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Multigenerational Effects and Demographic Responses of Zebrafish (*Danio rerio*) Exposed to Organo-Bromine Compounds

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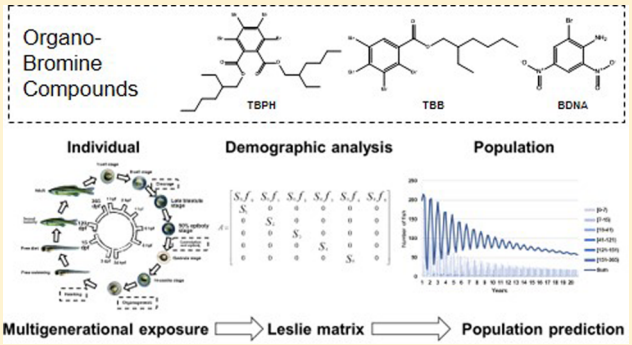
Supporting Information

ABSTRACT: Long-term exposure to toxic chemicals often has deleterious effects on aquatic organisms. In order to support appropriate environmental management of chemicals, a mathematical model was developed to characterize the effects of chemicals on multigenerational population dynamics in aquatic animals. To parametrize the model, we conducted a multigenerational laboratory toxicity test in zebrafish (*Danio rerio*) exposed to 2-bromo-4,6-dinitroaniline (BDNA). Long-term exposure to BDNA considerably reduced the fecundity of adult zebrafish (F_0 and F_1) and caused deformities in the offspring (F_2). Life history data, including changes in fecundity and population growth, were then integrated into the model to predict population dynamics of zebrafish exposed to two novel brominated flame retardants, bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (TBPH) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB). The model predicted that the fecundity of adult zebrafish would be significantly impaired after exposure to 90.36 μM TBPH and 99.16 μM TBB. Thus, prolonged exposure to such levels over multiple generations could result in population extinction within 20 years. Our results provide an intensive temporal perspective to investigate a keystone that connects with individual response to chemicals, population dynamics, and ultimately ecosystem influences.



INTRODUCTION

Long-term exposure to environmental pollutants can negatively affect aquatic organisms[1] and can even cause population decline and extinction.[2,3] However, it is often challenging to link specific pollutants with ecological changes in a given community.[2,3] In Lake Tai, one of the most severely polluted freshwater reservoirs in China,[4,5] the species richness of fish has dramatically decreased, falling from 106 species recorded between 1959 and 1985 to only 50 in 2008.[6,7] This decline is likely due to several factors, including environmental pollution.[8] To develop effective regulations and policies to maintain community diversity, it is necessary to predict population-level effects of pollutants and to understand the likely efficacy of various intervention plans.[2] Models of population dynamics could help fill the data gap due to insufficient field data. Traditional models that predict population dynamics influenced by demographic and environmental events assume that ecological status remains constant. However, community ecosystems are highly dynamic and influenced by the populations of the species they contain. Thus, to predict population dynamics it is important to assess the relationships between certain changes in individuals and their potential consequences in the population.

Population dynamics are governed by changes in environment-driven demographic rates, such as maturity, fecundity, and survival.[2,9] Classic demographic analysis provides an estimated rate of exponential population growth or decline based on a fixed set of life table parameters. However, information on the life history parameters of specific species is rare. Therefore, many studies use empirical relationships to obtain parameters for demographic analysis. Some of the

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empirical relationships only provide a single value for each of the demographic rates,[10−12] whereas others provide stage-specific values.[13] If a demographic rate (e.g., survival rate) is based on a single value, then the survival rate is assumed to be equal at all life stages, from infancy to adulthood. Meanwhile, the empirical relationships generating stage-specific survival rates are not based on severely depleted populations and only describe normal survival. Neither of these two types of relationships reveals the population fluctuations resulting from individual decline. Thus, a multigenerational exposure experiment is required to obtain information on individual life cycles affected by long-term pollutant exposure. The results can be used to construct a model that better predicts population dynamics trends.

Some of the substances that might affect these trends, including flame retardant products, have potentially deleterious long-term environmental consequences.[14,15] Although the widely used polybrominated diphenyl ethers (PBDEs) were associated with a number of environmental hazards and have been phased out of production,[16−18] novel brominated flame retardants (NBFRs), which are less environmentally persistent or have lower endocrine toxicity,[19,20] are increasingly used as alternatives. Several mixtures of additive flame retardants developed as PBDE replacements, such as Firemaster 550 (35% TBB and 15% TBPH), Firemaster BZ-54 (70% TBB and 30% TBPH), and DP-45 (TBPH only),[21,22] contain two NBFRs, bis(2-ethylhexyl)-2,3,4,5-tetrabromophthalate (TBPH) and 2-ethylhexyl-2,3,4,5-tetrabromobenzoate (TBB). Both TBPH and TBB have been listed as high production-volume chemicals by the United States Environmental Protection Agency[23] and have been detected in various abiotic and biotic matrices worldwide.[19,24,25] However, there are no data regarding their long-term effects on ecosystems. 2-Bromo-4,6-dinitroaniline (BDNA) is a relatively new substance that is an important intermediate for synthesizing the brominated azo dye Disperse Blue 79 and a major decomposition product of Disperse Blue 79 in aquatic environments, but there is little publicly available information on its toxicity.[26] Using the Ames *Salmonella* assay, significant mutagenicity of BDNA was detected at an environmentally relevant concentration of 1.25 mg/L by Peng et al. (2016). To establish a direct relationship between chemical stressors and ecological impact, a comprehensive toxicity assessment related to multigenerational exposure is required.

