes) were always higher when weighed the second time, and had a mean difference of 40 ug (n=9). This difference of 40 ug is greater than the precision limits of the balance, and introduced error into the growth results. The Wilcoxon paired-sample test (Zar, 1984) showed that the first set of animal total weight measurements were significantly different from the second set of measurements (P=0.05).

DISCUSSION

Acute Toxicity

The 96-h median lethal concentration (LC50) reported in the present study 816 ng TBT/L (391 - 1,487 ng TBT/L, 95% confidence limits) falls within the confidence interval of the 96-h LC50 of 1,100 ng TBT/L (680 - 1,400 ng TBT/L, 95% confidence limits) reported by Goodman et al. (1988). The results from these studies are in good agreement and suggest that the LC50 for TBT with M. bahia has been reasonably accurately determined. Additionally, the results of both of these studies are based on measured exposure concentrations from flow-through tests at similar temperatures between 25°C and 27°C. Both tests were begun with M. bahia which were less than 1-day-old, and both LC50 estimates were derived using the binomial method.

Despite the agreement between studies, certain aspects of the experimental design and chemical analyses employed in the present test are preferrable. The experiments of Goodman et al. (1988), may have been biased because the animals were not exposed to TBT in independent replicates at each test concentration. Mysids of three age groups were exposed to TBT treatments in separate Nitex retention chambers, but all three ages were exposed in the same aquaria at each concentration. Therefore, the results of Goodman et al. were based on 2 "pseudoreplicates" at each test concentration (Hurlbert, 1984), while 3 true replicates were used at each test concentration in the present study. In the present study, test water from one replicate at each TBT concentration was sampled daily for chemical analyses. Goodman et al. sampled from one replicate at each TBT concentration only twice during the test. Even though the TBT concentration at the highest exposure concentration was quite variable, the increased frequency of sampling and the use of an internal standard quantification

method in the present study may have produced more accurate estimates of the mean test concentrations than those in the tests of Goodman et al..

Bushong et al. (1987) reported that acute sensitivity to TBT under flow-through conditions differed considerably among a variety of crustacean species tested. Differences in LC50 values ranged from 500-600 ng TBT/L (72-h) for Eurytemora affinis to >31,000 ng TBT/L (96-h) for the grass shrimp, Palaemonetes pugio. Hall et al. (1988), also reported on the estuarine copepod, E affinis. In flow-through acute toxicity tests, a 72-h LC50 of 600 ng TBT/L (0.0 - 3,300 ng TBT/L, 95% confidence interval) was reported based on measured exposure concentrations. While M. bahia is not the most acutely sensitive to TBT among crustaceans, it falls in the group at the low end of the range of reported sensitivities.

Experiments with two species of mysids produced lower LC50 estimates with TBT than the LC50 found for M. bahia in the present test. In an acute, static-replacement toxicity test with TBT as leachate and neonates (<24-h post-release) of H. costata, a 96-h LC50 of 420 ng TBT/L (360 - 490 ng TBT/L, 95% fiducial limits) was reported based on measured exposure concentrations (Davidson et al., 1986). Although this LC50 value is surprisingly low as compared to that for M. bahia, the methods employed seem to indicate that an accurate LC50 was obtained. Test water in replicates at each dose was replaced every 24 hours, and chemical measurements were made at the beginning and end of this 24 hour period. Although mortality was not dose dependent prior to 96 hours, behavioral observations confirmed the effect levels resulting after 96 hours. In unpublished work from the Naval Oceans System Center, juvenile mysids, Metamysidopsis elongata, were found to be the most sensitive to TBT of a variety of species tested, with a 96-h LC50 of 500 - 1000 ng TBT/L (depending on life stage) based

on nominal concentrations in static-replacement tests (Hall & Pinkney, 1985). These results are interesting because the reported range of LC50's might have been even lower if they were based on measured TBT concentrations; toxicant concentrations typically decrease in the interval between water replacement in static tests.

Bivalves and crustaceans appear to be two of the more sensitive groups to TBT exposure, and early developmental stages of both of these groups have generally been found to be more sensitive than older life stages (Hall & Pinkney, 1985). For example, Bushong et al. (1987) reported that young Gammarus sp. had a 96-h LC50 of 1300 ng TBT/L, while the adults displayed a value of 5300 ng TBT/L. Clam embryos, Mercenaria mercenaria, were reported to have a 48-h LC50 of 1130 ng TBT/L (Roberts, 1987). In contrast, the planktonic larval stage (straight-hinge) of the clam was more resistant to TBT exposure. Roberts (1987) reported a 48-h LC50 of 1650 ng TBT/L, and while Laughlin et al. (1989) could not calculate an LC50 from their results, the 48-h LC50 was predicted to lie between 1000 and 2500 ng TBT/L. While juvenile M. elongata were found to be more sensitive to TBT than older life stages (Hall & Pinkney, 1985), Goodman et al. (1988) reported that age is not a large factor in the acute sensitivity of M. bahia to toxic compounds. The 96-h LC50 values for 1-, 5-, and 10-day old mysids were within a factor of two, and confidence bounds overlapped in exposures to either malathion or TBT.

28 Day Toxicity

Survival

The most severe effects of TBT were seen outside of the optimal temperature range for M. bahia. McKenney (1987) has reported that optimal survival of M.

bahia through 28 days was obtained at 23-24°C. This is consistent with the results of the present study in which the highest survival was found at 23°C. The 20 and 29°C test temperatures employed in the present study are not within the optimal range of temperatures for survival, and interacted with TBT concentration to produce detrimental effects on survival. In contrast, there were no significant TBT effects at the intermediate test temperatures of 23 and 26°C.

Although no significant differences were detected between the control and any TBT exposure concentration at 20°C, a 28-d LC50 value of 472 ng TBT/L (287-1516 ng TBT/L, 95% confidence limits) was calculated from survival results at 20°C. Daily survival data at this temperature showed that the toxic effects of TBT had been exerted by day 19 at the two highest exposure treatments. Although TBT had an affect on survival, the uptake rate at 20°C apparently did not produce body burdens of TBT high enough to significantly reduce survival at any dose level.

A 28-d LC50 estimate could not be calculated at 29°C, but examination of the probit transformed proportional mortality data suggests that the LC50 lies below 472 ng TBT/L. Despite the low proportional survival in controls at 29°C, there was a significant difference between survival for the control and at the highest TBT exposure concentration (539 ng TBT/L). Generally, the uptake rate of toxic chemicals is greater at increased temperatures (Cairns et al., 1975a, 1975b), so it is not surprising that the most significant effects on survival were seen at the highest test temperature. The explaination for this may be that the increased diffusion or active uptake at higher temperatures are associated with higher rates of water and solute movement across gills and other cell membranes (Cairns et al., 1975a, 1975b). Survival at the 273 ng TBT/L treatment was significantly different from that of the control only on day 28. One explanation is that it may

have taken the entire duration of the test for the body burden of TBT to reach significantly lethal levels at 273 ng/L. Another explanation is that there was a slight decrease in variance between replicates at 273 ng TBT/L on day 28, allowing for the detection of a statistically significant effect. Overall, the significant decrease in survival at 539 ng TBT/L was most convincing.

