Environmental Toxicology

Life-Cycle Studies with 2 Marine Species and Bisphenol A: The Mysid Shrimp (Americamysis bahia) and Sheepshead Minnow (Cyprinodon variegatus)

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Abstract: Bisphenol A (BPA) is a high production volume compound primarily used to produce epoxy resins and polycarbonate plastic. Exposure to low concentrations of BPA occurs in freshwater and marine systems, primarily from wastewater treatment plant discharges. The dataset for chronic toxicity of BPA to freshwater organisms includes studies on fish, amphibians, invertebrates, algae, and aquatic plants. To broaden the dataset, a 1.5-generation test with sheepshead minnow (Cyprinodon variegatus) and a full life-cycle test with mysid shrimp (Americamysis bahia) were conducted. Testing focused on apical endpoints of survival, growth and development, and reproduction. The respective no-observed-effect concentration (NOEC) and lowest-observed-effect concentration (LOEC) values of 170 and 370 μg/L for mysid and 66 and 130 μg/L for sheepshead were based on reduced fecundity. The hazardous concentrations for 5% of the species (HC5) values of 18 μg/L were calculated from species sensitivity distributions (SSDs) with freshwater-only data and combined freshwater and marine data. Inclusion of marine data resulted in no apparent difference in SSD shape, R² values for the distributions, or HC5 values. Upper-bound 95th percentile concentrations of BPA measured in marine waters of North America and Europe (0.024 and 0.15 μg/L, respectively) are below the HC5 value of 18 μg/L. These results suggest that marine and freshwater species are of generally similar sensitivity and that chronic studies using a diverse set of species can be combined to assess the aquatic toxicity of BPA. Environ Toxicol Chem 2018;37:398–410. © 2017 The Authors. Environmental Toxicology and Chemistry Published by Wiley Periodicals, Inc. on behalf of SETAC.

Keywords: Marine toxicity tests; Marine water; Hazard/risk assessment; Species sensitivity distributions; Surface water

INTRODUCTION

Bisphenol A (BPA; CAS 80-05-7) is an important high production volume compound used mainly as an intermediate in the production of epoxy resins and polycarbonate plastic. The occurrence of low concentrations of BPA in aquatic systems primarily results from wastewater treatment plant discharges [1]. Smaller amounts of BPA may occur in uncaptured or untreated landfill leachates [2] and leakage from septic systems [3]. It has been shown to degrade rapidly in fresh and marine waters and sediments, with half-lives from 1 to less than 40 d depending on the microbial acclimation in the test system, as demonstrated in river and seawater die-away studies [1,4,5].

Most wastewater treatment plants discharge to freshwater rivers and streams, although some receiving waters are actually estuarine or coastal marine locations. In addition, freshwater receiving streams generally flow toward and eventually into estuarine and coastal waters. Surface water monitoring programs in North America and Europe have reported low but detectable concentrations of BPA in both fresh and marine waters [6].

The chronic toxicity of BPA to freshwater organisms has been examined in studies that cover all the taxonomic groups specified by the US Environmental Protection Agency (USEPA).
for development of national water quality criteria [7] and by the European Chemicals Agency when using a species sensitivity distribution (SSD) [8], including fish, amphibians, pelagic and benthic invertebrates, plants, algae, and diatoms [9–24]. These studies employed partial life-cycle, full life-cycle, or multi-generation exposure regimes. Few toxicity data on BPA are available, however, for estuarine or marine species (limited to a study with the marine diatom [Skeletonema costatum]) [24]. We therefore considered it useful to develop data that support evaluation of the toxicity of BPA to estuarine/marine species.

The purpose of the present study was 3-fold. First, chronic toxicity studies with 2 marine species, the sheephead minnow (Cyprinodon variegatus) and the mysid shrimp (Americamysis bahia), were performed using standardized testing protocols to add to the small available marine organism dataset. Second, the data from the chronic studies with 2 marine organisms were combined with a much larger toxicity database of both freshwater and marine data from the chronic studies with 2 marine species were used to initiate the test. During the present study, mysids were fed brine shrimp (<48 h old (post hydration) and brine shrimp enriched with Selec® twice daily. Sheephead minnow embryos (<30 h old) were purchased from Aquatic Biosystems, acclimated to 28 °C dilution water, and held at test temperature until they were placed in the exposure system at Springborn Smithers Laboratories. Newly hatched larvae were fed live brine shrimp nauplii 3 times daily for approximately the first 28 d. A small amount of flaked food was also offered daily after day 14. Juvenile–adult fish were fed twice daily, one feeding of frozen brine shrimp and one feeding of flaked fish food. Fish were not fed during the 24 h prior to study termination.

Testing protocols

Procedures for the full life-cycle study with mysid shrimp followed USEPA Office of Pollution Prevention and Toxics (OPPTS) guideline 850.1350 and ASTM International standard methods [25,26]. Procedures for the 1.5 generation study with sheephead minnow followed US EPA OPPTS draft guideline 850.1500 [27]. These methods were developed specifically for their respective species and provide guidance for husbandry, test design, salinity, feeding, endpoints to be measured, and data analysis. These requirements were defined during development of the guidelines and were based mainly on the ability of multiple laboratories to obtain repeatable results. All studies were conducted in conformance with the Principles of Good Laboratory Practice of the Organisation for Economic Co-operation and Development [28].