Risk assessment using contemporary strategies has largely relied on the results of short-term laboratory acute toxicity tests using species for which limited guidelines exist; even tests based on chronic exposure still only cover a short period of an organism's life cycle.[27] As conventional tests focus on a few apical outcomes, they might not provide a mechanistic understanding or knowledge regarding the modes of action for sublethal end points, and severely limit the useful information obtained in one species with one chemical for extrapolation to other species or other chemicals. At the population level, one toxicity outcome that is of considerable concern for the environment is the occurrence of cumulative toxic effects, potentially mediated through chemical interference with epigenetic regulation in the organisms. However, for such effects, relatively little mechanistic information is available, and reliable testing strategies for both individual toxicity and multigenerational effects have not yet been developed. In this context, Adverse Outcome Pathways (AOP) provide a defensible framework to systematically collect, organize, and evaluate existing data, making it available for use by environmental evaluators and managers.[27,28] On the basis of similarities in molecular mechanisms that lead to similar toxicological outcomes, AOPs also provide a rational framework for extrapolating chemical effects, allowing the broader integration of available information and generation of robust and testable hypotheses.[29] Utilizing this information, risk assessment could specifically focus on clarifying whether the predicted effects did occur, and whether they led to toxicity outcomes, in information-poor situations.

To this end, the objectives of the present study were (1) to complement and improve the life history information for multigenerational zebrafish exposed to BDNA; (2) to demonstrate trends in population development affected by population stage structure via a demographic analysis model; (3) to predict population dynamics of zebrafish exposed to TBPH and TBB using demographic models; and (4) to explore the influence of these compounds on populations. A demographic analysis model was constructed using a Leslie matrix based on multigenerational toxicity data (e.g., survivorship, hatching, and cumulative fecundity at each generation) of a model species, the zebrafish (*Danio rerio*), exposed to environmentally relevant concentrations of BDNA. We then conducted a 21-day subchronic exposure experiment for TBPH and TBB. These data were used to construct a demographic analysis model to predict the long-term trend (20 years) in the population with continued exposure to TBPH or TBB.

MATERIALS AND METHODS

Animal Care. Adult zebrafish (4 months old, AB wild type) were obtained from the Institute of Hydrobiology, Chinese Academy of Sciences (Wuhan, China). They were maintained in an automatic culturing system (Zhongkehai Recycling Water Aquaculture System Co., Ltd., Qingdao, China) under static-renewal conditions (27 ± 1 °C, 14:10 light:dark) and fed to satiation with pellet food three times a day. All procedures involving zebrafish were approved by the Institution Animal Care and Use Committee of Nanjing University for laboratory animal use.

Multigenerational BDNA Exposure Protocol. We designed a multigenerational BDNA (J&K Scientific Ltd., Shanghai, China) exposure protocol covering three generations, including a Fish Short-Term Reproductive Assay (OECD 229),[30] a Fish Juvenile Growth Test (OECD 215),[31] and a Fish Early Life Stage Toxicity Test (OECD 210 and 236).[32,33] The procedures are summarized below.

F_0 Short-Term Reproduction Test (21 Days). A test solution of BDNA was applied as a model material. On the basis of BDNA concentrations detected in sediment of 0.1−1.9 mg/kg[34] with 3% total organic carbon (TOC),[35] and a K_{oc} value of 871.4 L/kg predicted by EPI Suite (EPIWEB 4.1, U.S. EPA), the predicted range of BDNA concentrations in a realistic aquatic environment was 3.83−72.68 μg/L in surface water. Therefore, four concentrations of BDNA (0.5, 5, 50, and 500 μg/L) and a BDNA-free control group were used. All stock solutions of BDNA were prepared in Milli-Q water and diluted with aerated distilled water containing 60 mg/L instant ocean salt (Sunsun Group Co., Ltd., Zhejiang, China). The total concentration of the calcium and magnesium ions in this solution was 2.5 mmol/L. The Ca:Mg and Na:K ion ratios were 4:1 and 10:1, respectively. The acid capacity K_s 4.3 of this solution was 0.8 mmol/L. Solutions were then stored at −20

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°C and diluted to the final concentrations immediately before use.

First, 50 male and 50 female adult zebrafish (four months old) with a body mass of 0.3−0.6 g were separately placed into 5 L beakers (five fish in each beaker, 20 beakers total) filled with dechlorinated water and allowed to acclimate at 27 ± 1 °C and 14:10 light: dark cycles for 7 days prior to experimentation. During this period, fish were fed with untreated food three times a day and the water was replaced once a day. Mortality was below 10% during the acclimation period. The populations were then divided into subgroups of five males or five females each in separate 5 L beakers. Each beaker was treated with 0, 0.5, 5, 50, or 500 μg/L BDNA, and each group was evaluated in duplicate. After 21 days of exposure, fish in each group were placed in embryo incubators (5 males and 5 females) for 5 days to produce offspring (F_1). The number of eggs produced and the cumulative fecundity were counted on days 1, 3, and 5. After the collection of embryos, males and females in each breeding tank were separated again.

F_1 Fish Life Cycle Growth Test (150 Days). During embryonic development (0−3 days), a set of randomly obtained embryos (each treated group included 100 embryos, in triplicate) was transferred to a 25 mL beaker. Offspring (F_1) were exposed to the same concentration of BDNA as the parent generation, F_0 (average survival rate of each group: 82.54%, 97.20%, 85.37%, 85.68%, and 83.90%, respectively). When embryos of the F_1 generation hatched, the larvae were transferred to 250 mL beakers. Each group was fed paramecium nutrient solution (1 mL)[36] from the ninth day after fertilization, then fed brine shrimp after 50 days. Males and females were separated into 5 L beakers at sexual maturity (100−120 days, Supporting Information, SI, Table S6). Fish in each group were put into the embryo incubator as described above to collect their offspring (F_2). This part of the experiment lasted up to 150 days. Water samples were collected daily and the concentrations of BDNA were determined using liquid chromatography−electrospray ionization (−)-tandem mass spectrometry. Detailed methods and row data are available in the SI (Text S1, Tables S1 and S2).

F_2 Early Life-Stage Toxicity Test (7 Days). A total of 50 F_2 embryos were collected and randomly divided into two treatment groups—exposure groups and recovery groups. Embryos in the exposure group received continued exposure to BDNA, whereas those in the "recovery" group were exposed to dechlorinated water for 7 days. Each treatment and control pair was conducted in triplicate.

