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ACUTE TOXICITY OF THE HERBICIDE BROMOXYNIL TO *DAPHNIA MAGNA*

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Abstract—The acute toxicities of technical-grade bromoxynil octanoate (BO) and two commercial formulations, Buctril® and Bronate®, to <24-h-old neonate *Daphnia magna* (Straus) were determined in soft, hard, and oligosaline water. In addition, effects of life stage, feeding, aging the herbicide, and exposure duration on BO toxicity to daphnids were investigated. Regardless of formulation, life stage, and water quality, BO was found to be extremely to highly toxic to daphnids in standard tests; 48-h EC50 values ranged from 41 to 161 μg/L. Bromoxynil octanoate was the most toxic to neonates in soft water and the least toxic in hard water. The acute toxicities of the three bromoxynil herbicides to a given age group of daphnids were similar within the same water type. Overall, neonates and 7-d-old adults were more sensitive than 14- or 15-d-old adults to each herbicide. Feeding daphnids during the toxicity test significantly decreased BO toxicity compared to not feeding them. Aging BO (as Buctril) in hard water decreased its toxicity, and the rate of deactivation was rapid, with an estimated half-life of biological activity of 13 h. Daphnids immobilized by exposures to toxic BO concentrations for ≤6 h recovered their mobility, whereas exposures of 18 and 24 h to BO produced toxic effects in daphnids similar to those exposed for 48 h. These results indicated that standard continuous exposure tests may not adequately predict the acute toxicity of BO to freshwater animals in the field.

Keywords—Acute toxicity Bromoxynil *Daphnia magna* Test condition effects

INTRODUCTION

Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile, mol. wt. 276.9) is a postemergent herbicide registered for use on corn, wheat, and other small grains to control certain broadleaf weeds [1]. Bromoxynil-based herbicides are widely used in the Northern Prairie Wetlands Region of North America [2,3] because the predominant crop in the region is wheat [4]. Buctril and Bronate are the commercial formulations of bromoxynil recommended for use in North Dakota [5].

The extensive use of bromoxynil-based herbicides in the region has led to growing concerns about the movements of these chemicals into wetlands and their effects on indigenous invertebrate populations that are an important food source for waterfowl. Wetlands are subject to inputs of agricultural chemicals via overspray, aerial drift, treatment of dry basins, and postapplication runoff as a result of their proximity to cultivated fields. Furthermore, most of these chemicals are applied in spring and early summer, which coincides with the breeding season of waterfowl [4]. The diet of breeding dabbling ducks (*Anas* spp.), particularly laying hens, consists largely of invertebrates, of which cladocerans such as *Daphnia* spp. constitute a substantial portion of their ration [6]. Thus, pesticide contamination of wetlands may adversely affect waterfowl reproduction by reducing the abundance of invertebrate food organisms [4].

To determine the potential hazard of these herbicides to key waterfowl food organisms, their toxicity to aquatic invertebrates and environmental exposure concentrations must be estimated [7]. The toxicities of pesticides are usually determined in the laboratory with prescribed species tested under standard conditions. However, pesticide toxicity to aquatic biota depends on a number of factors, including species, size and life stage (age) of the test animals, formulation vehicle, physicochemical characteristics of the water, and intrinsic properties of the pesticide [8,9]. Moreover, pesticide contamination of surface waters is usually episodic. Consequently, the exposure of aquatic organisms to these pesticides varies in concentration, duration,

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and frequency. The effects of brief and intermittent exposures to pesticides have received little attention in laboratory toxicity studies. Most toxicity tests with pesticides have incorporated only a few of these factors in their design, and therefore, the results may not be predictive of field situations. Clark et al. [10,11] reported that laboratory tests must simulate field exposure regimes to adequately predict the effects of pesticide use in the environment.

