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EVALUATION OF THE EFFECTS OF WATER DISINFECTION BY-PRODUCTS, BROMOCHLOROACETIC AND DIBROMOACETIC ACIDS, ON FROG EMBRYOGENESIS

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*Adverse developmental effects of two haloacetic acids, bromochloroacetic acid (BCA) and dibromoacetic acid (DBA), were determined by using the Frog Embryo Teratogenesis Assay—*Xenopus *(FETAX).* Xenopus *embryos (150–400/concentration group) were exposed to 0, 8000, 10,000, 12,000, or 14,000 ppm BCA or 0, 10,000, 12,000, 14,000, or 16,000 ppm DBA for 96 h beginning from stage 8 (mid-blastula) to stage 46 (when primary organogenesis is complete). BCA produced 29, 83, and 100% mortality at 10,000, 12,000 and 14,000 ppm, respectively. Incidence of malformations among surviving embryos at 96 h for 10,000 and 12,000 ppm BCA were 8.4 and 68%. Thus LC50 and EC50 for BCA were between 10,000 and 12,000 ppm. DBA did not produce any significant mortality or malformation at any of the concentrations tested. In summary, BCA affected development of* Xenopus *embryos only at high concentrations, while DBA did not affect* Xenopus *development at the concentrations tested.*

Chlorination is commonly used to disinfect drinking water. The use of chlorine to disinfect public water began in the early 1900s. Due to the efficiency, low cost, and ease of this technique, the use of chlorine spread quickly through the United States (Faber, 1952). Consequently, the incidence of water-borne diseases was greatly reduced (Faber, 1952; Barzilay et al., 1999). However, in the 1970s chlorination by-products were identified in treated waters. This raised concern over the safety of the chlorination process (Rook, 1974; Bellar et al., 1974). Since that time epidemiological and toxicological studies have linked exposure to disinfection by-products with cancer, particularly bladder and rectal cancer (Morris et al., 1992; Boorman et al., 1999; Nieuwenhuijsen et al., 2000), as well as developmental and reproductive toxicities (Reif et al., 1996; Nieuwenhuijsen et al., 2000; Graves et al., 2001).

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Dichloroacetic acid (DeAngelo et al., 1999), but not monochloroacetic acid or trichloroacetic acid (DeAngelo et al., 1997), has been reported to produce liver cancer, indicating that the type of by-product is critical in hepatocarcinogenicity. Because of the relatively recent discovery of these disinfection by-products, there are gaps in the current knowledge of their toxic effects.

Most epidemiological and toxicological studies focus on the trihalomethanes, the most prevalent class of chlorinated disinfection by-products. The haloacetic acids, the second most common type of disinfection by-products, have come under scientific scrutiny only recently. The chlorinated acetic acids have received most of this attention; their toxic effects (Bhat et al., 1991; Smith et al., 1992; Toth et al., 1992; Nartosky et al., 1996) and metabolic fate (Austin & Bull, 1997) have been studied in rodents. However, brominated acetic acids are more common than chlorinated species where bromine levels are high in source waters. When levels of bromine are low (0.07–0.1 mg/L), chlorinated haloacetic acids range between 1.0 and 7.3 μg/L while brominated haloacetic acids range between 0.5 and 1.5 μg/L. However, when bromine levels are higher (2.8–3 mg/L), chlorinated haloacetic acids are found at concentrations of 0.6–1 μg/L while brominated haloacetic acids levels are between 7.8 and 19 μg/L (Krasner et al., 1989).

It has been reported that brominated acetic acids are potentially more toxic than chlorinated species to developing rodent fetus (Hunter et al., 1996; Nartosky et al., 1996; Giller et al., 1997). Similar comparative studies for amphibian species are scant. Tribromoacetic acid (Bantle et al., 1999), but not dibromoacetic acid (DBA) (Reimers, 1999), was found to produce malformations in *Xenopus* embryos. The objective of the present study was to evaluate and compare the developmental effects of bromochloroacetic acid (BCA) and DBA using the Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX).