Short-Term Reproduction Test of Adult Zebrafish Exposed to TBPH and TBB (21 Days). The predominant pathway for fish exposure to TBB or TBPH (both purchased from AccuStandard Inc., New Haven, U.S.A.) is via food intake. TBPH and TBB tend to be distributed in environmental organic carbon in accordance with Henry's Law Constant of 3.02×10^{-2} pa·m^3/mol and 6.44×10^{-1} pa·m^3/mol, and log K_{ow} of 10.10 and 7.7, respectively. In an aquatic environment, they are found in sediment and plankton and accumulated from lower trophic levels. Therefore, TBPH- or TBB-treated food was used for the exposure tests. Food preparation followed Saunders et al.[37] Briefly, three TBPH (5.67, 22.59, and 90.36 μM) and three TBB (5.46, 24.79, and 99.16 μM) solutions were prepared in Dichloromethane (DCM, ROE Scientific, Inc., Newark, U.S.A.) and hexane (Merck KGaA, Darmstadt, Germany) (v:v = 1:1). For each solution, commercial pellet food was soaked in 150 mL of the solution in a flask, which was shaken for 2 h to ensure thorough mixing. The mixtures were then air-dried (stirred for 30 min every 6 h) in a dark fume hood for 48 h. The resulting concentrations were 4.00, 15.95, and 63.80 μg/g dry weight (dw) for TBPH and 3.00, 13.63, and 54.53 μg/g dw for TBB. The lowest concentrations were comparable to the TBPH and TBB concentrations measured in a sample of house dust from California, U.S.A. (260−3800 and 100−5900 ng/g dw, respectively).[38] The medium concentrations were based on an acute exposure test of TBPH and TBB in zebrafish embryos/larvae (SI Text S2 and Figure S1). The highest concentrations were comparable to average concentrations of sewage sludge from U.S. wastewater treatment plants (TBPH: 64−33 500 ng/g dw and TBB: 3430−89 900 ng/g dw).[39] Control food spiked with DCM/hexane was prepared the same way. Concentrations of TBPH or TBB in food pellets were measured using Thermo Scientific Trace 1300-ISQ LT single quadrupole mass spectrometry. Detailed methods and row data are shown in the SI (Text S1, Tables S3 and S4).

Exposure protocols were based on the Fish Short-Term Reproductive Assay, OECD test 229.[30] First, 15 male and 15 female adult zebrafish (0.3−0.6 g body weight) were placed in 5 L beakers filled with dechlorinated water and allowed to acclimate at 27 ± 1 °C and 14:10 light:dark cycles for 7 d. Fish were fed with untreated food three times a day and water was replaced daily. Mortality was lower than 10% during this period. Next, two groups of fish were fed TBPH or TBB-treated foods for 21 days, up to approximately 20% of their body mass. The control fish were fed untreated food. After 21 d, 4 females and 2 males from each group were randomly selected and relocated to 1 L incubation tanks to obtain offspring embryos. A total of 50 embryos from each treatment were rinsed, transferred to glass plates, and maintained in dechlorinated water for 7 days. Each treatment or control was conducted in triplicate. The number of eggs collected in each tank was normalized to the number of females per tank. Individual mortality and hatching delays were observed during the exposure period.

Leslie Matrix and Demographic Analysis. The Leslie matrix developed by Leslie and Lewis[40,41] is a matriarchal-based model, which projects population growth over time based on the fecundity and survival rates of individual life stage classes. Stage-specific survivorship and fecundity data were obtained from our BDNA multigenerational experiment (SI, Table S5). Parameters of first maturity (a_{mat} = 0.3 year) and lifespan (a_{max} = 1 year) were set as certified values. The model distinguishes various life stages of zebrafish based on mortality and integrates six life-history traits: embryogenesis and hatching period [0−7 dpf), reliance on yolk sac for nutrition [7−15 dpf), initiation of active eating [15−41 dpf), juvenile period [41−121 dpf), sexual maturity [121−151), and reproduction [151−365 days).[42,43] In addition, two spawning periods were set to calculate the fecundity of stage-specific adults: sexual maturity at 121−151 days and mature adulthood at 151−365 days.

The fertility of female fish at each stage (F_i) was estimated by the following:

$$F_i = f_i \times P_i \times S \quad (1)$$

where f_i represents the production of zebrafish offspring per female, P_i represents the proportion of female fish after sexual

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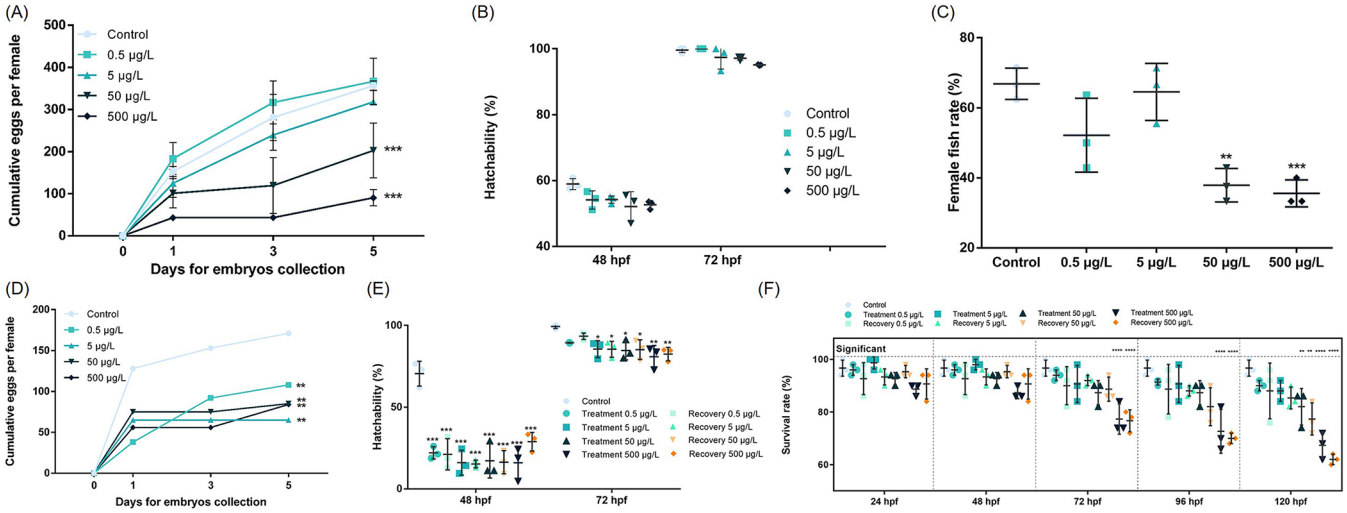