Test substance and preparation of stock solutions

The BPA (CAS 80-05-7) used for both tests was from the same lot (80070138; purity 99.68%), purchased from Acros Organics NV, and analytically characterized by Research Triangle Institute (Research Triangle Park, NC, USA). Concentrations reported in the present study were adjusted for purity of the test substance.

Stock solutions were prepared by placing a measured quantity of BPA in a volumetric flask and bringing it to the appropriate volume with deionized water. To facilitate dissolution, the stock solutions were adjusted to approximately pH 11 by adding sodium hydroxide (NaOH). The resulting stock solutions were sonicated until the solutions contained no undissolved material and approximately 2.5 mg BPA/mL.

Dilution water and test chemical-dilution water delivery system

Natural seawater was used as dilution and control water during these tests. Seawater was pumped from the Cape Cod Canal (Bourne, MA, USA) from approximately 1 to 4 m offshore at a depth of approximately 0.5 m. In the laboratory, the seawater was adjusted to a salinity of 20 ± 3‰, filtered, and intensively aerated for approximately 48 h within a holding reservoir prior to use. The seawater used for the mysid shrimp test had a salinity range of 20 to 21‰ and a pH range of 7.4 to 8.2, and that used for the sheephead minnow test had a salinity range of 18 to 22‰ and a pH range of 6.9 to 8.7. Representative samples of the dilution water source were analyzed monthly for total organic carbon (TOC) concentration. The TOC concentration of the dilution water source ranged from 0.92 to 0.98 mg/L during the tests.

The exposure system for both tests consisted of an intermittent-flow proportional diluter with a dilution factor of 50%. The diluter cycled in a consistent and regular pattern throughout the studies. The function of the diluter system (e.g., dilution water flow rates, stock solution consumption) was monitored daily, and weekly analysis of the exposure solutions for BPA concentration was used to verify proper operation of the diluter system.

Mysid shrimp life-cycle study

The exposure system consisted of 12 flow-through aquaria randomly positioned in a water bath maintained at 25 ± 2 °C. The test area was illuminated at a light intensity of 460 to 820 lux,
with a 16:8-h light:dark cycle. Retention and pairing chambers, used to house mysids in the test aquaria, were glass Petri dishes with an attached 15-cm-high screen collar to allow for solution movement through the chamber, with a flow turnover rate of 7 to 15 aquarium volumes/d. The solution volume in each of the retention chambers fluctuated from 390 to 710 mL, and in each of the pairing chambers fluctuated from 140 to 250 mL. The maximum organism loading was 0.0026 g of biomass/L of test solution/d for the first 15 d and 0.0013 g of biomass/L/d for the remainder of the exposure.

The test was performed with a dilution water control and 5 different BPA concentrations (38, 75, 150, 300, and 600 μg/L) that were selected based on the results of range-finding studies with mysids, performed to determine the appropriate range of concentrations to be tested in the chronic study. At the start of the study, 15 juvenile mysids (<22 h old) were impartially selected and placed in each of the 24 retention chambers. During the first 11 d of the study, each exposure aquarium contained 2 retention chambers, yielding 30 mysids/replicate vessel, and 2 replicate vessels were included per treatment level, resulting in 60 organisms for each treatment level and the control. On day 11, the 30 mysids in each exposure aquarium were redistributed in their aquarium such that there was one retention chamber and 10 pairing chambers with 1 male and 1 female mysid in each pairing chamber. Unpaired mysids were maintained in the retention chambers. Male mysids from the retention chambers were used to replace dead males from the paired groups. Females that died in the pairing chambers were not replaced.

To observe the mysids during the exposure period, each retention or pairing chamber was gently lifted from the aquaria daily. During this procedure, the water level in each chamber was reduced by allowing water to drain through the screen. The chamber was placed on a light table, and the number of dead and living test organisms, the number of offspring produced by each individual female, and any abnormal appearance or behavior were recorded. Dead parental mysids and offspring were recorded, removed, and discarded when observed during the test. Mortality was defined as lack of movement after gentle prodding with a glass pipette.

On test day 28, all mysids were euthanized by immersion in ice-cold deionized water. The mysids were carefully removed, blotted dry on absorbent paper, and separated into male and female groups for each replicate exposure level. Individual body length was measured to the nearest 0.05 mm. Male and female mysids were then transferred to aluminum pans, dried in an oven at 100°C for 21 h, and then placed in a desiccator. Individual total dry body weight to the nearest 0.01 mg was determined.

Reproductive success was calculated for each replicate aquarium (treatment and control) as the total number of offspring produced per female, the total number of offspring produced per female per reproductive day, and the number of reproductive females in each replicate. The number of female reproductive days was determined as the number of days that an individual female was alive, counting from the day that offspring were first observed in any control or treatment pairing chamber. Day 14 represented reproductive day 1.

**Sheephead minnow life-cycle study**

The flow-through aquaria were randomly placed in a temperature-controlled water bath maintained at 28 ± 1°C. The flow to the aquaria provided for 7.6 volume replacements every 24 h. The test was performed with a dilution water control and 6 different nominal BPA concentrations (9.4, 19, 38, 75, 150, and 300 μg/L) that were selected based on the results of range-finding studies to determine the concentrations to be tested.