MATERIALS AND METHODS

Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX)

The FETAX is a 96 h developmental toxicity test that uses whole embryos from the South African clawed frog, *Xenopus laevis* (ASTM, 1991). It was originally developed as a quick, cost-effective, and reliable assay to identify chemicals that could be teratogenic in humans (Bantle et al., 1994a). Subsequently, it has also been utilized to identify chemicals that may be producing developmental malformations and declines in amphibian populations (Burkhart et al., 1998). Comparisons between FETAX and mammalian teratogenesis studies demonstrate that FETAX can reliably predict chemicals that pose a threat to mammalian development (Dawson et al., 1989; Dresser et al., 1992; Bantle et al., 1994a, 1994b, 1996a, 1999; Fort et al., 1998, 2000).

In performing the studies reported herein, while ASTM guidelines for FETAX were adhered to for generation and sorting of embryos, the following exceptions were made for culture conditions: (a) 100-mm Pyrex glass petri

dishes instead of stipulated 55-mm polystyrene dishes (to avoid possible contamination of leachates from plastic); (b) 1 dish per concentration with 50 embryos/dish having 20 ml medium instead of recommended 2 dishes each with 25 embryos in 10 ml medium; and (c) 2 dishes for control group instead of 4. Clutches from individual mating pairs were maintained separately, and definitive tests were performed on at least three clutches for each haloacetic acid (three for DBA and seven for BCA). Data for all definitive tests for each chemical were pooled and reported as a single experiment (described later).

Animals and Animal Care

Proven breeder *Xenopus laevis* frogs were purchased from Xenopus One (Dexter, MI). Male and female frogs were housed separately in tanks containing 10 m*M* NaCl solution and maintained at 19–23 °C on a 12:12 h light:dark cycle. Frogs were fed Aquamax Grower 600 (PMI Nutrition International, Inc., Brentwood, MO) on alternate days.

Animal Breeding and Embryo Collection

Xenopus laevis frogs were bred according to the guidelines provided by the American Society for Testing and Materials (ASTM, 1991). Briefly, sexually mature frogs were injected with human chorionic gonadotropin (Intervet, Inc., Millsboro, DE) into the dorsal lymph sac (500 IU for males, 900 IU for females; 4 breeding pairs per experiment). Each breeding pair was placed in 1.5 gal of FETAX solution (6.25 g NaCl, 0.96 g $NaHCO_3$, 0.30 g KCl, 0.09 g $CaCl_2$, 0.60 g $CaSO_4 \cdot 2H_2O$, 0.75 g $MgSO_4$ in 10 L deionized water, pH = 7.6–7.9) in a 2.5-gal tank equipped with a plastic mesh bottom to facilitate collection of embryos. Each clutch was collected separately and treated with 2% w/v L-cysteine (pH 8.1) to remove the jelly coat from eggs. Embryos were examined under a dissecting microscope to determine viability and stage of development according to Nieuwkoop and Faber (1967) and ASTM standards (1991). Only normal, cleaving embryos at the mid-blastula (stage 8) to early gastrula (stage 11) were selected for the experiment.

Chemicals

BCA (CAS number 5589-96-8, lot 03507BI, purity 97%) and DBA (CAS number 631-64-1, lot 03807JS, purity 97%) were purchased from Aldrich (Milwaukee, WI). DBA was dissolved in deionized water to a concentration of 1 g/ml. BCA is liquid at room temperature and was left unaltered until stock solutions were made. Stock solutions were prepared every 48 h by diluting DBA or BCA in FETAX solution at pH 7.4–7.9, and solutions were stored in amber glass bottles at room temperature.

Experiment I: BCA

Seven clutches from different breeding pairs were kept separate and embryos were randomly divided into 5 treatment groups—0 ($n = 700$), 8000 ($n = 350$), 10,000 ($n = 350$), 12,000 ($n = 350$), and 14,000 ($n = 350$) ppm BCA—and

2 positive control groups—5.5 ppm ($n = 350$) and 2500 ppm 6-aminonicotinamide ($n = 350$). BCA concentration levels were chosen based on a preliminary concentration-response study. Positive control concentration levels were selected based on EC_{50} and LC_{50} for 6-aminonicotinamide suggested by the ASTM Standard Guide for Conducting the FETAX (ASTM; 1991). LC_{50} was defined as the lowest concentration that killed 50% of embryos cumulatively during the 96-h test period. EC_{50} was defined as the lowest concentration that produced at least one malformation in 50% of embryos surviving at 96 h.