The beginning of the decrease in survival for the two highest TBT concentrations at 29°C roughly coincided with the appearance of the first brood in females at that temperature (day 11-12), and survival continued to decrease throughout the remainder of the test. This may indicate increased sensitivity at the onset of reproduction with M. bahia, although survival began to decrease well before the onset of reproduction (appearance of marsupia) at 20°C.

Even though the effect of temperature on survival was not fully evaluated in the present study (the upper and lower thermal tolerance limits were not identified), results from this test are useful for comparison to results from other studies. At each test temperature, control survival after 28 days in the present study was higher than that predicted by McKenney (1987). The highest survival found by McKenney was approximately 50% through 28 days in a temperature range of 21-25°C (20°/$_{oo}$ S). These findings are probably related to the static replacement methods employed. The high control survival (0.85-0.88) at low and intermediate temperatures in this flow-through study compares well with survival in the controls of other flow-through studies with M. bahia at similar temperatures and test durations (McKenney, 1986; Lussier et al., 1985; Gentile et al., 1982a, 1983).

Proportional survival was low (0.63 and 0.45) at the two high temperatures as compared to the low test temperatures in the present study. A similar situation was found by McKenney (1987) who reported between 35 and 50% survival at

temperatures between 25 and 30°C. The low percent survival in that study was probably the result of the static-replacement methods employed. Other than the paper by McKenney, no tests have been found in the literature in which M. bahia was exposed to temperatures greater than 25°C through 28 days. The relatively low control survival at the high temperatures in the present study is probably related to increased algal or bacterial growth, similar to the situation reported in the thermal tolerance test.

Chronic effect concentrations at or below the 28-d LC50 for M. bahia found in the present study have been reported in the literature for other crustacean species. In life-cycle tests (63 days) with another species of mysid, H. costata, all animals exposed to 480 ng TBT/L died within the first seven days of exposure (Davidson et al., 1986). Although mortality did not prove to be dose-dependent, animals exposed to 380 ng TBT/L showed a significantly different survival than animals exposed to lower doses in that test. Bushong et al. (1990) found that copepod, Acartia tonsa, nauplii (<48-h-old) are very sensitive to TBT relative to other estuarine organisms. TBT concentrations of 230 and 240 ng TBT/L were found to significantly reduce survival relative to controls (ie. LOEC) in 6-day exposures. Exposure of Gammarus oceanicus to TBT at concentrations as low as 300 ng TBT/L for eight weeks significantly reduced survival of larvae exposed to TBT compounds (Laughlin et al., 1984). U'ren (1983) reported the toxicity of TBT to Acartia tonsa, with a 144-h EC50 (moribund or dead) of 400 ng TBT/L. TBT concentrations of less than 100 ng/L significantly reduced survival of the copepod, E. affinis, in one chronic test (Hall et al., 1988a). In a second chronic experiment, Hall et al. found adverse effects on survival were seen at approximately 200 ng TBT/L after 13-d, but there was no significant effect on survival at 100 ng TBT/L. These chronic TBT toxicity levels for E. affinis are

among the lowest measured concentrations reported to significantly affect survival of an estuarine zooplankton species, but sublethal effects (e.g. growth and reproduction) have been documented at lower levels of TBT (Bushong et al., 1987; Hall et al., 1988a) and are discussed below.

Bivalves have shown LC50's for TBT below that found for M. bahia in the present study. Some responses which have been observed at low levels of TBT are mentioned below. Beaumont & Budd (1984) reported that about half of the veliger larvae of the common mussel, Mytilus edulis, subjected to 100 ng TBT/L were dead by day 15, while control survival was high. Most of the surviving larvae were moribund and had grown significantly more slowly than controls. Maguire (1987), reporting on the work of others, gave 12-d and 8-d LC100 (100% mortality) values of 171 and 390 ng TBT/L with spat of the pacific oyster, C. gigas. This work was weakened by the fact that neither analytical methods nor type of exposure were reported and is, therefore, of limited value. Condition indices in adult oysters showed that general health of oysters, C. virginica, is markedly reduced by TBT concentrations of 100 ng/L and higher (Hendersen 1986). Hendersen (1986) also exposed marine fouling communities, including some bivalves, to TBT concentrations of 40 to 2500 ng/L over a period of three months. TBT concentrations of 100 ng/L and higher caused significant reductions of numbers in individual species and species diversity.

Reproduction

Temperature effects dominated the time to appearance of the first brood and first brood release, while TBT concentration did not play a significant role in regulating these responses. In general, broods appeared faster and were released sooner at high test temperatures. The upper and lower thermal tolerance limits

were not identified for either of these reproductive endpoints, but results from this test are useful for comparison to results from other studies. At each test temperature, time to appearance of embryos was similar to results obtained in the thermal tolerance experiment. At 20°C, the time to appearance of the first brood in controls at 20°C (18 days) in the present test was consistent with the results of Gentile et al. (1982a) and Gentile et al. (1983) at similar temperatures. The pattern of delayed reproductive development at low temperatures is supported by the results of Thompson (1984). Thompson reported that temperature had a significant effect on time required for Neomysis americana of both sexes to reach maturity. Raising the temperature 10°C from either 10 or 20°C caused a 50-60% reduction in age at maturity. Thompson also reported that temperature had a highly significant effect on mean incubation time. Broods developed slightly faster (1.25 times) at 25°C than at 20°C, and in another trial developed at least 6.5 times faster at 20°C than at 10°C.

Examination of control treatments from toxicity tests with M. bahia in the literature provides a picture of the effects of temperature on first brood release. At temperatures of 20-21°C the first brood was released in 20-28 days, the average being about 25 days (Gentile et al., 1982a, 1982b, 1983; Lussier et. al., 1985). As expected, this is longer than the mean number of day to first brood release in controls at 23°C in the present study; no broods were released at 20°C. First brood release has been reported in 22 days at 23°C and 18 days at 24°C (Lussier et al., 1985); the former compares well with the results at 23°C (23 days) in the present study. McKenney (1986) reported first brood release in 19 days at 25°C which was considerably longer than the 15 days at 26°C observed in the present study.

Temperature was the most predictive component describing variance in total

number of offspring released and number of offspring per FRD. As was mentioned previously, calculating the number of offspring per FRD is the preferred method of data analysis because it takes into account more sources of variability in the data than simply looking at total offspring production. The dominant effect of temperature on offspring production was seen by the significantly lower offspring production at 20 and 23°C than at 26 and 29°C. In control treatments, both total offspring production and number of offspring per FRD increased with increasing temperature to a maximum at 29°C. The trade-off, however, was that survival through 28 days decreased at temperatures above 23°C to a minimum at 29°C. The sublethal effects of TBT exposure on reproductive output became evident at the highest temperature. The interaction of temperature and TBT concentration explained a significant amount of the variation in offspring per FRD and total offspring production, particularly at 29° C. This interaction produced effects which were not evident with TBT concentration alone, namely a significant reduction in the number of offspring per FRD at 273 ng TBT/L. In work with another species of mysid, Davidson et al. (1986) stated that if a significant number of females cannot release viable juveniles, then the overall recruitment to the population may be reduced and the population may suffer an effect. To a lesser extent, TBT concentration alone also explained a significant amount of the reduction in offspring per FRD.

