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Life-cycle toxicity of dibutyltin to the sheepshead minnow (*Cyprinodon variegatus*) and implications of the ubiquitous tributyltin impurity in test material

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Dibutyltin (DBT) is used in the plastics polymerization process as a catalyst in polyvinyl chloride (PVC) products and is the primary degradation product of tributyltin (TBT), an antifoulant in marine paint. DBT and other organotin compounds make their way into the environment through antifoulants, PVC processing plants, and PVC products maintained in water and water-handling systems. A flow-through saltwater life-cycle toxicity test was conducted to determine the chronic effect of DBT to the sheepshead minnow (*Cyprinodon variegatus* Lacepede), an estuarine species. Embryos were monitored through hatch, maturation, growth, and reproduction in DBT concentrations of 158, 286, 453, 887, and 1510 μg l^{-1}. Progeny were monitored for survival as embryos and fry/juveniles, and growth for 30 days post-isolation. Mean length of parental generation fish was significantly reduced on day 30 at DBT concentrations ≥887 μg l^{-1}, setting the lowest observable effect concentration (LOEC) at 887 μg l^{-1} and the no observable effect concentration (NOEC) at 453 μg l^{-1}. Fecundity, as egg viability, was significantly reduced at the LOEC. Survival of parental and progeny generation embryos and mean length, wet weight and dry weight of progeny generation juveniles were not significantly affected at concentrations ≤LOEC. TBT, a toxic impurity in DBT reversibly produced in DBT by the process of comproportionation, was also monitored throughout this study. Comparing measured levels of TBT in this study with levels exerting toxic effects in an earlier TBT life-cycle study with *C. variegatus* suggests biological responses in this study were likely due to the TBT impurity and not to DBT alone. Results indicate that TBT impurity as low as 0.1% may have a significant influence on the perceived toxicity of DBT and that spontaneous production of TBT in DBT may be the major source of biological toxicity of DBT. Copyright © 2003 John Wiley & Sons, Ltd.

KEYWORDS: dibutyltin; *Cyprinodon variegatus*; tributyltin; life-cycle toxicity; PVC additives; toxicant impurities; antifoulants

INTRODUCTION

Dibutyltin (DBT) is used in the stabilization of the plastics polymerization process, and as a catalyst in polyvinyl chloride (PVC) products.[1] Approximately 70% of the total annual world production of non-pesticidal organotin compounds are used in the thermal and UV stabilization of PVC. Approximately 27 000 tons of DBT and monobutyltin (MBT) is used each year as stabilizers and catalysts. These organotin compounds make their way into the environment through PVC processing plants and PVC products maintained in water and water-handling systems.[2] Although not used as a pesticide, DBT also finds its way into environmental systems as the primary degradation product of tributyltin (TBT), an active ingredient used as an antifoulant in marine paint.[3]

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TBT is a highly toxic substance shown to exhibit acute and chronic effects to both marine and freshwater nontarget organisms.[2,4] DBT and other organotins have been identified in sewage and waste treatment facilities,[5] in foods,[6] fish products,[7] containers used in food storage[8,9] and in drinking water.[10] Residues of DBT and other organotins have been identified in the tissues of fish, birds, terrestrial mammals, and humans.[11] DBT has been shown to be a potential health risk as a thymolitic and immunotoxic agent in rats, mice, and humans,[6,12] in addition to being a teratogen in rats[13–15] and the marine tunicate, *Styeka plicata*.[16] With an ever-increasing demand on the production of plastic products, the potential exists for increasing environmental occurrence of organotins. Currently, marine and freshwater organisms are subject to exposure to DBT and other organotins in water, sediments, and diet in coastal waters contaminated by organotins.[9,17–22] The persistent nature of these compounds suggests chronic exposure to aquatic organisms and the need for long-term studies encompassing all critical life stages to assess the potential ecological impact more accurately.

A flow-through saltwater life-cycle toxicity test was conducted at the Gulf Coast Research Laboratory (GCRL) in Ocean Springs, MS, to determine the chronic effect of DBT to the sheepshead minnow (*Cyprinodon variegatus* Lacepede). The purpose of this study was to determine the long-term effect of DBT on survival, growth, reproduction, and progeny under flow-through conditions.