The full life-cycle exposure was initiated by randomly placing 50 embryos into each of 28 incubation cups with screen bottoms, one cup in each of 4 replicate aquaria in each treatment level and control (see Supplemental Data for a schematic diagram of the experimental design). Embryos in each cup were counted daily, and dead embryos were discarded. At the completion of the hatching period, percentage of hatching success was calculated for each replicate aquarium ([number of live fry ÷ number of eggs incubated] × 100). On test day 4, 25 of the newly hatched fry in each incubation cup were impartially selected and placed in their respective growth chambers, with the exception of replicate B of the highest treatment level tested. The eggs in this replicate were inadvertently lost during biological observations. Larvae from replicates A and C of this treatment level that would have been discarded after hatch were pooled, and 25 larvae were chosen to be continued in replicate B. Growth chambers were made with a glass bottom and screened sides.

On test days 4 and 55, the larvae in each of these groups were photographed using a modified version of the McKim and Benoit method [29], to measure the total length of individual fish and to accurately count the fish for determining percentage of survival. At approximately 55 d post hatch (dph), the juvenile stage was considered complete because most males demonstrated territorial behavior, and male and female secondary sexual characteristics were evident. At this time, the aquaria were separated into 3 compartments by screening. Spawning groups (2 males and 5 females) from each test aquarium were placed in one section of the corresponding spawning chamber in all test vessels, where they were held for 22 d. The other 2 compartments were for surplus first-generation (F0) adult fish and the second-generation (F1) embryos and larvae. Spawns were removed and counted daily. Females killed as a result of male aggression during spawning were not replaced in the spawning group; however, males that died were replaced, to maximize egg fertilization success.

Exposure of F0 fish was terminated at 111 dph. Each fish was individually measured for total length and wet weight (blotted dry), and dissected to verify sex and gonadal condition. Reproductive success was calculated based on the number of eggs per number of females alive per spawning day. For example, if on any given day during the 22-d spawning period a total of 78 eggs was counted in the spawning tray and 4 females were alive on that day, then the reproductive success for that day would be 78 ÷ 4, or 19.5. The mean reproductive success at each
treatment level represents 22 consecutive d of egg production for each group.

Exposure of F1 fish was initiated by incubating groups of 50 embryos on the day they were spawned by placing them in the third section of the replicate tanks. Following hatching of the embryos, F1 exposure was continued by impartially placing a group of 25 newly hatched larvae (per replicate aquarium) into a corresponding larval growth chamber. Following 27 to 29 d of post hatch exposure, each group was euthanized with tricaine methanesulfonate. At the end of the study, percentage of survival was recorded, and individual lengths and wet weights were determined as previously described. Data were compiled on the effects of exposure on hatching success, survival, growth (total length and wet wt), and reproductive success of F0 fish (eggs/female/day) and the hatching success, survival, and growth (total length and wet wt) of their progeny (F1).

**Analytical methodology**

Analytical confirmation of the exposure solution BPA concentrations was performed by high-performance liquid chromatography (Hewlett-Packard) with fluorescence detection (excitation wavelength = 230 nm; emission wavelength = 308 nm). Separations were achieved on an Agilent SB-C18 column (75 x 4.6 mm), with a mobile phase 0.05:100 phosphoric acid in purified reagent water: acetonitrile, a flow rate of 1.4 mL/min, and an injection volume of 500 μL. The limit of quantitation was 0.952 μg/L, and the average recovery, during the validation study, was 101 ± 1.92% for BPA in natural seawater with a salinity of 32‰.

To confirm exposure equilibration, diluter stock and test solution samples were collected and analyzed before test initiation. During the fish study, one replicate of each treatment level was sampled at test initiation, at hatch, and weekly thereafter from one alternating replicate of each treatment level and control. For the mysid study, samples were removed from alternating replicate solutions of each treatment level and the control on days 1, 7, 14, 16, 17, 21, and 28. Day 16 and 17 measurements were taken to confirm maintenance of test concentrations after an increase in turnover rate. Each exposure solution sample was collected from the approximate midpoint of the test aquaria. During the in-life periods of the exposure, samples of the stock solution being used at the time of analysis, as well as BPA-fortified quality control samples, were also analyzed concurrently with test solution samples.

**Statistical analysis**

Analyses were performed using the mean organism response in each treatment group rather than individual response values. All statistical analyses were conducted at the 95% level of certainty except in the case of the Shapiro–Wilk and Bartlett’s tests, in which the 99% level of certainty was applied. Data were checked for normality using the Shapiro–Wilk test and for homogeneity using Bartlett’s test. Statistical significance compared with the control was determined by Williams’ test or the Kruskal–Wallis test for growth and reproduction data following analysis of variance. In the sheepshead test, Fisher’s exact test (2-tailed) was used for the survival and hatching data, and Williams’ test was used in the mysid test. TOXSTAT® Ver 3.5 [30] was used to perform the computations.