Total reproductive output, uncorrected for number of females or duration of the test, in the controls of the present study was lower than that in control treatments of other toxicity studies with M. bahia. In tests conducted at 20 to 21°C (Gentile et al., 1982a, 1982b, 1983) the total number of offspring released ranged from 53 to 80 juveniles. Gentile et al. (1982a) reported an extremely high total of 338 in controls of one experiment at 20°C (30 $^{\circ}/_{oo}$ S) after 36 days. The

animals in these tests must have been cultured at 20°C, and thus fully acclimated. The animals in the present test were cultured at 24-26°C, and, as was mentioned previously, there was no offspring production at 20°C in the present study. The only obvious explanation for these differences is that the test duration was longer (35-51 days) in the tests of Gentile et al. as compared to 28 days in the present study.

In a series of experiments, Lussier et al. (1985) and Gentile et al. (1983) reported reproductive output as the total number of juveniles per "available female reproductive day." The number of offspring per female reproductive day (FRD) in the present study was higher than the number of offspring per "available female reproductive day" reported in the studies mentioned above, but the values cannot be related to each other directly. Gentile et al. and Lussier et al. based their calculations on results with individual females, while calculations in the present tests were based on observations for whole replicates.

In work with the mysid, Neomysis intermedia, Toda et al. (1984) also reported on reproductive output. Similar to the effect of temperature in the present study, they reported that daily specific reproductive rate increased exponentially with an increase in temperature from 10 to 25°C. The average number of eggs produced per day showed a clear increase with temperature increase from 0.8 eggs per day at 10°C to two eggs per day at 25°C.

For the majority of toxicants examined in life-cycle toxicity tests using M. bahia, a reduction in reproductive success at sublethal concentrations has proven to be the most sensitive criterion for chronic biological effects (McKenney, 1987). When exposed to a variety of toxicants at concentrations which are ultimately lethal, individual M. bahia females produced fewer young than unexposed animals (McKenney, 1982). A smaller percentage of the females in the population

became reproductively active and contributed young to the population when exposed. Modifications in the energy metabolism of individual mysids exposed to toxicants has been related to decreases in total production of young. McKenney (1985) reported that usage of proteinaceous substrates for energy metabolism during the maturation of M. bahia was altered by exposure to high sublethal concentrations of thiobencarb (S-[4-chlorophenyl] methyl diethylcarbamothionate). Elevated O:N ratios during the maturation of these thiobencarb-exposed mysids suggest a greater reliance on energy-rich lipid substrates to support the elevated rates of oxidative metabolism, resulting in less lipid material being available for gamete production. Similar relationships exist between energy metabolism and reduced reproductive success of M. bahia exposed to endrin (McKenney, 1982). Changing patterns in substrate utilization were correlated with low reproduction rates, suggesting that high lipid catabolism of mysids maturing at sublethal concentrations of a toxicant may be responsible for inhibition of reproduction in adult mysids exposed to TBT.

While only a few studies have examined the sublethal reproductive effects of TBT on crustaceans, effects have been found at TBT concentrations as low as the Lowest Observable Effect Concentration (LOEC) (273 ng TBT/L) reported at 29°C in the present study. In experiments with the mysid, H. costata, an MATC of 140 ng TBT/L (LOEC= 190 ng/L) was determined, above which the release of juveniles was significantly reduced (Davidson et al., 1986). The increase in adverse effects after females began releasing juveniles was attributed to increased sensitivity of these mysids during the release of young. Laughlin et al. (1984) reported that TBT exposure of Gammarus blocked the release of young following several weeks of exposure to 300 ng TBT/L. During exposure to sublethal concentrations of TBT, egg production rates of Acartia tonsa (number of eggs per

female per day) were reduced by 37% relative to the control at 100 ng TBT/L (based on nominal concentrations, Johansen & Mohlenberg, 1987). Egg production was significantly reduced after exposure for 72, 96, and 120 hours at 100 ng TBT/L, but at 10 and 50 ng TBT/L, the decrease in egg production rate became significant only after 120 hours. This difference in the delay time for egg production in response to TBT exposure may be attributable to toxic effects related to accumulated TBT.

Low TBT exposure concentrations have been shown to impair reproduction in other groups of animals as well. Exposure to 240 ng TBT/L leachates inhibited larval production of the European oyster, Ostrea edulis, (Thain, 1986). At this concentration of TBT there was no sexual differentiation in these hermaphroditic organisms. TBT concentrations well below 100 ng/L have been reported to cause sublethal toxic effects in the dog-whelk, Nucella lapillus. A reproductive abnormality termed "imposex" (the imposition of male sexual characteristics on females) has been reported in both field and laboratory studies at exposures levels of 20 ng TBT/L (Bryan et al., 1986). Experimental concentrations exceeding 5 ng TBT/L have caused blockage of the oviduct and sterilized females before they could reproduce (Bryan et al., 1989). With the mud snail, Ilyanassa obsoleta, Bryan et al. (1989) also reported that the frequency of imposex was 100% and the intensity of penis expression was high at TBT concentrations exceeding 20 ng/L. In all of the studies examining imposex, however, the data are all from casual field observations with minimal chemical measurements which precludes establishing a clear cause-and-effect relationship (Salazar & Champ, 1988). Severe imposex can apparently affect a population but the degree of imposex and the TBT concentration that induces these effects remain unclear. In studies with N. obsoletus, it has been reported that other chemicals like organoleads might

induce the same phenomenon and that imposex does not necessarily have any significant impact on the population (Salazar & Champ, 1988).

Growth

Temperature had the dominant effect on dry weights of female mysids. Differences in mean dry weights at different temperatures were less pronounced for males than for females. In general, mean dry weights at all TBT treatments were higher at the intermediate temperatures (23 and 26°C) and mean dry weights generally decreased at the extreme temperatures of 20 and 29°C. McKenney (1987) reported that after 21 days, individual mysids at favorable food densities weighed on average between 690 and 723 ug (25±1°C, 20 $^{o}/_{oo}$ S). Mean dry weights of all animals in the present test were generally above this range at 23 and 26°C, and within or below this range at 20 and 29°C.

TBT concentration did not have a significant effect on mean dry weight at any test temperature, even at the extremes of temperature. One possible explanation is that energy was invested in growth and not reproduction under extreme temperature conditions. Homogeneity of variance tests revealed homoscedasticity among TBT exposure concentrations at 20 and 29°C, and the coefficient of variation (CV) for mean dry weight data at each TBT-temperature treatment was generally small (<0.15). Therefore, the absence of significant TBT effects does not seem to be attributable to inherent variance in these data.

There was a confounding factor which affected the dry weight results of this test. A significant difference was found between repeated animal total weight measurements. When the weighing pans with animals were removed from the drying oven, they were allowed to cool to room temperature in a dessicator. They were then allowed air exposure time in the weighing room before dry weight

measurements were taken. While dried animals tend to absorb moisture from the air upon cooling, previous experience with this procedure indicated that the weights would stabilize after about an hour of equilibration time. Apparently, under the prevailing conditions, sufficient time was not allowed for equilibration, and the weights were not stable when weight measurements were made. Individual weighing pans probably increased in weight in proportion to time of air exposure and number of animals in the pan.