The study was initiated with less than 24-h-old embryos (F_0 generation) isolated from culture, monitored through hatch, growth, maturation, and reproduction, and concluded 30 days post-isolation of the F_1 generation. The F_0 generation was evaluated for embryo survival, survival and growth of juveniles after exposure for 30, 61, and 90 days, and survival, reproduction, and growth of F_0 adults. Embryo survival, juvenile survival, and growth 30 days post-isolation were evaluated for the F_1 generation. Significant effects were observed at lower DBT concentrations for F_0 than F_1 generation fish, yielding an overall lowest observable effect concentration (LOEC) at 887 $\mu g\ l^{-1}$ and a no observable effect concentration (NOEC) at 453 $\mu g\ l^{-1}$.

It is suggested that some overall biological effects resulting during exposure to DBT may be due to the persistent and unavoidable impurity, TBT, in the test compound and that this impurity may have been the root cause of some toxic effects encountered in previous DBT toxicity tests.[23] Although the influence of TBT as an impurity on the toxicological evaluation of DBT has been suggested in earlier studies, to our knowledge this is the first direct correlation of TBT concentrations and toxic responses in a DBT life-cycle study to TBT concentrations and toxic responses obtained in a previous study with the same organisms using only TBT.[24] This comparison lends strong support to the conclusion that some of the observed biological consequences arising from DBT exposure in this study and other DBT studies are due to TBT impurity.

MATERIALS AND METHODS

Test substance

The test substance, DBT dichloride, NB No. 5813-1-3, was received on 26 November 1990 from Atochem North America, Woodbridge, NJ. The Analytical Chemistry Section of GCRL determined the percentage distribution of total butyltins to be 99.84% DBT, 0.087% TBT and 0.077% MBT. We have no evidence that the analytical process itself is responsible for either the TBT or MBT. Tetrabutyltin (TTBT) was not detected in the analysis of test substance. Exhaustive purification of the test compound failed to yield a test substance completely free of TBT and MBT. These latter two compounds are formed from the reversible comproportionation reaction of DBT ($2DBT \leftrightarrow TBT + MBT$) and, as such, are not entirely avoidable.[25] Based upon our experience, these impurities cannot be reduced to levels much below 0.1% DBT. The inevitable presence of these impurities, particularly TBT, is significant because of high TBT toxicity. Concentrations of DBT and other butyltins in ionic form (DBT^{2+}) are reported in micrograms of DBT per liter of seawater.

Test organism

Sheepshead minnow embryos were obtained from a continuous culture maintained at GCRL. Adults selected as spawners were maintained in a static recirculating system at 15‰ salinity. The temperature in the system was raised from room temperature ($\sim 24 \pm 2\,^{\circ}C$) to $30 \pm 2\,^{\circ}C$ for 2 weeks prior to collection of study embryos. Embryos were naturally spawned onto several cylindrical filter sponges, each approximately 6 cm in length, placed in the tank the afternoon before study initiation. Embryos were less than 24 h old at initiation of the life-cycle toxicity test.

Dilution water

Salt water used for culture and testing was filtered (10 μm) natural seawater collected from Santa Rosa Sound near Pensacola, FL. The dilution water was adjusted to a salinity of approximately 15‰ with unchlorinated well water from the GCRL facility. Prior to introduction into the test system, diluent water was passed through both a carbon filter and ultraviolet sterilizer. Chemical characterizations of both dilution waters found no detectable organochlorine and organophosphate pesticides, polychlorinated biphenyls (PCBs) or organotins.

Test methods

The test system included a modification of a Mount and Brungs[26] diluter system using a dilution factor of 0.5. The system also included 12 glass exposure aquaria ($90 \times 45 \times 26\ cm^3$) designed to contain approximately 57 l of test solution or dilution water by maintaining an overflow level of 14 cm. A dilution water control, and nominal test DBT concentrations of 92, 184, 368, 735, and 1470 μg DTB l^{-1} were run in duplicate during the study. The concentrations selected were based upon the results of preliminary embryo/juvenile exposures.

Test solutions were prepared fresh with each diluter cycle using Hamilton precision liquid dispensers (PLD-II) to deliver DBT stock solution to the toxicant mixing box of the diluter. At each cycle the diluter delivered 1 l of test solution to splitter boxes which dispensed 0.5 l to each of two replicates per treatment.