**SSDs**

Aquatic studies [9–24] were assembled that assessed the toxicity of BPA across a broad range of taxa including fish, amphibians, numerous invertebrates, algae, and aquatic plants. In developing this database, studies previously collected and critically reviewed by Staples et al. [31,32] were used as a starting point. Further systematic literature searching was performed to identify any additional relevant published or unpublished aquatic studies. To be consistent, following identification of potentially relevant studies, a data quality evaluation was conducted to identify those considered “valid” according to the methods described by Staples et al. [31]. All studies employed long-term exposures covering critical portions of the life cycle including early life stages, full life cycles, and exposure across more than one generation. No-observed-effect concentrations (NOECs) and effect concentrations (usually EC10 values) for all apical endpoints related to growth and development, reproduction, and survival were assembled from all studies. When there was more than one valid study for a species, the study with the lowest NOEC or EC10 value and longest duration was used to represent that species.

Although BPA is known to bind weakly to the estrogen receptor of mammals [33] and can induce the production of vitellogenin in male fish [13,14], indicative of exposure to an estrogen-like compound, most of the effects observed in the large database of chronic studies are not unique to endocrine activity. Toxicity studies that measure apical endpoints related to an individual organism’s and population’s ability to survive, grow, develop, and reproduce integrate the cumulative effects of exposure to a substance, including effects on the endocrine system. Thus, the extensive database of chronic studies for BPA provides ecologically relevant NOEC or EC10 values for use in hazard characterization.

An SSD was constructed from the assembled set of chronic NOEC and EC10 values. The model used to calculate SSDs for BPA, ETx 2.0, was obtained from The Netherlands National Institute of Public Health and the Environment (RIVM) [34] and is an SSD method for evaluating aquatic toxicity data included in regulatory guidance [35]. This program was used to calculate a lower 5th percentile concentration from the distribution of chronic NOEC values (i.e., an HCS value). The SSD assumes a normal distribution of species sensitivity that is log transformed to perform the calculations. The assumption that the toxicity dataset fits a log-normal distribution is tested using the Anderson–Darling test for normality.

**Surface water concentrations of BPA**

Published literature and unpublished reports from government agencies with monitoring data for BPA from estuarine and
maritime waters in North America and Europe were assembled, evaluated for reliability, and statistically analyzed following the methods given in Klecka et al. [6] to calculate summary statistics that describe the full distribution of concentrations for each region. Many unpublished governmental reports lacked some analytical details but were deemed of sufficient reliability to be included in the monitoring database. The statistical analyses accounted for concentration data that were censored (e.g., below analytical detection limits), for values that were only presented as summary statistics (e.g., ranges or means), and for location (e.g., multiple samples taken at one location and single samples taken at other locations). These statistical procedures enabled the development of distributions of estuarine and marine surface water BPA concentration data from which summary statistics were calculated for each region. The summary statistics (i.e., median and upper 95th percentile concentrations) were directly compared with the HC5 values obtained from the SSD. The list of references reporting concentrations of BPA in estuarine and marine waters for North America and Europe that were used is given in the Supplemental Data.

RESULTS

Mytid shrimp life-cycle study

Analytical determination of the BPA concentrations in the exposure solutions showed arithmetic time-weighted mean concentrations that ranged from 48 to 61% of nominal and defined the treatment levels tested as 18, 41, 74, 170, and 370 µg/L. Effect concentrations reported are based on the mean measured concentrations (expressed as time-weighted averages). Analysis of 19 of the 21 quality control samples was consistent with the predetermined recovery range, ranging from 85.6 to 104% of the nominal fortified levels. The 2 samples out of the acceptable range (80–120%) were 70.0 and 78.4% of the nominal level, possibly because of spiking, handling, or instrument errors. Based on these results, it was determined that satisfactory precision and quality control were maintained during the analysis of exposure solutions. Volume turnover was increased from 7 to 15 aquarium volumes/d because of an observed loss in test material on day 14, after the pairing chambers were added to the test system, thus increasing surface area for possible adsorption.

Daily monitoring for all solutions throughout the test period established that dissolved oxygen concentration was maintained between 5.6 and 7.3 mg/L (~80–100% saturation), the pH ranged from 7.3 to 8.1, temperature ranged from 26 to 27 °C, and salinity ranged from 20 to 22‰.

At test termination, the mysids in the control met the performance criteria of the USEPA guideline (>70% survival of F0 mysids between pairing and test termination, >75% of the females in the control released young, and the control organisms produced >3 offspring/female) [25]. As shown in Table 1, there were no statistically significant differences (Williams’ test) in overall survival or in male- or female-specific survival for any treatment compared with the control. The NOEC for survival was 370 µg/L.

Growth as assessed by total body length and dry weight was not affected by exposure to BPA (Figure 1). Mean number of offspring per female among mysid exposed to the 370 µg/L treatment level was significantly different (Williams’ test, \( p < 0.05 \)) from controls (Figure 2). No other statistically significant changes from control performance were determined. Based on time-weighted average concentrations and the most sensitive endpoint analyzed (number of offspring per female), the lowest-observed-effect concentration (LOEC) for the study with mysid shrimp was 370 µg/L BPA, and the NOEC was 170 µg/L.