Overall, weights increased slightly (mean of 40 ug), but significantly, between weighings. Although this confounded the interpretation of the growth results, pans were weighed with no attention given to temperature or TBT treatment, thus adding no bias to the weight measurements for any particular treatments. This weighing procedure may have moderated the importance of the error. It is unlikely that TBT treatment effects were masked by this error in dry weight measurements. Significant differences in female dry weight were found between control treatments, so large weight differences between TBT treatments should also have been detected.

The effect of temperature on growth has been investigated with other species of mysids. Kuhlmann (1984) reported that the growth rate of Neomysis integer was greatest at 21°C and decreased at both higher and lower temperatures. Toda et al. (1984) reported that, with excess food present, the growth rate of juvenile Neomysis intermedia increased exponentially with increasing temperature up to 20°C. The growth rate in juveniles leveled off above 25°C, and dropped at 29°C. Pezzack & Corey (1982) reported no growth at 4°C with adult Neomysis americana; growth rates increased exponentially with temperature up to 25°C. Similarly, Thompson (1984) reported an exponential relationship between juvenile growth rate and increasing temperatures below 20°C for N. americana. In

contrast, adult growth rates were generally unaffected by short term changes in temperature.

Each the species of mysids mentioned above displays the same general pattern of growth in response to temperature as was observed in the present study. However, the minimum temperature required for growth is higher for M. bahia than for any of these other species. Female mean dry weight was significantly reduced from the optimal at 20°C for M. bahia, while the more temperate species, N. americana, for example, displayed exponential growth even below 20°C. Strong temperature dependence may be characteristic for many species of mysids, particularly temperate species, and it is very likely that their growth rates and generation lengths in nature are mainly controlled by temperature (Toda et al., 1984).

Few studies have examined the effects of TBT on growth in crustaceans, and as the present study indicated, this does not appear to be a particularly sensitive sublethal response. Subadults of the mysid, H. costata, showed no growth inhibition at 310 ng TBT/L or less after 21 days (Davidson et al., 1986). Adult females displayed no growth inhibition at 380 ng/L or less after 63 days. Growth effects were observed only at 1000 ng TBT/L in lobster (Homarus americanus) larvae, based on nominal concentrations in static renewal tests (Laughlin & French 1980). Hall et al. (1988d) reported that after 24 days of exposure to TBT, the weight of Gammarus sp. in control treatments was 2.8 times greater than the weight of those organisms exposed to 579 ng TBT/L. A significant difference from controls was reported at 49 ng TBT/L, but no significant differences were found at higher TBT concentrations, indicating that factors other than TBT exposure may have been responsible for the results. Laughlin & Linden (1984) reported slight decreases in growth for the Baltic amphipod, Gammarus

oceanicus, upon exposure to TBT, although responses at TBT levels below 1000 ng/L were not marked.

The most severe sublethal growth effects of TBT have been observed with bivalves. Lawler & Aldrich (1987) reported that after 48 days, TBT exposure levels less than 20 ng/L caused growth retardation in C. gigas spat. Waldock and Thain (1983) reported that C. gigas spat grew poorly at 150 ng TBT/L, and adults exposed to TBT concentrations of 150 ng/L for 56 days displayed reduced growth and shell thickening. Maguire (1987), reporting the results of others, showed slow growth and high mortality in C. gigas spat after 10- and 12-d at 49 and 73 ng TBT/L, respectively. Juvenile mussels (<20mm), Mytilus edulis, exposed to 70, 80, and 200 ng TBT/L had significantly reduced growth rates after 63 days (Salazar & Salazar 1987). Following seven weeks of exposure to TBT leachates, Thain (1986) reported a reduction in growth of bivalve spat (C. gigas, M. edulis, and V. decussata) at 240 ng TBT/L. Adult bivalves are less sensitive than juveniles, but growth effects have been observed. For example, in tests with adult mussels, M. edulis, lasting 66 days, a decrease in shell length was found at 310-730 ng TBT/L by comparison to the control (Valkirs et al., 1987). The significance of these results is somewhat questionable, however, because significant differences in shell lengths were based on changes of only 1% in shell length.

IV. GENERAL DISCUSSION

TBT and Temperature

Detrimental effects of TBT were seen at both the highest and lowest test temperatures in this study. Sublethal concentrations of TBT and temperatures within the zone of tolerance of M. bahia were chosen with the intent of investigating the interactive effects of these factors. As discussed above, their interaction increased the susceptibility of M. bahia to TBT. Low temperature (20°C) reduced the lethal concentration of TBT, and high temperature (29°C) significantly influenced both lethal and sublethal concentrations of TBT. Although the detrimental effects of TBT at high temperatures were expected, based on a knowledge of the effects of temperature on acute toxicity with invertebrates, the low levels of TBT at which significant effects were observed are notable. A temperature within the zone of tolerance of this animal and a sublethal level of TBT contamination, neither of which would produce a significant response on its own, produced a significant reduction in reproductive output in M. bahia. The reported LOEC of 273 ng TBT/L is among the lowest reported in the literature for macroinvertebrates (Hall et al., 1988a).

Data on the influence of water quality on organotin toxicity are scarce. Only one study has been found in the literature which examines the effect of temperature and salinity on response to an organotin compound. Wright & Roosenberg (1982) examined the acute toxicity of trimethyltin to larval Uca pugilator. There was a consistent increase in susceptibility to the toxicant with increasing temperature and diminishing salinity. At 20°/$_{oo}$ S, 48-h LC50's were

200 ng TMT/L at 10°C, 40 ng TMT/L at 20°C, and 30 ng TMT/L at 28°C. In other words, an increase in temperature from 20° to 28°C resulted in a 25% decrease in the dose required to produce 50% mortality in 48-h, and an even greater decrease in dose resulted from a temperature increase from 10° to 20°C. Wright & Roosenberg stated that these data serve to emphasize the importance of temperature-salinity considerations to toxicity, particularly in shallow estuarine areas likely to be a source of trialkyltin compounds.

In the present life-cycle test with M. bahia, a temperature difference of 3°C between 26 and 29°C had a marked effect on toxicity through 28 days. TBT exposure at 26°C had no effect on survival and reproductive output, but both survival and the number of offspring per FRD were significantly reduced at low TBT levels at 29°C. This represents a more significant effect on toxicity than the effect of temperature on the 48 hour LC50 reported by Wright & Roosenberg (1982). The present test points to the need for long-term (e.g. life cycle) testing when examining the interaction of environmental factors with organotin toxicity. This may be true for most environmental contaminants.

A few studies have examined the effects of temperature on the acute toxicity of pollutants to mysids. As was the case for M. bahia, an increase in temperature generally results in an increase in toxicity. McLusky & Hagerman (1987) examined the effects of temperature and salinity on the toxicity of chromium, nickel, and zinc to the mysid, Praunus flexuosus. In exposures up to 300 hours, an increase in temperature from 5 to 15°C (at a given salinity) led to reduced median survival times at a given metal concentration for all metals. Similarly, Voyer & Modica (1990) found that an increase in temperature from 20° to 25°C led to sharply reduced 96 hour LC50's for M. bahia exposed to cadmium. Temperature increases within the thermal optimium for survival of M. bahia yielded

progressively greater mortality, decreasing median tolerance by a factor of three.