Solubility of DBT in 15‰ water was found to be too low for preparing usable stock solutions; therefore, stock solutions were prepared by adding 11.25 g of DBT to a glass carboy containing 45 l of distilled water, and mixing for approximately 3 h with a chemical mixing pump (Voigt-England, Model D 12-C). Care was exercised to keep quantities of DBT below saturation levels to keep accompanying TBT concentrations at a minimum. The stock solution was later transferred through a 10 μm filter into another 45 l carboy, from which it was dispensed during each diluter cycle. The dilution water control received a volume of distilled water greater than or equal to the volume of the DBT-amended stock solution delivered to any exposure aquarium.

Test water temperature was maintained at $30 \pm 1\,^{\circ}C$ with a heated recirculating water bath. A 16 h light and 8 h dark photoperiod with a 15 min dimmer system to simulate dawn and dusk was maintained. Light intensity across treatments was 393 ± 54 lx, as measured 2.5 cm above the water surface.

The biological methods described below were developed from methods described by Hansen *et al*.[27] and Rexrode and Armitage.[28]

Parental generation embryo and juvenile survival

The test was initiated when 25 microscopically confirmed embryos (i.e. F_0 generation) were impartially added to each of two retention chambers per treatment replicate (50 embryos per replicate; 100 embryos per treatment). Retention chambers were 150 mm Petri dish bottoms with an attached 15 cm tall 40-mesh (475 μm pore size) nylon collar. Embryos were removed daily, rinsed with dilution water, and counted. Dead embryos were discarded and live embryos were returned to the retention chamber.

After hatching, juvenile fish were fed twice daily with brine shrimp (*Artemia salina*) nauplii hatched from cysts obtained from Aquarium Products, Glen Burnie, MD. The only exception was on day 5, when the fish were fed twice with nauplii hatched from cysts obtained from Neptune Industries, Salt Lake City, UT.

Survival of juvenile fish was monitored daily and any changes in physical appearance or behavior recorded. On day 30, juveniles were removed, photographed for length determinations, and impartially thinned to 25 fish per treatment replicate prior to returning them to their treatment of origin. Fish not returned to the treatment chambers were anesthetized and wet weighed (towel dried).

Maturation and growth

Following thinning, fish were monitored for survival, growth, and physical and behavioral effects as they matured to adults. On days 61 and 90 of the study, all fish were removed from each treatment replicate and photographed for length determinations.

While maturing, during reproduction, and up to test termination, fish were fed three times daily. Each feeding consisted of a single food type, either brine shrimp nauplii (Aquarium Products, Glen Burnie, MD), frozen adult brine shrimp (San Francisco Bay Brand Frozen Brine Shrimp, Newark, CA), or commercial flake food (Stress Flakes, Novalek, Hayward, CA). All foods were analyzed and found to contain less than detectable limits of DBT, MBT, TBT, TTBT, organochlorine and organophosphate pesticides, and PCBs.

Reproduction

The reproductive phase of the study was initiated after observation of physical (dimorphic coloration, etc.), and behavioral (territorialism by males, spawning) characteristics displayed by sexually mature fish. Reproductive data were collected by isolating spawning groups in each of the treatment replicates. Each spawning group was formed by sequestering a sample of up to five mature fish from a given tank into a spawning chamber placed into their tank. Since each tank could accommodate only one spawning chamber at a time, it was necessary to conduct multiple spawning trials to provide as much reproductive information as possible.

On day 107, when greater than 50% of the fish in all concentrations could be accurately sexed, the first of three spawning groups was selected. The second spawning group was selected on day 128, and the third and final spawning group on day 149. During each trial, spawning groups were formed in each of the treatment replicates with sufficient numbers of surviving fish. There were no spawning groups selected from one of the two $1510\,\mu g\,l^{-1}$ DBT treatment replicates during reproduction because surviving fish did not reach sexual maturity by study termination.

Spawning chambers were of glass construction $20 \times 35 \times 22\,cm^3$ with a 0.5 cm Teflon® mesh bottom to allow eggs to pass out of the chamber. Two days following the placement of adult fish in spawning chambers, a 0.5 mm mesh screen tray was placed under each chamber to collect spawned eggs. During each trial, eggs were harvested daily for 14 consecutive days, rinsed with dilution water, counted, and then assessed microscopically for viability. At the end of the egg collection period, all spawners were removed from their respective chambers, measured to the nearest 1 mm in standard length, and weighed (towel dried) to the nearest milligram. Dry weight determinations were obtained after placing fish in a drying oven at $60\,^{\circ}C$ overnight and transferring them to a desiccator for cooling.

F_1 generation

The progeny generation was initiated with eggs harvested during the reproductive phase of the study. During spawning trials, embryos were isolated in retention chambers and placed in the same treatment replicate from which they were spawned.