Sheepshead minnow life-cycle test

Analyses of the exposure solutions during the 115-d test resulted in time-weighted averages ranging from 75 to 88% of the nominal treatment levels, and defined the measured concentrations as 7.1, 17, 31, 66, 130, and 250 µg/L. Recoveries of all but 2 of the 66 quality control samples were consistent with the predetermined recovery range, ranging from 81.1 to 119% of the nominal fortified levels. The 2 samples out of the acceptable range (80–120%) were 125 and 139% of the nominal level, possibly because of spiking, handling, or instrument errors. Based on these results, it was determined that satisfactory precision and quality control were maintained during the analysis of exposure solutions. In general, exposure concentrations were consistent within sampling intervals and followed the intended dose-gradient throughout the test; however, similar challenges as experienced in the mysid test in the maintenance of stable concentration when surface area was increased were noted. In the sheepshead minnow study, adjustments made to the quantity of BPA delivered to the diluter system addressed these fluctuations. Effect concentrations reported are based on the mean measured concentrations (expressed as time-weighted averages).

Throughout the 115-d test, the water quality of the test solutions remained within acceptable ranges for the survival, growth, and reproduction of the sheepshead minnow. Dissolved oxygen concentration and pH ranged from 5.1 to 8.3 mg/L (>75% saturation) and 6.7 to 8.2, respectively. Salinity ranged from 19 to 22‰, and exposure solution temperatures ranged from 26 to 29 °C. Temperature deviated from the stated 28 ± 1 °C on test day 15 in the upper water bath and test

| Time-weighted mean BPA conc. (µg/L) | 28-d survival (%) | Male survival (%)^a | Female survival (%)^a |
|--------------------------------------|-------------------|---------------------|---------------------|
| Control 80 (0.54) | 85 (2.9) | 89 (15) |
| 18 86 (9.6) | 82 (16) | 97 (4.2) |
| 41 80 (3.7) | 80 (4.5) | 89 (3.0) |
| 74 81 (6.2) | 76 (8.4) | 100 (0.0) |
| 170 90 (2.5) | 92 (12) | 91 (13) |
| 370 86 (4.5) | 100 (0.0) | 92 (11) |

^a Survival by sex was assessed from pairing until the end of the 28-d exposure (Williams’ test, \( p = 0.05 \)).
day 17 in the lower water bath, when the min/max thermometer registered 26°C. Heaters and water baths were adjusted to bring the temperature back to the intended range. Because control performance met the specified criteria and the temperature was within the tolerance for this species, the deviations in temperature are not expected to have had a negative impact on the results of the study.

The F₀ embryos hatched by the fourth day of incubation at all treatment levels and the control. Statistical analysis (Fisher’s exact test) determined no significant difference in hatching success among embryos exposed to any of the treatment levels tested compared with the control (Table 2). Following 28-dph exposure, F₀ mean larval survival at all treatment levels ranged from 94 to 99%, which was not statistically different from the control (96%), based on Fisher’s exact test. The mean total length of larval fish exposed to BPA ranged from 21.2 to 22.2 mm, and statistical analysis (Williams’ test) determined no significant difference in mean total length among larval fish exposed to any of the treatment levels tested compared with the control (21.3 mm; data not shown).

At 55 dph, mean F₀ fish survival ranged from 92 to 99% for all treatment levels, which was not statistically different from the control (96%), based on Fisher’s exact test. The mean total length of F₀ fish ranged from 32.9 to 34.1 mm and was determined to not be statistically different (Williams’ test) compared with the control (33.9 mm; data not shown).

At test termination (day 115), F₀ mean survival at all treatment levels ranged from 93 to 99%, which was not statistically different from the control (96%), based on Fisher’s exact test. Mean male and female total length of BPA-exposed fish ranged from 45.9 to 47.1 mm and 39.2 to 41.0 mm, respectively, neither of which was statistically different (Williams’ test) compared with controls (46.8 and 40.4 mm, respectively; Figure 3). Mean wet weights of male and female fish exposed to BPA ranged from 1.87 to 2.02 g and 1.06 to 1.39 g, respectively and were not statistically different (Williams’ test) compared with the controls (1.97 and 1.16 g, respectively; Figure 3).

Reproductive performance during the present study is shown in Figure 4A. The number of eggs per female per day was 12, 13, 12, 14, 8, and 9 among fish exposed to the 7.1, 17, 31, 66, 130, and 250 μg/L treatment levels, respectively. Statistical analysis (Williams’ test) determined a significant difference in the number of eggs per female per day among females exposed to the 130 and 250 μg/L treatment levels compared with the control. However, the mean percentage of F₁ hatching success (Figure 4B) and time to hatch for the F₁ embryos (Figure 4C) were not statistically significantly different from controls (Williams’ and Kruskal–Wallis tests, respectively). Following 28 d of post hatch exposure, F₁ larval survival ranged from 89 to 98% and was unaffected at all treatment levels tested compared with the control (98%) based on Fisher’s exact test. Statistical analysis (Williams’ test) of mean total length and mean wet weight of F₁ larval fish determined no significant difference compared with the control (Figure 5).

A NOEC of 250 μg/L, the highest concentration tested, was determined for all survival, growth (length and weight), time to hatch, and hatching success endpoints assessed with both F₀ and F₁ sheephead minnows exposed to BPA. Values for NOEC and LOEC of 66 μg/L and 130 μg/L, respectively, were established based on F₀ reproductive success (eggs/female/d).