Figure 1. Dotted line plot (A) shows 5 days cumulative production per female of F_0 generation exposed to 0.5, 5, 50, or 500 μg/L of BDNA in 21 days. Box plot (B) reveals F_1 hatching rate with exposure to BDNA or in the control at 48 h post fertilization (hpf) and 72 hpf. Box plot (C) illustrates F_1 female fish proportion exposed to BDNA or in the control group at sexual maturity (120 days post fertilization, dpf). Dotted line plot (D) reveals 5 days cumulative production per female of F_1 exposed to BDNA in 150 days. Box plot (E) shows F_2 hatching rate upon exposure to BDNA and in the recovery and control groups at 48 hpf and 72 hpf. Box plot (F) illustrates the proportion of survival in F_2 zebrafish at 24, 48, 72, 96, and 120 hpf. Each cross curve or middle line of the box represents the mean value, and the upper (lower) bound represents the standard deviation (SD). Asterisk (*) represents a significant difference compared with the control by one-way ANOVA with Tukey's multiple range tests. * Represents $p < 0.05$; ** represents $p < 0.01$; and *** represents $p < 0.001$.

maturity, and S represents the survival rate from birth to sexual maturity.

To complete a Leslie matrix analysis, the life history information was organized in a projection matrix $\boldsymbol{A}$:

$$\boldsymbol{A} = \begin{bmatrix} F_1 & F_2 & F_3 & F_4 & F_5 & F_6 \\ S_1 & 0 & 0 & 0 & 0 & 0 \\ 0 & S_2 & 0 & 0 & 0 & 0 \\ 0 & 0 & S_3 & 0 & 0 & 0 \\ 0 & 0 & 0 & S_4 & 0 & 0 \\ 0 & 0 & 0 & 0 & S_5 & 0 \end{bmatrix} \quad (2)$$

where S_i represents the survival from stage i at time t to stage $i + 1$ at time $t + 1$.

The number of females at each stage at time t was calculated as follows:

$$N_t = [n_{1,t}, n_{2,t}, n_{3,t}, n_{4,t}, n_{5,t}, n_{6,t}]^T \quad (3)$$

where n_{ij} is the number of animals of stage i at the start of year j.

The numbers for the following years are determined by the following:

$$N_{t+1} = AN_t \quad (4)$$

or

$$N_{t+1} = A^t N_1 \quad (5)$$

The intrinsic rate of population increase ($r_{\text{intrinsic}}$) is defined as the instantaneous rate of population growth, which is the natural logarithm of the largest eigenvalue (λ) of matrix A:

$$r_{\text{intrinsic}} = \ln(\lambda) \quad (6)$$

The equations and matrices were solved by coding in MATLAB version R2016b. Details and related codes are shown in SI Text S3.

Statistical Analyses. Statistical analyses were conducted using GraphPad Prism 6 (GraphPad Software Inc., CA, U.S.A.). A Kolmogorov−Smirnov test was used to evaluate the distribution normality of lethality and hatching rates. Levene's test was used to evaluate homogeneity of each variable. Differences among groups were determined by single-factor analysis of variance (ANOVA) followed by Tukey's multiple comparisons. $p \leq 0.05$ was considered significant. Monte Carlo-type stochastic simulations were employed using PopTools (PopTools version 3.2.5. Available on the Internet. URL http://www.poptools.org) to add a probabilistic estimation of the variability of model predictions based on the variability of the input parameters. The input parameters in the matrix were defined by normal distribution before projecting the stochastic matrix. The 95% confidence interval (CI) of each prediction was obtained based on 1,000 iterations.

RESULTS AND DISCUSSION

Chronic Effects of BDNA on Growth and Sexual Maturation of Offspring. No notable mortality (<10% per group), abnormal behavior, or body surface damage were observed in adult zebrafish exposed to each concentration of BDNA for 21 days in the F_0 test. However, the 5-day cumulative fecundities of females exposed to median and high concentrations (50 or 500 μg/L) were significantly reduced ($p < 0.05$) compared with the control (Figure 1A). During the early life stage (0−7 days) of F_1 offspring, there was no significant difference in mortality rate compared with the control (SI Table S5). No significant developmental delay, pericardial edema, or spine curvature were noted. Hatch rates were 54.1 ± 2.3%, 54.2 ± 0.9%, 52.1 ± 3.7%, and 52.7 ± 1.0% for fish exposed to 0.5, 5, 50, and 500 μg/L at 48 hpf,

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respectively, similar to the rate in the control group (58.9 ± 1.4%, Figure 1B), suggesting that BDNA did not cause developmental toxicity in the parent zebrafish (F_0) or affect the growth of F_1 embryos or larvae. However, effects of BDNA on juvenile fish were observed at the onset of sexual maturity 90−120 dpf (days post fertilization). The average proportion of females in the treatment groups was significantly smaller, 37.9 ± 3.9% and 35.6 ± 3.1% in the groups exposed to 50 and 500 μg/L, respectively ($p < 0.01$), compared with the control (66.9 ± 3.6%), t (Figure 1C and SI Table S6). The average egg production per female significantly decreased from the control (171.2) to 108.0, 64.5, 85.2, and 84.3 in the 0.5, 5, 50, and 500 μg/L treatments during the 5-day chemical accumulation period (Figure 1D). These findings demonstrate that BDNA caused significant reproductive toxicity by impairing the sex ratio and fecundity, which could threaten the development of normal population structure.