F_1 generation embryos and juveniles were treated in the same manner as described for the F_0 generation during the first 30 days of the study. Upon removal from the retention chambers at 30 days post-isolation, standard length was directly determined, and fish were weighed after being blotted dry. Dry weights were determined in the same manner as used for the parental fish.

Analytical chemistry

One 200 ml water sample was collected from one control replicate and each DBT concentration replicate once a week throughout the study for measurement of DBT concentrations. Samples were taken from below the water surface in an area not in contact with chamber walls. Water samples were collected in 200 ml volumetric flasks and processed on the day of collection.

DBT concentrations were quantified by a modification of the Uhler and Steinhauer[29] method. Samples were extracted three times with 0.05% tropolone in hexane. Combined extracts were first dried over anhydrous sodium sulfate, then reduced in volume via rotary evaporation. The internal standard, tripropyltin chloride, was then added in an amount consistent with DBT levels expected in the samples. Subsequent addition of *n*-pentylmagnesium bromide converted the butyltins to the corresponding fully substituted pentyl derivatives. The reaction was quenched with 5 M H_2SO_4, and the derivatives were purified by Florisil adsorption chromatography. Final volume reduction to 25 ml was followed by gas chromatography on a fused silica DB-5 capillary column contained in a Perkin–Elmer™ 8500 gas chromatograph with flame photometric detector modified with photocells and photometric filters chosen for optimum sensitivity to butyltins and combined with a Perkin–Elmer Nelson™ software supported computerized data system. Identification of butyltins was achieved by comparison of relative retention times with authentic standards of all four butyltins with quantitation following internal standard techniques. Detection limits for this procedure based upon ionic species were: 3.7 μg l^{-1} for TTBT; 1.3 μg l^{-1} for TBT; 1.2 μg l^{-1} for DBT; 2.6 μg l^{-1} for MBT.

The two high treatment concentrations were also monitored for TBT because it was anticipated that the TBT concentration in these treatments might approach the NOEC and LOEC levels for TBT determined in an earlier life-cycle study by Manning *et al.*,[24] i.e. 0.42 μg l^{-1} and 0.66 μg l^{-1} respectively. Accordingly, samples taken from the two high treatment concentrations at each sampling event were processed according to an alternate procedure, yielding a method detection limit for TBT below its NOEC level. The sample processing was the same as that used for DBT analysis, but with a final volume reduction to 200 μl and an internal standard adjusted to match the expected level of TBT. Method detection limits (ionic species) were: 0.034 μg l^{-1} for TTBT; 0.075 μg l^{-1} for TBT; 0.125 μg l^{-1} for DBT; 0.073 μg l^{-1} for MBT.

Statistical analysis

Since each DBT concentration was represented by duplicate aquaria, continuous data (i.e. growth and fecundity) were analyzed by a hierarchical or nested analysis of variance.[30,31] When the *F* value for the treatment effect was significant, all exposure concentration responses were compared with the control response using a two-tailed Dunnett's test.[31,32] Percentage viability of eggs collected during the reproductive segment of the study was also compared across all three spawning trials using percentage viable values across replicates, and across spawning trials as treatment observations with the EPA Dunnett's Program Version 1.1.[33]

Dichotomous data (embryo, juvenile, and parental survival) were analyzed using 2×2 contingency tables. The two-tailed Fisher exact test[31] was used to determine differences between replicates of each treatment group. Replicate responses were then pooled, and each treatment compared with the control using chi-square analysis of contingency tables.[31] The significance level used in all statistical tests was 0.05.

Quality control/quality assurance

The Quality Assurance Unit of GCRL inspected all critical phases during the course of the study to assure that equipment, personnel, procedures, and records conformed to laboratory standard operating procedures. The study was conducted in accordance with the Good Laboratory Practices standards promulgated by the US EPA–FIFRA.[34]

RESULTS AND DISCUSSION

Water chemistry

Generally, variability in measured concentrations appeared to decrease as concentrations increased. The geometric mean concentrations of DBT in each of the treatments ranged from 158 to 1510 μg l^{-1} with coefficients of variation ranging from 14.6 to 65.8%. TBT levels measured throughout the study were reasonably close to the expected theoretical amounts that would be found in DBT based upon impurity levels. The mean concentrations of TBT found in the 887 μg l^{-1} and 1510 μg l^{-1} DBT treatments were 0.44 μg l^{-1} and 0.64 μg l^{-1} TBT respectively. The measured TBT concentrations for these two treatments reflect closely the NOEC (i.e. 0.42 μg l^{-1} TBT) and LOEC (i.e. 0.66 μg l^{-1} TBT) for TBT determined in an earlier study.[24] Controls were free of butyltins (i.e. TTBT, TBT, DBT, and MBT) at all samplings (Table 1).