**Development of SSDs**

The RIVM ETx 2.0 program [34] was used to calculate HC5 values for the distribution of NOEC/EC10 values. Twenty-two chronic toxicity tests covering 17 distinct families of aquatic organisms and 17 different species [9–24] were used to develop SSDs (Table 3). Fourteen of the species within the dataset inhabit freshwater, and 3 species inhabit estuarine or marine water. Two
SSDs were prepared, the first using only freshwater species and a second using freshwater and estuarine/marine species combined (Figure 6A and B).

A key assumption in the SSD approach is that the distribution of species sensitivity follows the log-normal distribution. The RIVM ETx 2.0 program uses the Anderson–Darling test for normality of the distribution of effect concentrations [34]. For the datasets with freshwater-only and with both freshwater and marine organisms, the calculated Anderson–Darling statistics of 0.35 and 0.21, respectively, are well below the critical value of 0.75 at the $p = 0.05$ level of significance. Thus, the assumption of a normal distribution (log transformed) was confirmed for both SSDs. For the freshwater-only dataset ($n = 14$) and for the combined dataset ($n = 17$), the HCS values calculated from the SSDs are both 18 $\mu$g/L.

### Concentrations of BPA in estuarine and marine surface waters

Concentrations of BPA from estuarine and marine waters were obtained from 62 studies from Europe and 8 from North America, spanning the years 1997 to 2014 (Supplemental Data, TABLE 2: Sheepshead minnow mean (± standard deviation) $F_0$ hatching success and survival at day 28 and day 115$^a$

| BPA concn. (µg/L) | Hatching success$^b$ (%) | 28 d | 115 d |
|-------------------|--------------------------|------|------|
| Control           | 94 (1.6)                 | 96 (4.6) | 95 (6.0) |
| 7.1               | 86 (6.5)                 | 99 (2.0) | 99 (2.0) |
| 31                | 95 (2.5)                 | 94 (5.2) | 93 (6.0) |
| 31                | 90 (4.3)                 | 98 (2.3) | 95 (3.8) |
| 66                | 91 (2.0)                 | 96 (3.3) | 95 (3.8) |
| 130               | 87 (9.3)                 | 94 (6.9) | 93 (12) |
| 250               | 86 (3.5)                 | 98 (2.3) | 95 (3.8) |

$^a$ No effects on hatching success or survival were found (Fisher’s exact test, $p = 0.05$) during the life-cycle exposure to bisphenol A (BPA).

$^b$ All embryo groups at all concentrations and the controls, with the exception of replicate B of the 250 $\mu$g/L treatment level, which was inadvertently lost during biological observation, hatched by day 4 of incubation. This replicate was excluded from the analysis of hatching success.

![FIGURE 3](image.png)

**FIGURE 3:** Sheepshead minnows mean (± standard deviation) total length (mm) and wet weight (g) for males and females after 115-d exposure to bisphenol A (BPA). No effects were observed (Williams’ test, $p = 0.05$).

![FIGURE 4](image.png)

**FIGURE 4:** (A) Sheepshead minnow $F_0$ mean (± standard deviation) reproductive performance (numbers of eggs per female per day) after bisphenol A (BPA) exposure. Statistical significance tested shown (‘*’) using Williams’ test ($p < 0.05$). (B) No effects were found for mean (± standard deviation) $F_1$ percentage of hatching success (Williams’ test, $p = 0.05$) or $F_1$ 28-d survival (Fisher’s exact test, $p = 0.05$). (C) No effects were found for mean (± standard deviation) $F_1$ time to hatch (Kruskal–Wallis test, $p = 0.05$). All embryo groups in all treatments hatched within 5 to 6 d of incubation.
endpoints in mysids exposed to 18 to 370 μg/L BPA. These results suggest that mysids are somewhat more sensitive than other invertebrates such as Daphnia magna (NOEC for reproduction of 3160 μg/L) and the amphipod Hyalella azteca (NOEC for reproduction of 470 μg/L; Table 3).

Sheepshead minnow were exposed for 1.5 generations to 7.1 to 250 μg/L BPA, beginning with <30-h-old newly hatched F₀ embryos through F₀ adult stage, reproduction, and then hatch and grow out for 28 d of the F₁ juveniles. No effects were observed for larval survival or growth at any time during the 155-d exposure at all concentrations up to 250 μg/L. Reproductive performance was reduced in the top 2 concentrations, resulting in a NOEC of 66 μg/L. Exposure continued with F₁ offspring. No effects on F₁ hatching success, survival, or growth were found.

The results for the sheepshead minnow were somewhat different than were found in 2 long-term studies with fathead minnows (Pimephales promelas). In Mihaich et al. [14], adult fathead minnows were exposed to 1 to 640 μg/L BPA through F₁ hatch and larval growth. With the exception of reduced survival in males at 640 μg/L, no statistically significant effects were seen at any treatment level in males or females with respect to growth, fecundity, hatchability, and gonadosomatic index. The NOEC from this 164-d study was 160 μg/L, based on male survival. In a multigeneration test, fathead minnow (P. promelas) were exposed for 444 d over the course of 3 generations to 1 to 250 μg/L BPA that included F₀ reproducing adults, F₁ eggs grown to be reproducing adults, and F₂ eggs [13]. The NOEC for growth endpoints of 640 μg/L and higher showed few differences between age or generation. Reproductive NOEC values in F₀ and F₁ breeding pairs were 640 and 160 μg/L, respectively. Hatching success NOEC values for F₁ and F₂ were 160 and 16 μg/L, respectively. With sheepshead minnow, the lowest NOEC (66 μg/L) was for reproduction (eggs/female/d), and no adverse effect on hatching success was observed. The reasons for the differences in endpoint sensitivity are not known, but appear to be consistent with the overall variability between species, as seen in Table 3.