In contrast with the normal development of the F_1 generation, the F_2 fish exhibited a series of adverse outcomes (SI Figure S2). During the cleavage period (2 hpf), control embryos developed to a 64-cell stage with 3 regular tiers of blastomeres, whereas the exposed embryos were delayed in growth. Those exposed to 0.5 or 5 μg/L only developed to a 4-cell stage (2 × 2 array of blastomeres), and those exposed to higher levels (50 or 500 μg/L) only developed to a 2-cell stage (partial cleavage). During the pharyngula period (24 hpf), control embryos developed to 26 somites, whereas embryos exposed to 0.5 and 5 μg BDNA/L developed to 19−20 somites. Development stalled at the gastrula stage (5−10 hpf in normal development) in embryos exposed to 50 or 500 μg/L BDNA.

The zebrafish embryos and larvae of the F_2 generation in the recovery and control groups showed similar morphology. For instance, treatment groups had a significant reduction ($p < 0.01$) in average hatch rate (from 16.1 ± 8.2% to 22.0 ± 3.0%) compared with the control (70.5 ± 6.2%) at 48 hpf. Similar proportions were observed in the recovery groups (from 15.2 ± 1.9% to 28.9 ± 4.6%) (Figure 1E). The average hatch rates were 80.8 ± 5.6% to 89.4 ± 0.2% in the treatment groups and 82.5 ± 3.3% to 93.4 ± 1.6% in the recovery groups at 72 hpf. Mortality was consistent between the treatment and recovery groups at a high BDNA concentration of 500 μg/L from 72 to 120 hpf (Figure 1F).

Long-term exposure to BDNA likely has the strongest impact on adult fecundity (F_0 and F_1), with subsequent deformities in F_2 offspring. Female production and cumulative spawning revealed a clear dose-dependent relationship compared with fish that were never exposed to BDNA. To our knowledge, this is the first study to demonstrate that BDNA has the potential to disrupt sexual hormones and cause reproductive toxicity. This outcome was consistent with the decrease in egg production and proportion of females in the F_1 generation. Screening of chemicals with (anti) estrogenic activity based on the response to VTG induction is a standard method in aquatic environments.[44−46] For example, in a previous study, VTG production was confirmed to be directly associated with the quality and quantity of eggs, and ultimately the viability of produced embryos in teleosts.[47] Altered levels of VTG in fish, including zebrafish, reflect abnormal estrogen receptor binding and/or activation of the estrogen response element.[48−50] These findings indicate that BDNA might have antiestrogenic activity, resulting in endocrine disorders.

Pollutants' effect on fecundity have also been documented. Fecundity in the F_0 generation was significantly inhibited and did not recover fully by the F_1 generation.[37] For example, multigenerational exposure to benzo[a]pyrene in zebrafish affected survival and induced developmental deformities in offspring.[51] In the present study, no significant differences in mortality or disturbances in growth between control fish and those exposed to BDNA were observed in the F_0 generation, indicating that organisms are able to compensate somewhat for the long-term reproductive toxicity caused by pollutants, as qualitative changes in population patterns occur only when the compensation mechanisms are overwhelmed. In addition, early in the F_2 generation, we found a significant growth delay in BDNA-treated groups. This phenomenon did not improve in the recovery group, which indicates that F_2 offspring were affected by BDNA. Previous studies also reported that maternal exposure to pollutants can alter the quality and size of eggs and fitness of offspring.[52] Our results demonstrate that long-term exposure to BDNA directly affects the growth of zebrafish and may have irreversible effects on subsequent offspring via parental inheritance or maternal deposition.

Simulation of Zebrafish Population Dynamics Using a Stage-Specific Demographic Model. A basic demographic model, based on the current conditions (e.g., pressures of life table, Leslie matrix), shows the dynamics of population change.[53] We hypothesized that modeling based on long-term BDNA parameters could be used to predict the development of the zebrafish population under TBPH or TBB pollution pressure (SI Table S8). First, linear regression and *t*-test were employed to verify the accuracy of values predicted by the model based on values obtained over three generations exposed to BDNA, collected at 7, 21, and 150 d (SI Table S9). Then, for consistency among the three treatment parameters (BDNA, TBPH, and TBB), a probability density of coefficient of variation (CV) analysis was used to measure the dispersion of the probability distribution of survivorship data. The slope of the linear regression was 1.005 (0.99−1.01 at 95% confidence), and the R^2 value was 0.9994 (Figure 2A). The residual difference (δ) between the observed and predicted values was 1.76 (Figure 2B). These results demonstrate high agreement between the observed and predicted values at all six stages. In addition, the density in the CV analysis of survivorship data (collected at 7 and 21 d) was similar among BDNA, TBPH, and TBB (Figure 2C). None of the CV values exceeded 15%; thus, survivorship data among these compounds were not significantly different. This result supports our hypothesis that the BDNA-based model can predict zebrafish population changes under TBPH or TBB perturbation.

Influence of High Levels of TBPH and TBB on Fecundity and Early Development of Zebrafish. During the 21-day exposure to TBPH or TBB, no notable mortality, abnormal behavior, or body surface damage were observed in adult zebrafish (F_0). However, significantly lower fecundity was found in the two treatment groups fed 63.80 μg/g TBPH dw ($p = 0.0012$) and 54.53 μg/g TBB dw ($p = 0.0027$), compared with the control (Figure 3A). Females produced 116 ± 56, 144 ± 4, and 22 ± 2 embryos each in the groups exposed to low, medium, and high concentrations of TBPH, respectively, and 158 ± 37, 130 ± 24, and 61 ± 14 embryos each in groups exposed to TBB. In contrast, control females produced 180 ± 5 embryos each. Statistically significant delays in fertilization of embryos were found in the TBPH-H group at 60 to 72 hpf (p