Bromoxynil octanoate (BO, mol. wt. 403.1) is the active ingredient in Buctril and Bronate. It is one of the most acutely toxic herbicides to aquatic organisms used in the region; LC50s for *Daphnia* and freshwater fish are ≤170 μg/L [1]. However, no published data are available on the acute toxicity of these formulated products to aquatic organisms. The purpose of this study was to evaluate the acute toxicity of BO and its formulated products Buctril® and Bronate® to *Daphnia magna*. Acute toxicity studies were designed to assess the effects of water quality, age of daphnids, feeding, aging herbicide solutions, and pulse exposures on BO toxicity to daphnids. Also, postexposure recovery from bromoxynil intoxication and delayed toxicity were monitored in *D. magna* to further assess the effects of BO on aquatic invertebrates.

MATERIALS AND METHODS

Culture

The culture methods for *D. magna* were based on guidelines recommended by the American Society for Testing and Materials (ASTM) [12,13]. Daphnid brood stocks were cultured in 3.8-L glass jars containing 2 L of hard water (hardness 275 ± 5 mg/L $CaCO_3$, alkalinity 200 ± 10 mg/L $CaCO_3$, and pH 7.8–8.6) maintained at 20 ± 1°C in a temperature-controlled water bath with a photoperiod of 16:8 h light:dark. The cultures were started with 20 <24-h-old neonates ("neonates"), and the daphnids were transferred to fresh culture water twice a week.

Daphnids were fed 2×10^8 cells of the green algae *Selenastrum capricornutum* and 20 mg of a trout food–yeast mixture daily. The algae were cultured in Woods Hole MBL media [14] according to the methods of Miller et al. [15]. The trout food–yeast solution was prepared by mixing commercial salmon diet (BioDiet® Grower, Bioproducts, Inc., Warrenton, OR) and dry yeast (Red Star® Active Dry Yeast, Universal Foods Corp., Milwaukee, WI) in a four-to-one (w/w) ratio with hard water to achieve a final concentration of 5 ± 0.2 mg solids per milliliter.

Dilution water

Toxicity tests were conducted in the following dilution waters (Table 1): (a) ASTM soft water, which is recommended for use in acute toxicity tests with fish and invertebrates [16]; (b) blended hard water, which was representative of the major water quality characteristics of wetland feeding sites used by dabbling ducks in North Dakota [17]; and (c) oligosaline water, which was designed to simulate major water quality characteristics of oligosaline prairie lakes in North Dakota [17]. ASTM soft water and oligosaline water were reconstituted by adding appropriate quantities of reagent-grade mineral salts ($CaSO_4 \cdot 2H_2O$, $MgSO_4$, $NaHCO_3$, KCl, and

Table 1. Mean (±SD) characteristics of untreated dilution waters used in toxicity tests with *Daphnia magna*

Characteristic	Water type		
	ASTM soft	Blended hard	Oligosaline
pH	7.7 ± 0.1	8.3 ± 0.2	8.9
Conductivity (μmhos/cm at 25°C)	153.2 ± 2.6	828.7 ± 16.2	2,270
Alkalinity (mg/L $CaCO_3$)	30.1 ± 0.2	201.2 ± 1.6	619
Hardness (mg/L $CaCO_3$)	40.2 ± 0.3	275.6 ± 0.8	560
Calcium (mg/L)	7.1 ± 0.2	77.0 ± 1.3	30
Magnesium (mg/L)	5.4 ± 0.2	20.2 ± 1.0	118
Chloride (mg/L)	<1.0	11.3 ± 0.5	109
Sulfate (mg/L)	40.2 ± 1.9	221.0 ± 9.4	554
Potassium (mg/L)	1[a]	7[b]	58[a]
Sodium (mg/L)	13[a]	72[b]	301[a]
No. of tanks	6	12	1

[a]Nominal concentrations.
[b]Typical analysis of well water.

NaCl) to reverse osmosis–deionized (D.I.) water. Blended hard water was prepared by mixing well water with D.I. water, if needed, to achieve a hardness of 275 ± 5 mg/L $CaCO_3$. Water quality characteristics were determined according to standard methods [18,19].

Test chemicals

Technical-grade BO (95.1% purity) and two liquid commercial formulations, Buctril (33.4% BO) and Bronate (31.7% BO plus 34.0% 2-methyl-4-chlorophenoxyacetic acid; MCPA), were supplied by Rhône-Poulenc Ag Company, Research Triangle Park, North Carolina. Buctril and Bronate are formulated with petroleum distillates containing xylene (11.0 and 4.8% by weight, respectively), ethyl benzene (2.2 and 1.0% by weight), and other proprietary ingredients. Stock solutions of each formulation were prepared in glass-distilled acetone (OmniSolv, EM Science, Cherry Hill, NJ) because of the low water solubility of BO (80 μg/L; Rhône-Poulenc Ag Company, unpublished data).