Embryos were maintained at 22–24 °C, in FETAX medium (pH 7.4–7.6), at a density of 2.5 embryos/ml solution, and the medium was changed every 24 h. Mortality was recorded and dead embryos were removed every 24 h. At 96 h postfertilization, embryos were euthanized using 2% w/v 3-aminobenzoic acid ethyl-ester (CAS number 886-86-2, Aldrich, Milwaukee, WI) and fixed in 3% formalin. Stage of development of embryos (based on criteria defined by Nieuwkoop & Faber, 1967) and presence of any malformations (Bantle et al., 1996b) were recorded using a dissecting microscope (10–15× magnification).

Tadpoles were stained with 2% basic fuchsin and photographed using a Sony DXC ISA video camera fitted with a Nikon AF Nikkor lens. Length of the tadpole (from nose to tip of tail) was measured using Image Pro Plus (version 3.0). Tadpole length was used to determine the minimum concentration to inhibit growth (MCIG), defined as the concentration of chemical that significantly reduced tadpole length.

Experiment II: DBA

The protocol used for experiment II was the same as that of experiment I. Clutches from four different breeding pairs were used and, based on an initial range-finding test, the DBA concentration groups were: 0 ($n = 300$), 10,000 ($n = 150$), 12,000 ($n = 150$), 14,000 ($n = 150$), 16,000 ($n = 150$) ppm.

Statistical Analyses

Data were analyzed using StatView (version 5.0, SAS Institute, Inc., Cary, NC). Stage of development, length of the body, and incidence of malformations were analyzed using analysis of variance (ANOVA) with a Tukey/Kramer post hoc test. A level of significance of $p = .05$ was used for all tests. Percentage values were transformed using arcsine of the square root of the value divided by 100 to account for any inequalities in variance.

RESULTS

Experiment I: BCA

Mortality rates, average stage of development, body length, and incidence of malformations are presented in Table 1. The LC_{50} for BCA was found to be between 10,000 and 12,000 ppm. Average stage of development was significantly reduced at 10,000 and 12,000 ppm BCA compared to control.

TABLE 1. Effect of BCA on Survival and Development of *Xenopus* Embryos

BCA treatment	Mortality (%)[a]	Stage of development[b]	Body length (mm)	Malformations[c] (%)		Edema[c] (%)
				Axial	Gut	
0 ppm	2 (700)	45.9 ± 0.01	9.4 ± 0.04	0.0	2.2	1.4
8000 ppm	11 (350)	45.9 ± 0.02	9.1 ± 0.10[d]	1.3	3.5	0.3
10,000 ppm	29 (350)	45.8 ± 0.04[d]	8.6 ± 0.10[d]	0.8	6.0	1.6
12,000 ppm	83 (350)	45.2 ± 0.13[d]	8.2 ± 0.15[d]	2.0	66.0[d]	0.0
14,000 ppm	100 (350)	NA	NA	NA	NA	NA

[a] Cumulative mortality at 96 h; numbers in parentheses indicate number of embryos.
[b] Based on Neiuwkoop and Faber (1967).
[c] Percent of surviving embryos at 96 h.
[d] Significantly different ($p = .05$) from control using ANOVA and Tukey/Kramer post -hoc test.

Body length was significantly reduced at 8000, 10,000, and 12,000 ppm BCA compared to control (Figure 1). Therefore, MCIG was determined to be 8000 ppm BCA. Incidence of malformations increased significantly at 12,000 ppm BCA (68.0%). The most prominent malformations noted were axial deformities and gut malformations (Figure 2). EC_{50} was found to be between 10,000 and 12,000 ppm BCA.

Experiment II: DBA

Mortality rates, average stage of development, body length, and incidence of malformations are presented in Table 2. There was no increase in mortality for any level of DBA tested and thus LC_{50} could not be determined. Stage of development at 96 h was similar between DBA-treated and control tadpoles. DBA did not markedly affect the length of the tadpole at any concentration level tested and thus MCIG could not be determined. Malformations noted included optic lesions, gut malformations, and edema. However, the number of malformations in DBA-treated tadpoles did not differ from controls and thus EC_{50} also could not be determined. In all tests, positive control treatments (5.5 ppm and 2500 ppm 6-aminonicotinamide) resulted in 100% mortality by 96 h.