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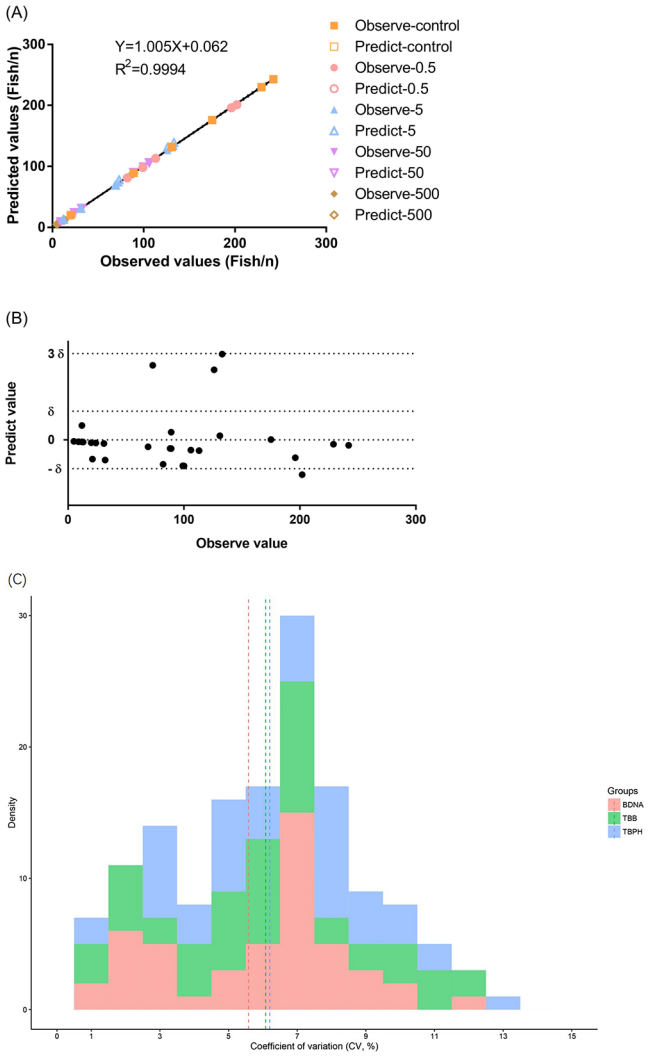

Figure 2. Scatter plot (A) shows the relevance between observed values (*x*-axis) and predicted value (*y*-axis) for different exposure concentrations of BDNA in the quasi three-generation exposure test. The trend line is a straight line with a slope of 1 and a *y*-axis intercept of 0. Scatter plot (B) shows the results of the residual analysis focused on the observed and predicted values. The value of δ is 1.76, and the dashed line represents $\pm\ \delta$ and 3δ, respectively. Stacking plot (C) illustrates the coefficient of variation (CV) distribution of offspring (F_1 generation) over 7 days for the survival rate after exposure to BDNA, TBPH, and TBB for 21 days.

= 0.0073) (Figure 3B). The proportion of survivors was comparable between the treatments and the control. No adverse effect was observed for any normal larvae until 144 hpf.

Dietary TBPH or TBB impaired reproductive function in adult zebrafish (F_0), with a clear dose-dependent relationship. Similar effects were observed in zebrafish exposed to di(2-ethylhexyl)-phthalate (DEHP), a structural analogue of TBPH.[54] A previous profile analysis reported significant differences between zebrafish exposed to the greatest concentration of TBPH/TBB mixture and those exposed to a solvent control.[37] Results from the short-term TBPH or TBB exposure likewise indicate that long-term exposure could decrease the reproductive success and sustainability of fish populations.

Long-Term High Exposure to TBPH or TBB Can Reduce Population Growth, Leading to Extinction. Cumulative fecundity (F_0) and survivorship data from the 21-day exposure test and early life-stage toxicity test (0−7 dpf) were employed in the Leslie matrix (SI Table S10). The survival parameters from larvae to adult (7−15, 15−41, 41−121, 121−151, and 151−365 days) were consistent with the findings from the control group of the BDNA experiment. The contribution to sensitivity of the dominant eigenvalue (i.e., the finite rate of increase) predominantly arose from survival before sexual maturity ([0,121)). The survival rate was predominantly responsible for sensitivity in the Leslie matrix, and fecundity did not have remarkable influence. Considering that the growth of larvae and/or juvenile fishes was affected to a greater extent by the compound or the external environment, this conclusion was considerably consistent with predictions based on common sense. In addition, the most sensitive period was 15−41 days (the period of active eating). This result was related to the larvae beginning to feed on exogenous food, which was apparently a sensitive stage. These parameters were used to predict population growth for zebrafish over 20 years. The highest TBPH and TBB concentrations decreased zebrafish spawning to 87.4% and 65.4% of control values, respectively. However, low or moderate concentrations caused no notable inhibition (Figure 3A and B). We next assessed the deterministic demographic model we built to the stochastic matrix drawn using Monte Carlo-type random simulation to predict how the zebrafish population-level will develop if they are chronically affected by TBPH or TBB. The population growth rates ($r_{intrinsic}$) showed that different effects of TBPH or TBB on individual-level end points could attenuate at the population level over the next 20 years. For example, at low exposure, zebrafish populations were predicted to fluctuate but increase gradually, by 0.01 or 0.02 for TBPH and TBB, respectively (Figure 4B and F, SI Table S11). In contrast, volatility or bluff-type decrease was predicted in populations that were exposed to medium or high concentrations. Populations in the TBPH-M ($r_{intrinsic}$ = −0.02) and TBB-M ($r_{intrinsic}$ = −0.01) groups would decline during the first decade and then stabilize over the next decade (Figure 4C and G). Populations were predicted to become extinct within 3−5 years at the highest level of TBPH ($r_{intrinsic}$ = −0.36) or TBB ($r_{intrinsic}$ = −0.14) (Figure 4D and H). In a stage-structured population analysis within the cumulative 2, 5, 10, and 20 years, deteriorating groups had aging populations. The proportion of the adult-aged population (150−365 days) in each treatment over 20 years was as follows: TBPH-H (6.9%) > TBB-H (3.4%) > TBB-M (2.2%) > TBPH-M (2.1%) > TBPH-L (2.0%) > TBB-L (1.9%) > control (1.8%) (Figure 5).