Test methodology

Before testing, daphnids were acclimated to the appropriate dilution water over a 4-h period by placing them in a 1:1 culture:dilution water mixture for 2 h and then into a 1:3 culture:dilution water mixture for 2 h.

Procedures for conducting acute toxicity tests with daphnids closely followed those recommended by the ASTM [12,16]. However, some procedures were modified because the effects of water quality, age of daphnids and test solutions, food, and exposure duration on BO toxicity to daphnids were investigated. All acute toxicity tests were conducted in 250-ml beakers containing 200 ml of solution. Temperature was maintained at 20 ± 1°C by immersing the beakers in a temperature-controlled water bath. The photoperiod was 16:8 h light:dark, and light intensities ranged from 818 to 958 lux.

Most tests consisted of exposing groups of 10 daphnids to a series of 10 BO concentrations ranging from 10 to 1,000 μg/L (which differed by 60% between treatments), an acetone solvent control, and an untreated dilution water control. In tests with aged BO solutions and short-term exposures (≤24 h), higher BO concentrations (≤13,000 μg/L) were used. Test solutions were prepared by pipetting appropriate amounts of freshly prepared stock solutions into the beakers. The concentration of acetone in the solvent control was 0.5 ml/L, which was equal to the highest concentration of acetone in any test solution. Except for tests conducted in aged solutions, daphnids were randomly placed in the test solutions within 30 min after adding the toxicant. Daphnids were not fed during acclimation or testing (exposure and postexposure period) except in tests on the effects of feeding.

The number of affected daphnids in each treatment was recorded every 24 h after the beginning of the test. The effect criterion was immobilization, which was defined as the lack of movement (except for minor movement of the appendages) in response to prodding with a gentle current of water during a 5-s observation period. In test solutions with "floaters" (i.e., daphnids trapped at the water surface), the floating daphnids were gently submerged with a drop of test solution and checked for immobility [12].

Dissolved oxygen (YSI [Yellow Springs, OH] model 58 DO meter) and pH (Orion [Boston, MA] model SA250 pH meter) were measured in the control, low, medium, and high treatments at 0 and 48 h of exposure and at 48 h postexposure. Temperature was measured twice daily in the water bath.

Effect of formulation, technique, and water type. Neonates were exposed to the three bromoxynil herbicides for 48 h under static and static-renewal conditions in ASTM soft water and blended hard water ("soft water" and "hard water," respectively) and under static conditions in oligosaline water. For a given dilution water type, all tests were conducted concurrently with neonates of the same cohort. In the renewal tests, daphnids were transferred to freshly prepared test solutions at 24 h of exposure. After 48 h of exposure, all daphnids from each treatment were placed in 100 ml of clean dilution water for 30 s and then transferred to another set of 250-ml beakers containing 200 ml of clean dilution water for the remainder of the 96-h test period. Control daphnids were transferred in the same manner as BO-treated daphnids. Observations on delayed immobility of mobile daphnids and recovery of immobile daphnids were made at 24 and 48 h postexposure.

Effect of life stage. Different-aged daphnids (7 and 14 or 15 d old) were tested with the three bromoxynil herbicides in soft and hard water. Daphnids were exposed to the herbicides for 48 h under static conditions and then transferred to clean water for the remainder of the 96-h test period. For tests conducted in a given water type, the adults were from the same cohort that was used in the first set of tests with neonates, except for 14-d-old adults tested with Bronate in hard water. The adults were reared as described above, except that the entire cohort was maintained in three jars and fed 4×10^8

S. capricornutum cells and 40 mg of trout food-yeast mixture (four times the normal ration) daily. The daphnids did not appear to be stressed by the high densities, as evidenced by the production of young after 8 d and no production of ephippia [12,13].