DISCUSSION

Notwithstanding the fact that ASTM guidelines for FETAX were not followed verbatim for the definitive tests reported herein, results from these studies correlate well with data generated in previous FETAX studies on haloacetic acids. In these studies, haloacetic acids produced few adverse effects on frog embryos even at high concentrations. Tribromoacetic acid was found to be teratogenic to frog embryos between 1400 and 10,000 ppm and lethal to half of exposed embryos at 9000–19,000 ppm (Bantle et al., 1999); while DBA did not induce malformations, it was lethal to half of exposed frog embryos at 11,750 ppm (Reimers, 1999). However, several studies using rodents found

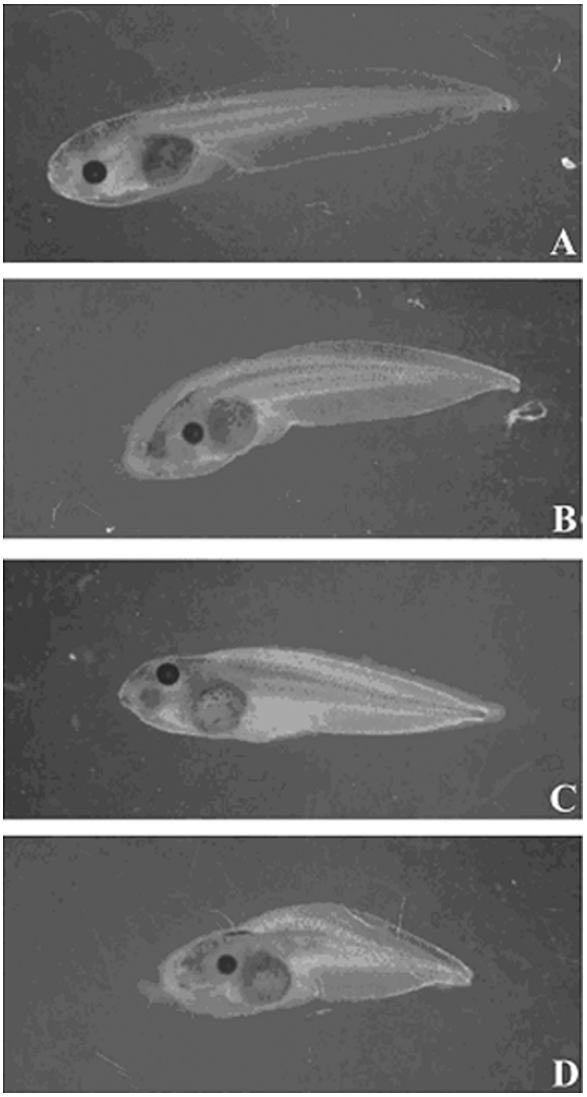

FIGURE 1. Photographs of 96-h *Xenopus* embryos illustrating variation in body length between control and BCA-exposed animals. (A) Embryo from the control group. (B) Embryo from the 8000 ppm BCA group. (C) Embryo from the 10,000 ppm BCA group. (D) Embryo from the 12,000 ppm BCA group. (A)–(D) 5× original magnification.

that haloacetic acids produce detrimental effects on reproduction and development at doses relatively lower than those used in the current study. Delayed parturition was noted in mice dosed with 0.23 mmol DBA/kg/d from gestation d 6 through 15 (Nartosky et al., 1996). Abnormal development of sperm, retention of step 19 spermatids, and atypical residual bodies were seen in