Biological results

F_0 generation survival

F_0 generation embryo survival was not affected by DBT concentrations ≤1510 μg l^{-1} and survival was 94% or greater in all treatments (Fig. 1). Survival of juveniles at day 30 was significantly reduced at 1510 μg l^{-1} DBT, but not lower

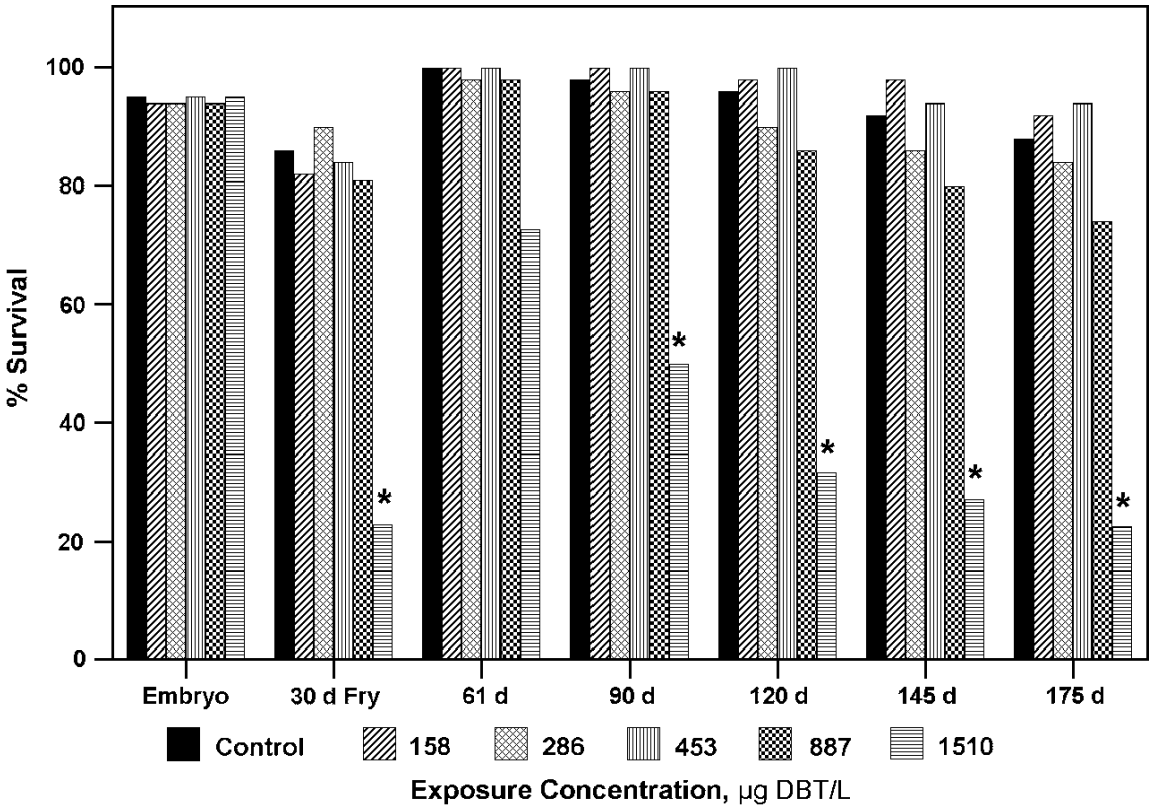

Figure 1. Survival of F_0 generation sheepshead minnow exposed to 158 to 1510 µg DBT/L at time periods from embryo to 175 days. Asterisks indicate significantly reduced relative to the control ($p < 0.05$).