Both Thompson (1984) and Hargreaves et al. (1982) examined the acute toxicity of naphthalene to Neomysis americana as a function of temperature. At least over some ranges, temperature had a significant effect on both the control mortality and the acute toxicity of naphthalene. Comparison of 96-h LC50 values indicated that the effect of increasing temperature 5°C from 20°C to 25°C in the summer was greater, with mysids at the higher temperature being significantly (1.6 times) more sensitive to naphthalene (Thompson, 1984). N. americana exposed to acclimation temperatures ranging from 14 to 25°C exhibited 96-h LC50s ranging from 0.8 to 1.4 ug naphthalene/L (Hargreaves et al., 1982). The LC50 was significantly lower at the highest temperature (greater toxicity), a trend observed for other crustaceans exposed to hydrocarbons.

Despite the general trend of increased toxicity at higher temperatures, the interaction of temperature and toxicity is not always predictable and there may be considerable justification for treating chemicals and animals individually (Cairns et al., 1975a, 1975b). Various toxicants have different physiological effects and temperature influences these processes in a variety of ways. Careful examination of the literature reveals variability in the effect of temperature on toxicity of pollutants to crustaceans. For example, both high and low temperatures influence the toxicity of zinc and mercury. McLusky et al. (1986) mentioned four studies in which two crab species displayed greater toxicity at low temperatures in both short- and long-term exposures to mercury. Conversely, with several other crustacean test species, mercury toxicity was seen to increase with increasing temperature. In yet other studies, exposures of crustacean species to different types of toxicants have revealed that both low and high temperatures are stressful as compared to intermediate values. This was the case for TBT and M. bahia in

the present study. Taken together, these recent studies support the conclusions of Cairns et al. (1975a, 1975b) mentioned above. The degree of temperature influence on toxicity and its direction depend on the species and the type of toxicant.

Potential Environmental Toxicity

Hall et al. (1988a) state that, in general, potential environmental hazards of organotin compounds could occur with various trophic groups after long-term exposures to sublethal concentrations. These compounds are bioaccumulated in aquatic biota and could, therefore, pose a risk to the food chain if they are passed from plankton to the higher trophic levels (Hall & Pinkney 1985). It must be kept in mind that laboratory toxicity tests do not replicate true environmental conditions. Most toxicity tests are conducted under controlled laboratory conditions in the absence of important environmental factors such as bottom sediment and suspended organic matter, which may affect the bioavailability of toxic substances (Salazar, 1986). Therefore, direct application of these data to predict biological responses under actual environmental conditions needs to be made cautiously. However, laboratory toxicity tests are important as measures of the relative toxicity of different pollutants, and results from these tests are acceptable conservative estimates of potential effects of toxicants on aquatic ecosystems.

Measured environmental TBT concentrations indicate that acute effects with all but the more sensitive species would probably be minimal in the environment. Hall et al. (1988b) reported that only the maximum TBT concentrations reported in the water column of Virginia and Maryland marinas would potentially be acutely toxic to some Chesapeake Bay copepods, bivalve embryos, and bivalve

larvae. However, mean summer TBT concentrations reported in some Maryland marina areas are high enough to be sublethally toxic to sensitive Chesapeake Bay copepods such as Eurytemora affinis (Hall et al., 1988a) after prolonged exposure. Bivalve embryos and larvae may be affected as well. Although environmental toxicity may be strongly influenced by periods of peak TBT concentrations, mean concentrations are more defensible estimates of long-term environmental exposure levels.

As was mentioned previously, reported 12-month average TBT concentrations ranged from 51-408 ng TBT/L in four marinas in the Maryland portion of Chesapeake Bay (Hall et al., 1987). Hall et al. reported that monthly trends in water column TBT concentrations in the various marinas showed that peak concentrations were reached during May and June. In the marina area (Port Annapolis Marina) with the highest 12-month mean TBT concentration (408 ng TBT/L), concentrations were high (261-998 ng TBT/L) from May through October. It is likely that these peak TBT concentrations resulted from launching of freshly painted boats and increased recreational boating activity during the spring and summer in these marina areas. Results from a four-month (May-August) spatial-temporal TBT monitoring study in Maryland waters revealed peak spring (June) concentrations of 1800 ng TBT/L for Port Annapolis Marina and 1170 ng TBT/L for Mears Marina (Hall et al., 1988c). Mean concentrations for the four month period were 435 ng TBT/L and 291 ng TBT/L, respectively, for the two marinas. Hall et al. also reported that in Back Creek, where these and other marinas were located, concentrations as high as 810 ng TBT/L were observed in the channel. A mean concentration of 222 ng TBT/L was reported for one Back Creek channel station. Following peaks in TBT concentration in early June in these areas, TBT levels were reduced significantly during late

summer and early fall (Hall et al., 1988c), most likely coinciding with decreased pleasure boat activity in these areas.

The potential for sublethal toxicity in the environment is great where high mean TBT concentrations coincide with increased water temperatures during the spring and summer months. The sublethal reproductive effects reported for M. bahia in the present study occur at concentrations (273 ng TBT/L) below the mean TBT concentrations given above for some Maryland marinas over a 4 month period (Hall et al., 1988c). M. bahia, however, is not a resident Chesapeake invertebrate. It is a subtropical species which is typically most abundant at 25 to 30°C. Chesapeake Bay invertebrates have lower thermal tolerance limits than M. bahia, and may be sublethally affected at temperatures typically found there during periods of elevated TBT levels. For animals of sensitivity similar to M. bahia, sublethal toxic effects are possible at the mean TBT concentrations reported in Chesapeake Bay during recent years.

A mysid species indigenous to Chesapeake Bay, Neomysis americana, yielded LC50's similar to those for M. bahia in acute exposures to three toxicants (Roberts et al., 1982). The LC50's for exposures to sodium lauryl sulfate, cadmium, and lannate differed by a factor of two or less. In most cases, 95% fidicial limits were broadly overlapping, supporting a lack of significant differences between the two species or between repeated tests. As mentioned previously, optimal survival and growth for N. americana occurs at temperatures below 25°C (Thompson, 1984), and one would expect that the optimal temperature for reproductive output in this species is lower than that for M. bahia. Thompson (1984) has shown that there is a significant change in the toxicant sensitivity of N. americana at temperatures above 20°C. Mysids at 25°C were more sensitive to toxicant exposure than mysids at 20°C, and sensitivity is predicted to be even

greater at temperatures above 25°C. Temperatures this extreme are easily reached during the periods of high mean TBT concentration in Chesapeake Bay. Although the interaction effect found between TBT and temperature for M. bahia cannot be assumed to apply to all organisms of similar sensitivity, it is well established that animals are more susceptible to toxicant stress near their limits of thermal tolerance. The mean TBT concentrations found in Chesapeake Bay during the spring and summer may be hazardous to indigenous invertebrates at the temperatures typically occuring at those times.

Recommendations for Future Research

Short-term chronic toxicity tests are suitable for testing many substances, but the results of the present test indicate that life cycle toxicity tests are still needed for substances of major ecological risk, like TBT. The mysid 7-day rapid chronic test (Lussier et al., 1986) is a widely-used, sensitive method which is useful in establishing water quality criteria and screening chemicals since results generally predict those from the life cycle test. Results presented by Lussier et al. (1988) show that for seven substances (63% of those tested), chronic values derived from the 7-day static-replacement tests were within a factor of 2.5 of chronic values obtained from life-cycle tests conducted simultaneously. However, the 7-day test was found to be a poor predictor of results from life cycle tests in some cases, a fact which one could not establish this without a side-by-side comparison of the tests.