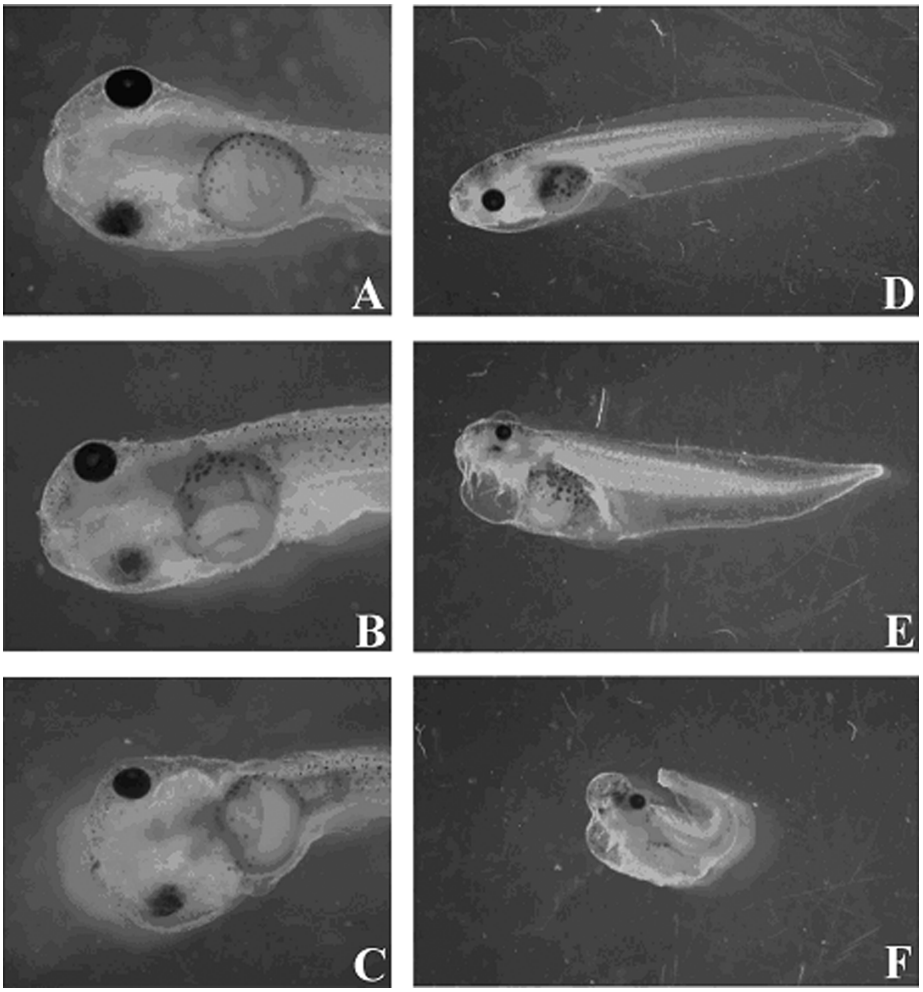

FIGURE 2. Photographs of 96 h control and BCA-exposed *Xenopus* embryos illustrating edema, gut, and axial malformations. (A) Control embryo showing normal gut coiling. (B) Embryo from the 10,000 ppm BCA group showing abnormal gut coiling. (C) Embryo from the 12,000 ppm BCA group showing an immature, malformed gut. (D) Embryo showing normal morphologic features. (E) Embryo showing edema in the cardiac and gut areas. (F) Deformed embryo showing axial malformation and edema in the cranial and cardiac regions. (A)–(C) 7.5× and (D)–(F) 5× original magnification.

adult rats dosed with 90 mg DBA/kg/d for 14 d (Linder et al., 1994) or with 50 mg DBA/kg/d for 31 d (Linder et al., 1995, 1997). Reduced caput sperm counts were found in rats dosed with 10 mg DBA/kg/d for 14 d (Linder et al., 1994). As few effects were noted in frog embryos in the current study, even at high concentrations, FETAX results do not appear to reflect the possible detrimental effects of haloacetic acids on mammalian development. However, it

TABLE 2. Effect of DBA on Survival and Development of *Xenopus* Embryos

DBA treatment	Mortality (%)[a]	Stage of development[b]	Body length (mm)	Malformations[c] (%)		Edema[c] (%)
				Axial	Gut	
0 ppm	4 (300)	46.0 ± 0.21	9.0 ± 0.48	0.3	3.0	1.7
10,000 ppm	5 (150)	45.9 ± 0.17	9.0 ± 0.45	0.0	2.7	0.7
12,000 ppm	5 (150)	45.9 ± 0.25	9.0 ± 0.48	0.0	2.0	2.0
14,000 ppm	7 (150)	46.0 ± 0.09	8.9 ± 0.51	1.3	2.7	1.3
16,000 ppm	4 (150)	46.0 ± 0.19	8.9 ± 0.48	0.0	4.0	2.0

[a] Cumulative mortality at 96 h; numbers in parentheses indicate number of embryos.
[b] Based on Neiuwkoop and Faber (1967).
[c] Percent of surviving embryos at 96 h.

should be noted that it is not known if chronic exposures to haloacetic acids adversely affect reproduction in amphibians, similar to that observed (e.g., impaired spermiogenesis) in mammals.