Table 1. Measured DBT concentrations during the 191 day study

DBT concentration (µg l^{-1}, ionic species)			
Nominal	Mean measured[a]	CV[b]	*N*[c]
Control	nd[d]	—	26
92	158	65.8	26
184	286	40.7	26
368	453	34.1	26
735	887	20.9	27
1470	1510	14.6	26

[a] Geometric mean of all measurements in both replicates.
[b] Coefficient of variation.
[c] Number of samples.
[d] Not detected, <1.2 µg l^{-1} DBT.

exposure concentrations. After thinning on day 30, F_0 generation survival was significantly reduced only in the 1510 µg l^{-1} DBT treatment at days 90, 120, 145 and 175. Though there was a reduction in survival of the F_0 generation exposed to 887 µg l^{-1} DBT as early as day 120 of the study, this effect was not statistically significant. Control survival from thinning to termination was 88%. For exposure times ≥90 days, the data in Fig. 1 indicate an apparent LC_{50} value DBT exposure between 887 and 1510 µg l^{-1}.

F_0 generation growth

The mean length of F_0 generation sheepshead minnows was significantly reduced relative to control fish at exposure day 30 for DBT concentrations ≥887 µg l^{-1}, but not for longer exposure times (Table 2). The mean length of fish exposed to 1510 µg l^{-1} DBT was significantly less than control fish at 30, 61, and 90 days of exposure. No significant reductions in overall wet weight of test fish exposed to DBT were found.

Reproduction

The reproductive phase of the study was initiated on day 107 with the selection of the first trial spawners. Spawning chambers generally housed three female and two male sheepshead minnows from the same treatment replicate. At the beginning of the reproductive phase of the study the 1510 µg l^{-1} DBT treatment had no mature fish in one replicate and only one mature male remaining in the second replicate. Therefore, this spawning group consisted of one male and three females. Following 14 days of egg collection, the total production of viable (i.e. fertilized) eggs ranged from 15.4 to 8.5 eggs per female per day, with no significant

Table 2. Standard length and mean wet weight of F_0 generation sheepshead minnows to day 90

Mean DBT concentration ($\mu g\ l^{-1}$, ionic species)	Rep.[a]	Mean standard length[b] (mm)			Mean wet weight[b] (mg)
		Day 30	Day 61	Day 90	Day 30
Control	A	16.4 ± 2.0 (38)[a]	25.1 ± 3.3 (25)	29.1 ± 3.9 (24)	143 ± 29 (10)
	B	15.8 ± 1.4	25.1 ± 2.5 (25)	29.3 ± 2.9 (25)	123 ± 37 (16)
	MEAN	16.1 ± 1.7	25.1 ± 2.9	29.2 ± 3.4	131 ± 35
158	A	17.0 ± 1.7 (38)	25.9 ± 2.2 (25)	29.2 ± 2.1 (25)	157 ± 48 (10)
	B	16.2 ± 1.7 (39)	25.5 ± 2.0 (25)	29.1 ± 2.3 (25)	131 ± 38 (11)
	MEAN	16.6 ± 1.7	25.7 ± 2.1	29.2 ± 2.2	143 ± 44
286	A	16.2 ± 1.4 (44)	25.1 ± 2.5 (24)	28.6 ± 2.5 (24)	126 ± 29 (16)
	B	16.1 ± 1.7 (41)	25.3 ± 3.7 (25)	28.7 ± 3.0 (24)	136 ± 34 (13)
	MEAN	16.2 ± 1.6	25.2 ± 3.1	28.7 ± 2.7	130 ± 31
453	A	16.0 ± 2.1 (40)	25.1 ± 2.6 (25)	28.3 ± 2.9 (25)	117 ± 38 (12)
	B	15.3 ± 1.9 (40)	25.3 ± 3.0 (25)	29.3 ± 3.5 (25)	115 ± 43 (12)
	MEAN	15.6 ± 2.0	25.2 ± 2.8	28.8 ± 3.2	116 ± 40
887	A	15.1 ± 2.0 (40)	24.2 ± 4.1 (24)	28.3 ± 4.4 (23)	114 ± 38 (12)
	B	14.9 ± 2.0 (36)	24.1 ± 3.9 (25)	27.7 ± 4.6 (25)	125 ± 48 (8)
	MEAN	15.0 ± 2.0[c]	24.1 ± 3.9	28.0 ± 4.5	118 ± 41
1510	A	8.4 ± 1.6 (7)	12.4 ± 2.5	13.9 ± 4.2 (4)	
	B	9.9 ± 1.9 (15)	16.7 ± 5.4 (11)	21.6 ± 6.2 (7)	

[a] Values expressed as mean plus/minus standard deviation with number of observations in parentheses.
[b] MEAN: overall means of treatment means and standard deviations.
[c] Value significantly different from control lengths ($\alpha = 0.05$).