Our matrix model analyzes changes in population growth resulting from changes to vital rates.[13,55] On the basis of the number of initial females and spawning (5, N_0), zebrafish populations exposed to continuous high concentrations of TBPH or TBB were likely to exhibit large population losses and undergo extinction within 20 years. A previous experiment on a whole lake showed similar reproductive failure in fathead minnows,[56] with a loss of young fish observed after a second season of EE_2 addition, but no loss in a reference lake. However, assessing impacts on population growth from impacts on single individual-level end points could result in underestimation or overestimation. In the field, the population growth rate is linked to population abundance and environmental carrying capacity. When the number of births is less than the number of deaths, the growth rate declines. But in a smaller population, more resources are available to each

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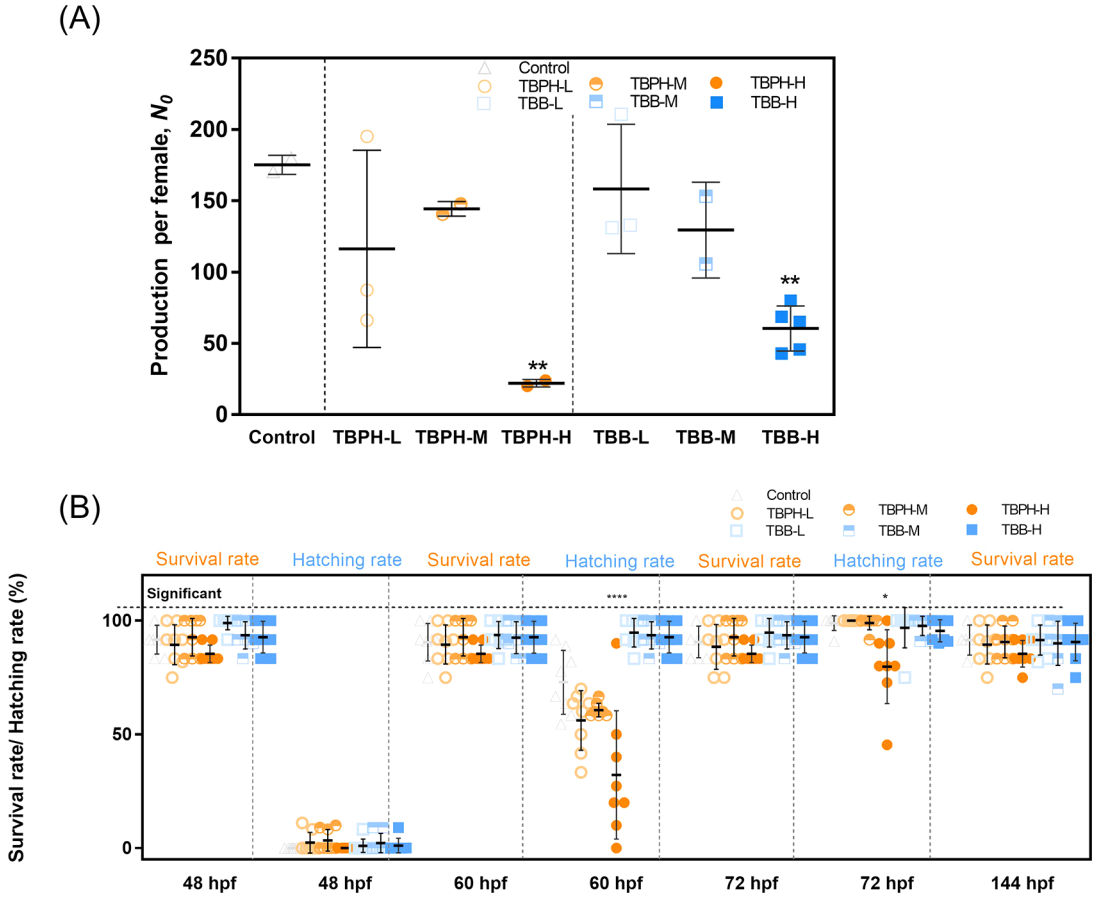

Figure 3. Box plot (A) shows the number of eggs produced per female in F_0 generation exposed to TBPH or TBB for 21 days. Box plot (B) reveals the proportion of survival and hatching in offspring zebrafish (F_1) at 48, 60, 72, and 144 hpf. The middle line of the box represents the mean value, and the upper/lower bounds represent the standard deviation (SD). Asterisk (*) represents significant difference compared with control group by one-way ANOVA with Tukey's multiple range tests. * Represents $p < 0.05$; ** represents $p < 0.01$; and *** represents $p < 0.001$.

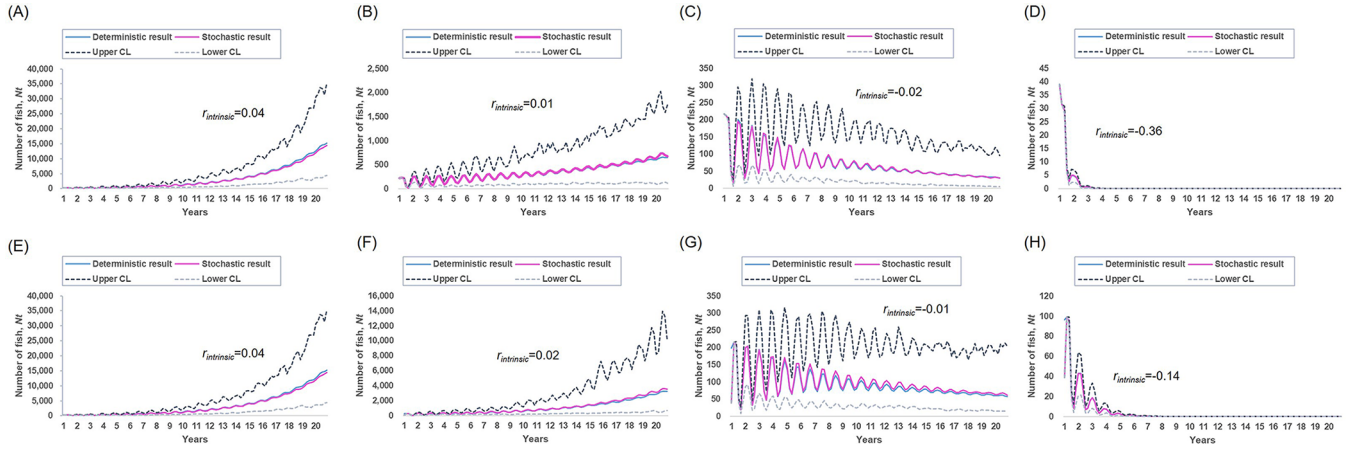

Figure 4. Population projection of the zebrafish in 20 years. Control groups (A and E) are compared with fish continuously exposed to 4 μg/g dry weight (TBPH-L, dw) (B), 15.95 μg/g (TBPH-M, dw) (C), 63.80 μg/g (TBPH-H, dw) (D), 3 μg/g (TBB-L, dw) (F), 13.63 μg/g (TBB-M, dw) (G), and 54.53 μg/g (TBB-H, dw) (H). $r_{intrinsic}$ represents intrinsic rate of zebrafish population growth. The deterministic result (blue solid line) represents the projection of model with state vital parameters (based on multigenerational exposure, time invariant), and the stochastic result (pink solid blue) represents the projection of model with time variation (Monte Carlo-type stochastic simulations based on survival rate normal distribution, μ (S_i), σ (0.025)). The dashed line (upper or lower CL) represents 95% confidence interval (CI) based on 1,000 iterations.