These mysid shrimp and sheepshead minnow data further expand an already rich database of acute and chronic aquatic studies with BPA (shown in Table 3). This database includes BPA aquatic studies over a broad set of freshwater and marine taxa, including fish, amphibians, plankton, benthos, mollusks, primitive sponges, and hydra, as well as algae and aquatic macrophytes. As with most chemicals, BPA has been assessed primarily in freshwater species. In addition to the 2 new marine studies reported, there is also a study with the marine diatom (Skeletonema costatum) that is of high enough quality to be included in the development of a combined freshwater and marine aquatic SSD [18,24].

Combining freshwater and marine data in an SSD approach allows for a more robust assessment and has precedent in both the scientific literature and regulatory guidance. As seen in Figures 6A and B, the inclusion of the marine NOEC/EC10 with the freshwater data results in no apparent difference in the shape of the SSD, in the R² values calculated for the distributions, or in the HC5 value of 18 μg/L that were calculated from each of the 2 SSDs. This finding suggests that the freshwater and marine data...
| Family (phylum) | Species | Endpoint | NOEC (LOEC) | Reference |
|----------------|---------|----------|-------------|-----------|
| Salmonidae (Chordata) | Rainbow trout *Oncorhynchus mykiss* | 28-d, juvenile growth test | 3640 (11 000) | [9] |
| Adrianichthyidae (Chordata) | Medaka fish *Oryzias latipes* | Multigeneration | 247 (1179) | [10] |
| | Medaka fish *O. latipes* | Mortality: 1820 | 355 (1820) | [11] |
| | Medaka fish *O. latipes* | Growth: 120 | Reproduction: 120 | [12] |
| Cyprinidae (Chordata) | Fathead minnow *Pimephales promelas* | 35-d ELS assay | 640 (1280) | [13] |
| | Fathead minnow *P. promelas* | Multigeneration | 640 | [13] |
| | Fathead minnow *P. promelas* | Growth: 640 | Reproduction: 16 | [14] |
| Cyprinodontidae (Chordata) | Sheepshead minnow *Cyprinodon variegatus* | 116-d life cycle | Mortality: 250 | Present study |
| | Guppy *Poecilia reticulata* | 30-d | 500 (5000) | [15] |
| Pipidae (Chordata) | African clawed frog *Xenopus laevis* | 90-d | Mortality: 500 | [16] |
| | (Amphibian) | Growth: 500 | Sexual differentiation: 500 | [17] |
| Daphniidae (Arthropoda) | Water flea *Daphnia magna* | 21-d | Mortality: 3160 | [18] |
| | (Planktonic) | | Reproduction: 3160 | [18] |
| Mysidae (Arthropoda) | Mysid shrimp *Americamysis bahia* | 28-d life cycle | Mortality: 370 (370) | Present study |
| | (Planktonic) | | Growth: 370 | [18] |
| Hyalellidae (Arthropoda) | Amphipod *Hyalella azteca* | Survival, growth, reproduction | Mortality: 1000 | [18] |
| | (Benthic) | | Growth: 1000 | [18] |
| Chironomidae (Arthropoda) | Midge *Chironomus riparius* | Reproduction: 490 (1000) | Growth and development | [19] |
| | (Aquatic insect) | Moulting delay: 100 | 1000 | [19] |
| Brachionidae (Rotifera) | Rotifers *Brachionus calyciflorus* | Life cycle, intrinsic rate of population increase | Population growth: 1800 (3600) | [18] |
| Ampullariidae (Gastropoda) | Prosobranch molluscs *Marisa cornuarietis* | 84-d adult survival and reproduction; hatchability and | 640 | [20] |
| | | 90-d juvenile growth trials | Mortality: 640 | [20] |
| | | Reproduction: 640 | Hatching: 640 | [20] |
| | | Juvenile growth: 640 | | [20] |
| | | 181-d adult survival and reproduction; hatchability and | 640 | [21] |
| | | 90-d juvenile growth trials | Mortality: 640 | [21] |
| | | Reproduction: 640 | Hatching: 640 | [21] |
| | | Juvenile growth: 25 (640) | Mortality: 42 (460) | [22] |
| Hydridae (Cnidaria) | Hydra *Hydra vulgaris* | Juvenile growth: 25 | Mortality: 42 | [22] |

(Continued)
can be combined for use in assessing the chronic aquatic toxicity of BPA in fresh and marine waters. From a regulatory perspective, European, US, and Canadian regulations and guidance [7,36,37] all allow for the use of freshwater data or a mixture of freshwater and marine data in the derivation of a marine predicted no-effect concentration. The SSDs that were prepared for BPA in the present study provide further support for these regulatory practices.