Effect of feeding. Neonates were fed 1.2×10^7 *S. capricornutum* cells and 2.0 mg of trout food-yeast mixture once daily during a 48-h exposure to Buctril under static and static-renewal conditions in hard water. This ration was one-half that used by Buhl [20] in flow-through chronic toxicity tests with Buctril and *D. magna*.

Effect of aging solutions. Neonates were exposed for 48 h to BO (as Buctril) solutions that were aged for 0.125, 0.25, 0.5, 1, 2, 3, 8, and 16 d in hard water to determine the time course of BO detoxification. In one set of tests, solutions that were aged for 0.125, 0.25, and 0.5 d were tested on the same day as freshly prepared BO (as Buctril) solutions. In the other set of tests, solutions aged for 1, 2, and 3 d were tested on the same day as freshly prepared BO (as Buctril) solutions, and solutions that were aged 8 and 16 d were tested on the preceding day.

Effect of exposure duration. Neonates were exposed to a series of BO (as Buctril) concentrations for 1.5, 3, 6, 18, 24, and 48 h under static conditions in hard water. After the prescribed exposure period had elapsed, daphnids were transferred to another set of 250-ml beakers containing 200 ml of clean water for the remainder of the 96-h test period. In a later set of tests, neonates were exposed to a similar series of BO (as Buctril) concentrations for either a single 12-h period, two 12-h periods, or a single 48-h period and then transferred to clean water for the remainder of the 96-h test period. In the double 12-h exposure test, neonates were exposed for 12 h, transferred to clean water for 12 h, reexposed for 12 h in freshly prepared BO solutions, and then placed in clean water for the remainder of the test. Observations on immobilization were made at the end of the exposure periods and at 24-h intervals during the test.

Statistical analysis

The EC50 values (based on immobilization) and 95% C.I.s were calculated for each observation period by the moving average-angle method [21], using a computer program prepared by the Environmental Monitoring and Support Laboratory of the U.S. Environmental Protection Agency (EPA; Cincinnati, OH). All EC50 values are expressed as nominal concentrations of BO. The criterion of nonoverlapping 95% C.I.s was used to determine significant differences ($p = 0.05$) between EC50 values. Deactivation indexes for aged BO (as Buctril) solutions were calculated by dividing the EC50 of an aged solution by the EC50 of the corresponding fresh solution, and the half-life of biological activity of BO was estimated by the method of Marking [22].

RESULTS

In all tests, DO concentrations were maintained at or above 70% saturation (6.2 mg/L), except for tests in which the daphnids were fed (≥50% saturation). The pH of the test solutions was within ±0.2 units of the control solutions. The mean pH of individual tests ranged from 7.8 to 7.9 for soft water, from 8.4 to 8.8 for hard water, and was 9.1 for oligosaline water.

None of the daphnids in the control treatments were immobilized or showed any overt signs of stress during the exposure and postexposure periods, except for two tests in which one neonate in the water control was immobile after 48 h in one test and one neonate in the solvent control was immobile after 24 h in the other test. Also, immobility of daphnids exposed to the three lowest BO concentrations (10, 17, 28 μg/L) was ≤10%, and for most tests it was 0%. These results indicated that the stress of transferring daphnids to clean water after the exposure period had elapsed was minimal.

Effect of formulation, technique, and water type

The acute toxicities of the three bromoxynil herbicides (based on percentage of BO) to neonate daphnids exposed under static and static-renewal conditions were similar within the same water type but varied considerably among water types (Table 2). For each herbicide, the 48-h EC50 values for static and static-renewal tests in the same water type only differed by a factor of ≤1.2. These results indicated that static exposures were comparable to static-renewal exposures in assessing acute BO toxicity to daphnids.