individual and there is less fishing pressure on each individual, so survival of older fish is increased. In contrast, larger populations experience increased fishing pressure on each individual and lower population growth. A similar pattern was observed in laboratory experiments that examined the effects of chemicals in terrestrial and aquatic animals.[2,9,53,57,58] Future studies should explore the effects of various conditions on population dynamics.

Extending the Analysis to Realistic Environment Management. The integrated laboratory test and modeling

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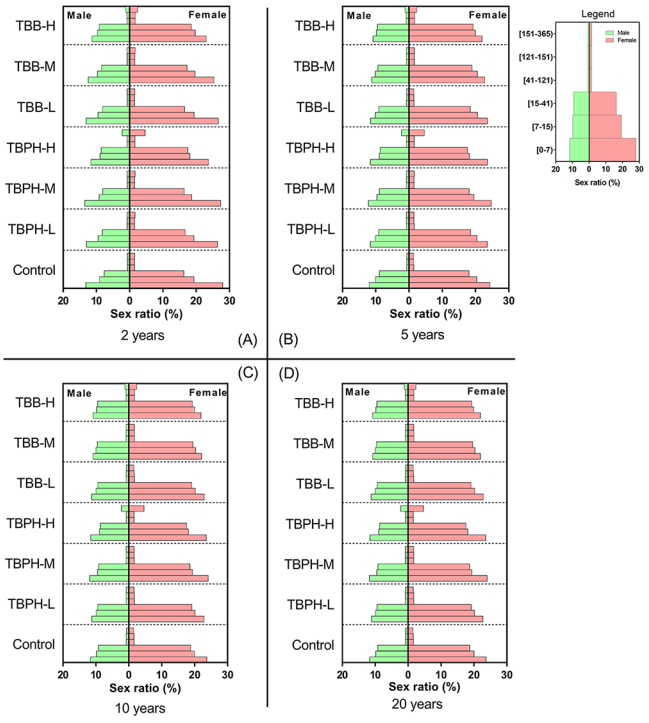

Figure 5. Stage-based structure analysis for zebrafish population within 2 (A), 5 (B), 10 (C), and 20 years (D), respectively. The structure projection intervals are for six life stages: 0−7 days, 7−15 days, 15−41 days, 41−121 days, 121−151 days, and 151−365 days.

approach developed in the present study overcomes the challenge of complicated long-term field observations and can be applied to address broader-scale and long-term ecotoxicity effects. Future investigations should conduct controlled pre- and postimpact tests to relate reduction in effluent toxicity with changes in species demographics, and to evaluate the effectiveness of remediation practices on contaminated sites.

Life-stage data for individual fish could be combined with population modeling to infer population demographics when data are lacking. The critical steps or checkpoints along the pathway should be measurable and have potential predictive value.[59] Individual-level responses can also reflect the toxicity and adversity associated with a class of chemicals, which helped us to expand our understanding of biological life history traits. To assess a compound for which data are limited, relationships between egg production and end points of interest (e.g., survival rate, fertilization, hatching) can be incorporated into a population model. Such models can be used to characterize the relationships between fecundity and end points of interest for compounds, which can then be applied to other compounds. In addition, changes in fecundity (including survival rate, egg production, and sex ratio) at the individual level can be associated with end points of interest such as depressed plasma vitellogenin and maternal deposition (eq 1). Utilizing the surveyed N_1 to adjust annual values of fecundity and survival rates over time step t, population changes after t years (N_{t+1}) (eqs 4 and 5) of chemical exposure can be predicted.

Different modes of action can cause different reproductive outcomes in terms of survival and fecundity. Models can only predict population trajectories when mechanisms of action from the toxic chemicals are well characterized. For example, 2,4,6-trichlorophenol (TCP) has acute toxicity in zebrafish (LC_{50} = 960 μg/L at 96 hpf).[60] Because its mode of action differs from that of BDNA, a demographic model based on BDNA exposure cannot predict population changes after TCP exposure. Thus, eqs 1, 2, 4, and 5 of the model can only be applied to toxicants that have similar effects on populations.

In summary, our study integrates individual-level measurements from multigenerational experiments and a simple demographic model to predict the long-term effects of toxicity on a population. This integrated approach can be used to develop research questions and management strategies for assessing ecological risk to stressed populations of fish and wildlife. Because the environment is complex, more comprehensive data on population structure and development based on density-dependent growth are needed. Nevertheless, molecular mechanisms and biochemical and subcellular responses remain useful for population-level assessments. These responses must first be linked to higher-level effects in individuals that translate to population-level effects. The framework of the adverse outcome pathway network can help identify and visualize these linkages and classify different modes of action. Modeling effects on the individual through adverse outcome pathways provides a keystone that links bottom-up toxicity information with top-down information on population dynamics, community interactions, and ultimately ecosystem influences.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.8b00569.

Analytical method and materials; optimized instrumental parameters; concentration of BDNA in water solutions; concentrations of TBPH or TBB; early life-stage toxicity test of zebrafish; Leslie matrix and MATLAB codes; number of zebrafish in generation F1; sexual differentiation of groups; life history information for each treatment group; and accuracy analysis for the demographic model (Texts S1−S3 and Tables S1−S11) (PDF)

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Notes

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