**TABLE 3: (Continued)**

| Family (phylum) | Species                   | Endpoint: NOEC (LOEC) | Reference |
|-----------------|---------------------------|-----------------------|-----------|
| Spongillidae    | Poriferan sponge          | Growth: 1600*/ (16,000)| [23]      |
| (Porifera)      | Heteromeyenia sp.         | 9-d                   |           |
| Selenastraceae  | Green algae              | 1360*                 |           |
| (Chlorophyta)   | Selenastrum capricornutum | 4-d                   | [24]      |
| Lemnaceae       | Duckweed                  | EC10 growth: 1360*    |           |
|                 | Lemna minor               | 7-d                   | [18]      |
| Skeletonematacea (Bacillariophyta) | Diatom          | Growth: 7800*/ (20 000) |           |
|                 | Skeletonema costatum      | EC50 cell count: 1100*|           |
|                 |                           | EC10 cell count: 400* | [24]      |
|                 |                           | EC50 growth rate: 2500|           |

*a All studies used for the development of species sensitivity distributions for long-term chronic studies with bisphenol A were screened for study quality according to the procedures described in Staples et al. [31] and adapted from regulatory guidance [7,8,35]. All concentrations are in μg/L.
*b The lowest no-observed-effect concentration from the longest duration study was used for this species.
*c Indicates marine study.
*d Median effect concentration calculated using probit analysis.
† Indicates the value used in the species sensitivity distribution developed for the dataset.
NOEC = no-observed-effect concentration; LOEC = lowest-observed-effect concentration; EC = effective concentration; ELS = early life-stage study.

**FIGURE 6:** Species sensitivity distributions for chronic freshwater-only data (A) and freshwater plus marine chronic aquatic data (B) with bisphenol A (BPA). NOEC = no-observed-effect concentration; EC10 = effect concentration, 10%.
When pooling data, it is advisable to examine any obvious sensitivity differences between similar marine and freshwater species. For example, van Wezel et al. [38] combined aquatic toxicity data for dibutylphthalate to freshwater and marine organisms to produce a single environmental risk limit, because there were no obvious differences in their toxicity. Similarly, Hall and Gardinari [39] focused specifically on plant data in developing a combined freshwater and marine SSD for an herbicide. In a series of marine risk assessments on industrial chemicals linked to chlorine production, datasets for both freshwater and marine organisms were compared and combined to develop regulatory limits when no sensitivity bias was observed [40–44].

Bisphenol A has 22 NOEC/EC10 values from 17 taxonomic families of aquatic organisms. Although only 3 of these are with estuarine/marine organisms, a comparison of species sensitivity reveals that for fish, the freshwater fathead minnow is more sensitive than the sheepshead minnow by up to a factor of approximately 4, whereas for crustaceans, the mysid shrimp is more sensitive than either freshwater daphnids or amphipods (Table 3). For crustaceans, daphnids are much less sensitive than mysids, by a factor of approximately 19, whereas amphipods and mysid are only different by a factor of approximately 3. An ordering of the NOEC/EC10 data for BPA shows that the 3 most sensitive species, fathead minnow, prosobranch snail, and hydra, are all freshwater organisms, suggesting that marine species are not necessarily more sensitive than freshwater species. In a comparison of freshwater and saltwater fish species from a large database, Hutchinson et al. [45] found that 91% of the EC50 and 93% of the NOEC values were within a factor of 10 for all substances. There was slightly more variability with invertebrates: 33% of the EC50 and 83% of the NOEC values were within a factor of 10. Similarly, no significant differences in marine and freshwater H5C values were observed in a comparison of 10 insecticides [46]. Del Signore et al. [47], in a critical review of SSDs and their uses for ecological risk assessment, concluded that there were no systematic or consistent differences in sensitivity between freshwater and marine taxa.

CONCLUSIONS

For BPA, chronic aquatic toxicity data are available for a broad set of freshwater and some marine taxa, including fish, amphibians, plankton, benthos, mollusks, primitive sponges, and hydra, as well as algae and aquatic macrophytes. The studies employed exposure regimes that covered early life stages, full life cycles, and multiple generations. The studies measured apical endpoints related to survival, growth and development, and reproduction. The NOEC/EC10 values from freshwater studies ranged from 16 to 7800 μg/L. The present study with the marine species sheepshead minnow (C. variegatus) and mysid shrimp (A. bahia) employed a 1.5-generation and full life-cycle exposure regimes, respectively, to assess their toxicity from long-term exposure to BPA. The SSDs were created from freshwater-only species and from all species combined and yielded similar HC5 values of 18 μg/L. The combined set of studies suggests that marine species are of generally similar sensitivity to freshwater organisms and that no one type of endpoint (survival, growth and development, or reproduction) was predominantly more or less sensitive to BPA exposure. The results of the present study suggest that data for freshwater and marine species can be combined into one dataset to assess their toxicity from long-term exposure to BPA. The SSD developed from the combined dataset yielded a lower bound HC5 value that is higher than the upper bound 95th percentile concentrations of BPA in North American and European marine waters.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3957.

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Disclaimer—The authors are either employed by companies that produce and sell bisphenol A (BPA), as well as certain other products that contain BPA, or are independent contractors that have provided consulting or testing services to both government and industry.

Data availability—Please contact the lead author directly for access to data at emihaich@nc.rr.com.

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