In the present test, only the effects on survival might have been adequately assessed by the mysid 7-day test. The 28 day LC50 (472 ng TBT/L) was reduced by almost a factor of 2 as compared to the 96 hour LC50 (816 ng TBT/L), but the confidence intervals for these estimates overlap. The mysid 7-day test would

probably have produced an LC50 within the confidence interval of these LC50's, and may have provided useful information from which LC50's for longer test durations could be extrapolated. On the other hand, the effect of TBT on reproductive output detected in the present test would not have been adequately assessed by the 7-day test. The earliest stage of reproductive development of appearance of the first brood, which is similar to the reproductive endpoint examined in the 7-day test, was not affected by TBT exposure at the levels tested. The number of offspring per FRD was significantly reduced at 273 ng TBT/L in the present test, while TBT exposure levels higher by nearly a factor of 2 (539 ng TBT/L) showed no effect on earlier reproductive responses. The use of short-term chronic tests should be avoided in some cases, depending on the compound being tested and the biological response of interest.

The results of the present test confirm the necessity of conducting toxicity tests with interacting environmental factors like temperature, and partcularly long-term, sublethal studies. However, considerable attention must be given to the methods of statistical analysis applied to these types of tests. Data from toxicity tests dealing with reproductive output and growth typically have high inherent variability, and the present study was further complicated by the inclusion of temperature as a test factor. These factors often make design of tests for analysis with factorial ANOVA models impractical. Regression analysis is widely used to analyze acute toxicity test data, and these analyses may also appropriately be applied to chronic toxicity test data (Stephan & Rogers, 1982). Voyer & Modica (1990) state that regression coupled with response surface techniques have utility in developing data bases for substances whose toxicity is water quality dependent.

The step-wise multiple regression analysis used in the present study to examine the number of offspring per FRD identified significant linear and quadratic

responses to temperature and TBT concentration. Due to the high variance associated with the biological response data in this study, however, the regression mentioned above was the only one which produced an acceptable predictive equation. On the other hand, Voyer & Modica (1990) were successful in using multiple regression analysis coupled with response surface techniques to obtain estimates of cadmium toxicity to M. bahia as modified by salinity. In those experiments, 96 hour flow-through tests were conducted to examine the linear, quadratic, and interactive effects of cadmium and salinity. Coefficients of variation (r^2) from multiple regression analyses were 0.90 and 0.92, and indicated a good fit of the regression models to the data. Voyer & Modica concluded that 96 hour LC50's, derived using response surface techniques, compared favorably with those determined by conventional acute toxicity test methods, and that the former experimental approach provides an economical means of evaluating the potential impact of several factors applied simultaneously.

LITERATURE CITED

Alderice, D.F. 1972. Factor combinations: responses of marine poikilotherms to environmental factors acting in concert. pp. 1659-1722 In, Marine Ecology. Volume 1. Environmental Factors, Part 3. edited by O. Kinne, Wiley Interscience, London.

ASTM. 1987. Standard Guide for Conducting Life-Cycle Toxicity Tests with Saltmeter Mysids. ASTM Designation E 1191-87, pp. 710-725, Annual Book of ASTM Standards, vol. 14.04.

Beaumont, A.R. & Budd, M.D. 1984. High mortality of the larvea of the common mussel at low concentrations of tributyltin. Mar. Poll. Bull. 15:402-405.

Bryan, G.W., Gibbs, P.E., Hummerstone, L.G. and Burt, G.R. 1986. The decline of the gastropod Nucella lapillus around south-west England: evidence for the effect of tributyltin from antifouling paints. J. Mar. Biol. Ass. U.K. 66:611-640.

Bryan, G.W., Gibbs, P.E., Huggett, R.J., Curtis, L.A., Bailey, D.S. and Dauer, D.M. 1989. Effects of tributyltin pollution on the mud snail, Illyanassa obsoleta, from the York River and Sarah Creek, Chesapeake Bay. Mar. Poll. Bull. 20:458-462.

Bryant, V., McLusky, D.S., Roddie, K. and Newbery, D.M. 1984. Effect of temperature and salinity on the toxicity of chromium to three estuarine invertebrates (Corophium volutator, Macoma balthica, Nereis diversicolor). Mar. Ecol. Prog. Ser. 24:139-153.

Bushong, S.J., Hall, L.W., Jr., Johnson, W.E., Hall, W.S. and Ziegenfuss, M.C. 1987. Acute and chronic toxicity of tributyltin to selected Chesapeake Bay fish and invertebrates. 64 pp. Final Report. The Johns Hopkins University, Baltimore, MD.

Bushong, S.J., Zeigenfuss M.C., Unger M.A. and Hall L.W., Jr. 1990. Chronic tributlytin toxicity experiments with the Chesapeake Bay Copepod, Acartia tonsa. Environ. Tox. Chem. 9:359-366.

Cairns, J., Jr., Heath, A.G. and Parker, B.C. 1975a. Temperature influence on chemical toxicity to aquatic organisms. J. Water Poll. Control Fed. 47:267-280.

Cairns, J., Jr., Heath, A.G. and Parker, B.C. 1975b. The effects of temperature upon the toxicity of chemicals to aquatic organisms. Hydrobiologia 47:135-171.

Davidson, B.M., Valkirs, A.O. and Seligman, P.F. 1986. Acute and chronic effects of tributyltin on the mysid, Acanthomysis sculpta (Crustacea, Mysidacea). pp. 1219-1225. In, Proc. Organotin Symposium of the Oceans '86 Conference, Washington D.C., Vol. 4, New York.

De Lisle, P.F. & Roberts, M.H., Jr. 1986. The effect of acclimation on salinity tolerance of the mysid, Mysidopsis bahia Molenock. Comp. Biochem. Physiol. 85A:383-387.

Gentile, J.H., Gentile, S.M., Hairston, N.G., Jr. and Sullivan, B.K. 1982a. The use of life-tables for evaluating the chronic toxicity of pollutants to Mysidopsis bahia. Hydrobiologia 93:179-187.

Gentile, S.M., Gentile, J.H., Walker, J. and Heltshe, J.F. 1982b. Chronic effects of cadmium on two species of mysid shrimp: Mysidopsis bahia and Mysidopsis bigelowi. Hydrobiologia 93:195-204.

Gentile, J.H., Gentile, S.M., Hoffman, G., Heltshe, J.F. and Hairston, N., Jr. 1983. The effects of a chronic mercury exposure on survival, reproduction, and population dynamics of Mysidopsis bahia. Environ. Toxicol Chem. 2:61-68.

Goodman, L.R., Cripe, G.M., Moody, P.H. and Halsell, D.G. 1988. Acute toxicity of Malathion, Tetrabromobisphenol-A, and Tributlytin Chloride to mysids (Mysidopsis bahia) of three ages. Bull. Environ. Contam. Toxicol. 41:746-753.

Hall, L.W., Jr. 1988b. Tributyltin environmental studies in Chesapeake Bay. Mar. Poll. Bull. 19:431-438.