In the current study, the EC_{50} and LC_{50} for BCA were found to be relatively high (between 10,000 and 12,000 ppm). BCA also produced a significant decrease in embryo length at 8000 ppm, suggesting that BCA is developmentally toxic to frogs at this concentration level. However, these concentrations are considerably higher than levels found in treated drinking water. Reported levels of total haloacetic acids in treated drinking water range between 13 µg/L (Krasner et al., 1989) and 160 µg/L (Uden & Miller, 1983). Therefore, BCA by itself may not be either teratogenic or developmentally toxic to frogs at levels normally found in treated water; however, since it is likely that disinfection by-products exist in water as mixtures and that synergies or additivities might occur among them, it is possible that nontoxic concentrations of individual compounds become toxic when in mixture.

In this study, no adverse effects were observed in DBA-exposed tadpoles. It is possible that DBA is developmentally toxic and/or teratogenic at much higher concentrations. However, even the concentrations tested in this study would not be encountered in treated water. Thus it can be concluded that DBA is not teratogenic or a developmental toxicant to tadpoles at levels normally found in treated water.

Although earlier rodent studies noted that brominated acetic acids are potentially more toxic than chlorinated species (Hunter et al., 1996; Nartosky et al., 1996; Giller et al., 1997), and that toxicity (hepatocarcinogenicity) depends on the type of chlorinated haloacetic acids (DeAngelo et al., 1997), previous amphibian studies indicated that chlorinated species are more toxic than brominated species (LC_{50} for dichloroacetic acid: 4060–9600 ppm [Fort et al., 1993; Bantle et al., 1999]; LC_{50} for tribromoacetic acid: 9000–17000 ppm [Bantle et al., 1999]; and LC_{50} for DBA: 11750 ppm [Reimers, 1999]). Similarly, the current study further indicates that haloacetic acids containing chlorine in addition to bromine may be more toxic to amphibians than purely brominated species.

Brominated haloacetic acids have been found to be more teratogenic to mouse embryos than their chlorinated analogs (Hunter et al., 1996). Previous frog studies have also suggested that tribromoacetic acid, although less toxic, is more teratogenic than purely chlorinated species (Bantle et al., 1999). Considering that the concentration levels tested in amphibian studies were much higher than those used in rodent studies, it should be noted that the data from amphibian studies, including the current study, do not mirror mammalian data. No developmental effects were observed in the current study with DBA even at levels as high as 16,000 ppm. BCA was able to produce developmental retardation at 8000 ppm. In 9-d-old mouse whole-embryo culture, DBA was able to induce neural tube defects at 110 μ*M* (~240 ppm) (Hunter et al., 1996). Mouse pups from mothers dosed with 2.8 mmol DBA/kg/d (~1200 ppm/d) often had kinked, short, or absent tails (Nartosky et al., 1996). However, due to metabolism of DBA in the dam (see Austin & Bull, 1997), the fetuses would have been exposed to much less chemical than the estimated 1200 ppm DBA. Thus, teratogenic effects of haloacetic acids in mammals are observed at substantially lower doses than in the frogs.

It should be noted that FETAX was not performed in the current studies in such a way (i.e., using an exogenous metabolic activation system; Fort et al., 1998) that it could yield data to enable comparisons between amphibians and mammals. Nonetheless, the observed differences between rodent and frog studies may be due to the nature of the chemicals studied and not the predictive value of FETAX for mammalian teratogens. *Xenopus* can tolerate brackish water and live in 40% sea water for several days (Tinsley & Kobel, 1996). Therefore, it is possible that *Xenopus* and their embryos are tolerant to chemicals in sea water such as chlorine and bromine.

Although FETAX is a valuable amphibian model system, mammalian teratogenesis assays will be needed to conclusively determine the teratogenic risk BCA and DBA pose to mammals. It can be concluded from this study that neither BCA nor DBA is teratogenic or developmentally toxic to amphibians at levels that normally occur in treated water or source waters naturally containing haloacetic acids.

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