Table 3. Fecundity of F_0 generation sheepshead minnows in three spawning trials

Mean DBT concentration ($\mu g\ l^{-1}$, ionic species)	Spawning trial[a]							
	First		Second		Third		Total[a]	
	No. (%)	No./F/d	No. (%)	No./F/d	No. (%)	No./F/d	No. (%)	No./F/d
Control	1193 (84.4)	14.2	1471 (95.1)	17.5	917 (90.9)	11.9	3581 (92.4)	14.5
158	740 (66.3)	8.8	1032 (93.9)	12.3	1152 (93.6)	13.7	2924 (86.2)	11.6
286	1297 (72.9)	15.4	1564 (88.3)	18.6	565 (90.8)	7.9	3426 (84.3)	14.0
453	1159 (73.7)	13.8	1415 (91.2)	16.8	1261 (94.5)	15.0	3835 (85.0)	15.2
887	571 (73.3)	8.5	882 (74.4)	13.2	709 (80.4)	10.8	2162 (79.5)[b]	10.8
1510	1[c] (0.01)	—	—[d]	—	—[d]	—	—	—

[a] The values tabulated are total viable eggs with amount viable (%), followed by viable eggs/female/day calculated with adjustments for the female mortality that occurred during the spawning trial.
[b] Value significantly different from controls.
[c] This chamber contained only three females and one male. The male died on day 1 of egg collection.
[d] There were no remaining adults in this treatment following the first spawning trial.

differences between DBT-exposed fish and controls for all DBT concentrations below 1510 $\mu g\ l^{-1}$ (Table 3). There was only one viable egg collected in the 1510 $\mu g\ l^{-1}$ DBT treatment prior to the death of the only male spawner on the first day of collection. Although differences in the percentage of viable eggs were observed between control and DBT-exposed fish, none of the differences was significant.

There were no mature fish remaining in the 1510 $\mu g\ l^{-1}$ DBT treatment for either the second or the third spawning trials. The total production of viable eggs per female per day ranged from 18.6 to 12.3 in the second trial. There were no significant differences in viable eggs produced per female per day and in percent viability between control fish and DBT-exposed fish.

Total eggs produced per female per day ranged from 15 to 7.9 in the third spawning trial. As with the other trials, differences in parameters measured between control and DBT-exposed fish were observed, but none was significant.

The only significant effect on reproduction determined from this study was to the percentage viability of eggs when controls were compared with DBT-exposed fish across all three spawning trials. Using percentage viable values across replicates, and across spawning trials as treatment observations, there was a significant reduction in egg viability in 887 μg l^{-1} DBT, as shown in Table 3.

F_1 generation embryo survival, juvenile survival, and juvenile growth in exposure concentrations

A sufficient number of embryos was not produced in 1510 μg l^{-1} DBT to allow for isolation and monitoring of the first-generation embryos. Embryos were successfully isolated in all other treatments. Survival of progeny embryos was ≥ 93% in all DBT concentrations and did not significantly differ from that of the control embryos (96%). Juvenile survival at 30 days post-embryo-isolation in these same treatments ranged from 78% to 93% in 453 μg l^{-1} and 887 μg l^{-1} DBT respectively, and was not significantly different from control treatment (86%; Table 4). Although a pattern of reduction in standard length, wet weight, and dry weight in concentrations >453 μg l^{-1} DBT was demonstrated, no statistically significant growth response was determined for progeny returned to their treatment of origin.

CONCLUSIONS

The mean length of F_0 generation sheepshead minnows was significantly reduced on day 30 at DBT concentrations ≥ 887 μg l^{-1}, thereby setting the LOEC for the study at 887 μg l^{-1} DBT and the NOEC at 453 μg l^{-1} DBT. Of the other biological parameters measured in this study (i.e. egg viability, survival of F_0 embryos and juveniles, survival of F_1 embryos, wet and dry weight of F_0 fish and length, wet and dry weight of F_1 fish) only egg viability was significantly reduced at LOEC, and then only when the egg viability was analyzed collectively across all spawning trials.