Hall, L.W., Jr., Bushong, S.J., Hall, W.S. and Johnson, W.E. 1988a. Acute and chronic effects of tributyltin on a Chesapeake Bay copepod. Environ. Toxicol. Chem. 7:41-46.

Hall, L.W., Jr., Bushong, S.J., Johnson, W.E. and Hall, W.S. 1988c. Spatial and temporal distribution of butyltin compounds in a northern Chesapeake Bay marina and river system. Environ. Monit. Assess. 10:229-244.

Hall, L.W., Jr., Bushong, S.J., Ziegenfuss, M.C., Johnson, W.E., Herman, R.L. and Wright, D.A. 1988d. Chronic toxicity of tributlytin to Chesapeake Bay biota. Water Soil Air Poll., 39:365-376.

Hall, L.W., Jr., Lenkevich, M.J., Hall, W.S., Pinkney, A.E. and Bushong, S.J. 1987. Evaluation of butyltin compounds in Maryland waters of Chesapeake Bay. Mar. Poll. Bull. 18:78-83.

Hall, L.W., Jr. & Pinkney, A.E. 1985. Acute and sublethal effects of organotin compounds on aquatic biota: An interpretive literature evaluation. CRC Crit. Rev. Toxicol. 14:159-209.

Hargreaves, B.R., Smith, R.L., Thompson, C.Q. and Herman, S.S., 1982. Toxicity and accumulation of Naphthalene in the mysid, Neomysis americana (Smith), and effects of environmental temperature. pp.391-412. In, Physiological Mechanisms of Marine Pollution Toxicity, edited by Vernberg, W.B. et al., Academic Press, N.Y.

Henderson, R.S. 1986. Effects of organotin antifouling paint leachates on Pearl Harbor organisms: A site specific flowthrough bioassay. pp. 1226-1233. In, Proc. Organotin Symposium of the Oceans '86 Conference, Washington D.C., Vol. 4, New York.

Huggett, R.J. & Westbrook, D.J. 1987. Project 7: TBT in marine areas and freshwater effluent. Cooperative State Agency Program, Annual Report F: (1986/87). The College of William and Mary, Va. Inst. Mar. Sci., Gloucester Pt., VA.

Johansen, K. & Moehlenberg, F. 1987. Impairment of egg production in Acartia tonsa exposed to tributyltin oxide. Ophelia, 27:137-141.

Kathman, R. D., Austin, W. C. Saltman, J. C. and Fulton, J. D. 1986. Identification Manual to the Mysidacea and Euphausiacea of the Northwest Pacific. Canadian Special Publication of Fisheries and Aquatic Sciences 93:1-411 pp.

Kinne, O. 1970. Temperature: animals-invertebrates. pp. 407-514. In, Marine Ecology. Volume 1. Environmental Factors, Part 1. edited by O. Kinne. Wiley Interscience, London.

Kuhlmann, D. 1984. Effects of temperature, salinity, oxygen and ammonia on the mortality and growth of Neomysis integer (Leach). Limnologica 15:479-485.

Laughlin, R.B. & French, W.J. 1980. Comparative study of the acute toxicity of a homologous series of trialkyltins to larval shore crabs, Hemigrapsus nudus, and lobster, Homarus americanus. Bull. Environ. Contam. Toxicol. 25:802-809.

Laughlin, R.B., Jr., Gustafson, R.G. and Pendoley, P. 1989. Acute toxicity of tributyltin (TBT) to early life history stages of the hard shell clam, Mercenaria mercenaria. Bull. Environ. Contam. Toxicol. 42:352-358.

Laughlin, R.B., Jr. & Linden, O. 1987. Tributlytin - Contemporary environmental issues. Ambio 16:252-256.

Laughlin, R.B., Nordlund, K. and Linden, O. 1984. Long-term effects of tributyltin compounds on the Baltic amphipod, Gammarus oceanicus. Mar. Environ. Res. 12:243-271.

Lawler, I.F. & Aldrich, J.C. 1987. Sublethal effects of Bis(tri-n-butyltin) oxide on Crassostrea gigas spat. Mar. Poll. Bull. 18: 274-278.

Lussier, S.M., Gentile, J.H. and Walker, J. 1985. Acute and chronic effects of heavy metals and cyanide on Mysopsis bahia (Crustacea: Mysidacea). Aquat. Toxicol. 7:25-35.

Lussier, S. M., Kuhn, A. and Sewall, J. 1986. Guidance manual for conducting seven day mysid survival/growth/reproduction study using the estuarine mysid, Mysidopsis bahia. pp. 86-124. In, Users guide to the Conduct and Interpretation of Complex Effluent Toxicity Tests at Estuarine/Marine Sites. edited by, S.C. Schimmel, Environmental Research Laboratory, U.S. Environmental Protection Agency, Narragansett, RI.

Lussier, S.M., Kuhn, A. and Comeleo, R. 1988. Comparison of results from 7-day and life-cycle tests with Mysidopsis bahia. p. 113. In, Abstracts, Presented at Ninth Annual Meeting of the Society of Environmental Toxicology and Chemistry, November 13 to 17, 1988, Arlington, VA.

Maguire, J.R. 1987. Environmental aspects of tributyltin. Appl. Organomet. Chem. 1:475-498.

Markle, D.F. & Grant, G.C. 1970. The summer food habits of young-of-the-year striped bass in three VA rivers. Chesapeake Sci. 11:50-54.

McKenney, C.L., Jr. 1982. Interrelationships between energy metabolism, growth dynamics, and reproduction during the life cycle of Mysidopsis bahia as influenced by sublethal endrin exposure. pp. 447-476 In, Physiological Mechanisms of Marine Pollutant Toxicity, edited by Vernberg, W.B., Calabrese, A., Therberg, F.R. and Vernberg, F.J., Academic Press, New York.

McKenney, C.L., Jr. 1985. Associations between physiological alterations and population changes in an estuarine mysid during chronic exposure to a pesticide. Belle W. Baruch Libr. Mar. Sci. 13:397-418.

McKenney, C.L., Jr. 1986. Influence of the organophosphate insecticide fenthion on Mysoidpsis bahia exposed during a complete life cycle. 1. Survival, reproduction, and age-specific growth. Dis. Aquat. Org. 1:131-139.

McKenney, C.L., Jr. 1987. Optimization of environmental factors during the life cycle of Mysidopsis bahia. EPA Environmental Research Brief, EPA/600/M-87/004, 6 pp.

McLusky, D.S., Bryant, V. and Campbell, R. 1986. The effects of temperature and salinity on the toxicity of heavy metals to marine and estuarine invertebrates. Oceanogr. Mar. Biol., Ann. Rev. 24:481-520.

McLusky, D.S. & Hagerman, L. 1987. The toxicity of chromium, nickel and zinc: effects of salinity and temperature, and the osmoregulatory consequences in the mysid Praunnus flexuosus. Aquat. Toxicol. 10:225-238.

Nimmo, D.R., Bahner, L.H., Rigby, R.A., Sheppard, J.M. and Wilson, A.J., Jr. 1977. Mysidopsis bahia: an estuarine species suitable for life-cycle toxicity tests to determine the effect of a pollutant. pp. 109-116. In, Aquatic Toxicology and Hazard Evaluation, ASTM STP 634, edited by Mayer, F.L. & Hamelink, J.L., American Society for Testing and Materials, Philadelphia.

Nimmo, D.R. & Hamaker, T.L. 1982. Mysids in toxicity testing - A review. Hydrobiologia 93:171-178.

Pezzack, D.S. & Corey, S. 1982. Effects of temperature and salinity on immature and juvenile Neomysis americana (Smith). Can. J. Zool. 60:2725-2728.

Prosser, C.L. 1973. Comparative Animal Physiology, 3rd ed., Saunders, Philadelphia, 966 pp.

Rexrode, M., 1987. Ecotoxicity of tributyltin. pp. 1443-1455. In, Oceans '87: Proceedings International Organotin Symposium, Halifax, Nova Scotia, Vol. 4, New York.

Roberts, M.H., Jr. 1987. Acute toxicity of tributyltin chloride to embryos and larvae of two bivalve mollusks, Crassostrea virginica and Mercenaria mercenaria. Bull. Environ. Contam. Toxicol. 39:1012-1019.

Roberts, M. H., Jr., Warinner, J. E., Tsai, C.-F., Wright, D. and Cronin, L. E. 1982. Comparison of estuarine species sensitivities to three toxicants. Arch. Environ. Contam. Toxicol. 11:681-692.

Salazar, M.H. 1986. Environmental significance and interpretation of organotin bioassays. pp. 1240-1245. In, Proc. Organotin Symposium of the Oceans '86 Conference, Washington D.C., Vol. 4, New York.

Salazar, M.H. & Salazar, S.M. 1987. Tributyltin effects on juvenile mussel growth. pp. 1504-1510. In, Oceans '87: Proceedings International Organotin Symposium, Halifax, Nova Scotia, Vol. 4, New York.

Salazar, M.H. & Champ, M.A. 1988. TBT and water quality: a question of environmental significance. pp. 1497-1506. In, Oceans '88 Proceedings, Baltimore, Vol. 4, New York.

SAS Institute Inc. 1985. SAS User's Guide: Statistics, Version 5 Edition. Cary, N.C.: SAS Institute Inc., 956 pp.

Saxena, A.K. 1987. Organotin compounds: Toxicology and biomedical applications. Appl. Organomet. Chem. 1:39-56.

Seligman, P.F., Grovhoug, J.G., Valkirs, A.O., Stang, P.M., Fransham, R., Stallard, M.O., Davidson, B. and Lee, R.F. 1989. Distribution and fate of tributyltin in the United States marine environment. Appl. Organomet. Chem. 3:31-47.

Smith, S.M., Hoff, J.D., O'Neil, S.P. and Weinstein, M.P. 1984. Community and trophic organization of nekton utilizing shallow marsh habitats, York River, VA. Fish. Bull. 82:455-467.

Sokal, R.R. & Rohlf, F.J. 1981. Biometry. 2nd edition, W.H. Freeman and Company, New York, 859pp.

Sprague, J.B., 1985. Factors that modify toxicity. pp. 124-163. In, Fundamentals of Aquatic Toxicology, edited by Rand, G.M. & Petrocelli, S.R., Hemisphere Publishing Corp., Washington D.C..

SPSSx 1985. SPSSx Graphics. SPSS Inc., McGraw-Hill Book Co., New York, 305pp.

SPSSX 1986. SPSSx User's Guide, 2nd edition. SPSS Inc., McGraw-Hill Book Company, New York, 988pp.

Stephan, C.E. 1977. Methods for calculating an LC50. pp. 65-84. In, Aquatic Toxicology and Hazard Evaluation, edited by F.L. Mayer & J.L. Hamelink, ASTM STP 634, American Society for Testing and Materials, Philadelphia.

Stephan, C.E. & Rogers, J.W. 1985. Advantages of using regression analysis to calculate results of chronic toxicity tests. pp. 328-338. In, Aquatic Toxicology and Hazard Assessment, edited by R.C. Bahner & D.J. Hansen, ASTM STP 891, American Society for Testing and Materials, Pihladelphia.

Thain, J.E. 1986. Toxicity of TBT to bivalves: Effects on reproduction, growth and survival. pp. 1306-1313. In, Proc. Organotin Symposium of the Oceans '86 Conference, Washington D.C., Vol. 4, New York.

Thompson, C.Q. 1984. The effects of season and temperature on growth, reproduction and hardiness of the opossum shrimp Neomysis americana (Smith). Ph.D. Dissertation, Lehigh Univ., 181 pp.

Toda, H., Takahashi, M. and Ichimura, S. 1984. The effect of temperature on the post-embryonic growth of Neomysis intermedia Czerniawsky (Crustacea, Mysidacea) under laboratory conditions. J. Plankton Res. 6:647-662.

Unger, M.A. 1988. Investigation of tributyltin water/sediment interactions. Ph.D. Dissertation, The College of William and Mary, 80 pp.

Unger, M.A., MacIntyre, W.G., Greaves, J. and Huggett, R.J. 1986. GC determination of butyltins in natural waters by flame photometric detection of hexyl derivatives with mass spectrometric confirmation. Chemosphere 15:461-470.

U'ren, S.C. 1983. Acute toxicity of bis(tributyl-tin) oxide to a marine copepod. Mar. Poll. Bull. 14:303-306.

Valkirs, A.O., Davidson, B.M. and Seligman, P.F. 1987. Sublethal growth effects and mortality to marine bivalves from long-term exposure to tributyltin. Chemosphere 16:201-220.

Vernberg, W.B. & Vernberg, F.J. 1972. Environmental Physiology of Marine Animals. Springer-Verlag, New York, 346pp.

Vernberg, F.J. & Vernberg, W.B. 1981. Functional Adaptations of Marine Organisms. Academic Press, New York, 347 pp.

Voyer, R.A. & Modica, G. 1990. Influence of salinity and temperature on acute toxicity of cadmium to Mysidopsis bahia Molenock. Arch. Environ. Contam. Toxicol., 19:124-131.

Waldock, M.J. & Thain, J.E. 1983. Shell thickening in Crassostrea gigas: Organotin antifouling or sediment induced? Mar. Poll. Bull. 14:411-415.

Westbrook, D.J., Travelstead, E.J., Espourteille, F.A., Rice, C.D. and Huggett, R.J. 1986. Tributyltin in whole water and sediment collected near marinas and the Hampton Roads area in the southern Chesapeake Bay. Final Report to The Johns Hopkins Univ. Applied Physics Laboratory, contract no. 507242-0. College of William and Mary, Va. Inst. Mar. Sci., Gloucester Pt., VA.

Wright, D.A. & Roosenberg, W.H. 1982. Trimethyltin toxicity to larval Uca pugilator: effects of temperature and salinity. Arch. Environ. Contam. Toxicol. 11:491-

Zar, J.H. 1984. Biostatistical Analysis, second edition. Prentice-Hall, Inc., Englewood Cliffs, NJ, 718 pp.

VITA

PRENTISS H. BALCOM

Born in New London, CT, 23 August 1961. Graduated from Norwich Free Academy High School, Norwich, CT in 1979. Earned B.S. in Biological Sciences from the University of Connecticut in 1983. Employed by the University of Connecticut Marine Sciences Institute from 1983 to 1985. Entered Masters program at the School of Marine Science of The College of William and Mary in 1986. Currently employed by the Malcolm Pirnie, Inc., Tarrytown, NY.