In evaluating the data generated during the course of the study presented here, a confounding factor was always of particular concern in the evaluation of DBT effects. It is known that TBT can be created by the process of comproportionation, which, in the case of DBT, would rearrange butyl groups from two molecules of DBT to yield a molecule of TBT and MBT. The occurrence of TBT in DBT as a highly toxic impurity may be unavoidable. The low level (0.087%) of TBT impurity in the test substance was estimated to produce TBT levels in treatment aquaria that could approach or exceed the NOEC for TBT (i.e. 0.42 μg l^{-1}) determined in an earlier study[24] in the DBT treatment solutions of 887 and 1510 μg l^{-1} DBT. Comparison of results of the two studies are presented in Table 5. The effects exhibited by the F_0 generation relative to TBT concentrations measured during each study were strikingly similar. Fecundity, expressed as a reduction in percentage viable eggs, was affected at essentially the same TBT concentration in both evaluations. The statistical significance of an effect on egg viability in the DBT study and not the TBT study was probably due to the stronger statistical power provided by the one additional spawning trial used in the DBT study.

The influence of TBT as an impurity on the evaluation of DBT has been discussed in previously published studies. Widdows and Page[23] determined clearance rate oxygen uptake, absorption efficiency and scope for growth in mussels exposed to DBT and TBT as related to tissue concentrations of DBT and TBT. Owing to differences in the mechanisms of toxicity of DBT and TBT, the effects of TBT as an impurity could be separated from those of DBT in that study. While examining the large difference in the toxicity of DBT relative to TBT in chronically exposed medaka (*Oryzias latipes*) and guppy (*Poecilia reticulata*), Wester *et al*.[35] concluded that the toxicity of DBT toward guppies in this study was due to the 0.33% TBT impurity present in the DBT used in the experiments. With medaka exposed to DBT, Wester *et al*.[35] observed an NOEC for mortality, growth and behavioral factors of 1800 μg l^{-1} DBT. The results of that study, and from an earlier study with the guppy,[36] were presumed to be influenced by TBT impurity of the DBT test substance used.

Comparison of measured levels of TBT in the present study with those exerting adverse effects in a prior TBT study[24] indicates that the biological responses observed here are likely due to low levels of TBT present as an impurity in the DBT used, and not by the DBT itself. Even a DBT analytical procedure designed for high DBT concentrations

Table 4. Survival, length and weight of F_1 generation sheepshead minnows isolated into the treatment of origin

Mean DBT concentration (μg l^{-1}, ionic species)	Survival (%)		Standard length[a] (mm)	Weight[a] (mg)	
	Embryo	Juvenile		Wet	Dry
Control	96	86	13.5 ± 1.3	69 ± 20	18.6 ± 5.4
158	100	92	13.8 ± 1.3	73 ± 21	20.2 ± 6.6
286	97	85	13.1 ± 1.8	66 ± 21	18.8 ± 5.9
453	93	78	12.6 ± 1.7	61 ± 26	17.4 ± 9.4
887	95	93	12.2 ± 1.9	52 ± 25	15.1 ± 7.7

[a] Mean ± standard deviation.

Table 5. Comparison of the effects of measured TBT to the sheepshead Minnow in two separate life-cycle tests[a]

	TBT concentration measured ($\mu g\ l^{-1}$)	
	TBT life-cycle test[b]	DBT life-cycle test
F_0 generation		
Survival	0.66 Significant effects	0.64 Significant effects
Growth	0.42 Apparent reduction in wet and dry weight	0.44 Apparent reduction in wet and dry weight Significant reduction in standard length on day 30
Fecundity	0.42 Apparent reduction in total and percentage viable (two trials)	0.44 Apparent reduction in total and percentage viable (three trials)[c]
F_1 generation		
Survival	1.3 Significant effect only in second trial survival	— No significant effect on survival at any treatment level[d]
Growth	— No significant or apparent reductions	0.44 Apparent reduction in length and weight

[a] Effects are listed with lowest concentrations yielding these effects and were either distinct (but not statistically significant), referred to as 'apparent', or were statistically significant, referred to as 'significant'.
[b] Manning *et al.*[24]
[c] Significant reduction in percentage viable across trials (three trials).
[d] When returned to original treatment.

in exposure water using very pure DBT test material could easily be insufficiently sensitive to detect TBT, because the concentration differences are *ca* 1000-fold. Without a separate TBT analysis, TBT would have been overlooked throughout this investigation, as indeed it may have been by other investigators who did not carefully measure TBT during DBT exposures. We believe the presence of TBT as an impurity at a level as low as 0.1% may have a significant influence on the perceived toxicity of DBT and that the spontaneous production of TBT in DBT may be the major source of biological toxicity of DBT. Any toxicity of the DBT produced at the exposure concentrations in this study was considered to be an undetectable increment to that produced by the more toxic TBT impurity present.

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