Comparison of 48-h EC50 values indicated that the bromoxynil herbicides were significantly more toxic to neonate daphnids exposed in soft water than in hard water, except for Buctril tested under static-renewal conditions (Table 2). The toxicity of technical BO and Buctril to neonates exposed in oligosaline water was intermediate to that in soft and hard water; differences in 48-h EC50s between oligosaline and soft or hard water were not significant. Bronate was equally toxic to neonates in oligosaline

Table 2. Acute toxicity of bromoxynil octanoate (BO) to neonate *Daphnia magna* exposed for 48 h under static and static-renewal techniques in different dilution waters at 20°C, then placed in clean dilution water for 48 h

Water type and herbicide	Technique[a]	EC50 (μg/L) Exposure 48 h	EC50 (μg/L) Postexposure 96 h
ASTM soft			
Technical BO	S	57 (45–70)[b]	51 (40–62)[b]
	SR	61 (49–75)	54 (42–65)
Buctril	S	57 (45–70)	51 (40–62)
	SR	51 (40–62)	43 (34–59)
Bronate	S	43 (35–56)	39 (32–49)
	SR	49 (38–60)	45 (37–59)
Blended hard			
Technical BO	S	105 (82–136)	96 (77–119)
	SR	101 (82–125)	94 (75–116)
Buctril	S	91 (72–111)	84 (65–102)
	SR	75 (61–97)	75 (61–97)
Bronate	S	94 (75–116)	94 (75–116)
	SR	87 (69–107)	87 (69–107)
Oligosaline			
Technical BO	S	81 (63–99)	81 (63–99)
Buctril	S	61 (47–78)[c]	61 (47–78)[c]
Bronate	S	90 (68–114)	88 (70–108)

[a]S = static exposure, SR = static-renewal exposure.
[b]95% C.I.s in parentheses.
[c]No partial effects; 95% C.I.s: lower limit = highest test concentration with 0% effects; upper limit = lowest test concentration with 100% effects.

and hard water but was significantly less toxic in these water types than in soft water.

In all tests, floaters were present in the three highest BO concentrations (360, 600, and 1,000 μg/L) at 24 h; however, no floaters occurred in any treatment after 24 h. Regardless of herbicide, no surface film was observed in any of the BO and control treatments. Floaters also occurred in some of the lower BO concentrations, depending on herbicide and water type. Test concentrations that produced floaters at 24 h immobilized ≥90% of the neonates at 48 h. Conversely, not all concentrations that immobilized ≥90% of the neonates produced floaters. Based on these findings, the floating response was not as sensitive as immobilization to acute BO stress in daphnids.

Regardless of herbicide and water type, none of the immobilized neonates recovered their mobility after being transferred to clean water. Moreover, the onset of delayed immobility among mobile daphnids was minimal, as evidenced by the small differences (≤1.2-fold) between 48- and 96-h EC50 values (Table 2). These results indicated that the 48-h EC50 values for these herbicides were close to their respective 48-h LC50s.

Effect of life stage

Neonates and 7-d-old daphnids were equally sensitive to each bromoxynil herbicide in soft water, and both age groups were significantly more sensitive than 14-d-old adults to Buctril and Bronate (Table 3). All three herbicides were equally toxic to a given age group, except that Buctril was more toxic than technical BO to 7-d-old daphnids.

In the hard-water exposures, 7-d-old adults were consistently the most sensitive age group to BO (Table 3). A comparison of 48-h EC50s for all three age groups indicated that 7-d-old adults were significantly more sensitive than 14-d-old adults to each herbicide, and they were also significantly more sensitive than neonates to Bronate. Neonates were about 1.4 to 1.5 times more sensitive than 14-d-old adults to each herbicide, but the differences in 48-h EC50s were not significant. As was observed in the soft-water tests, the toxicities of the three herbicides were similar within each age group.

In both water types, floaters only occurred with 7-d-old daphnids exposed to Bronate for 24 h at BO concentrations ≥130 μg/L. Except for two treatments, immobilized adults did not recover their mo-

Table 3. Acute toxicity of bromoxynil octanoate (BO) to different age groups of *Daphnia magna* exposed for 48 h under static techniques in ASTM soft and blended hard water at 20°C, then placed in clean dilution water for 48 h

Water type, herbicide, and life stage	EC50 (μg/L) Exposure 48 h	EC50 (μg/L) Postexposure 96 h
ASTM soft		
Technical BO		
<24-h-old neonates	57 (45-70)[a]	51 (40-62)[a]
7-d-old adults	65 (53-81)	57 (45-70)
15-d-old adults	70 (53-99)	70 (53-99)
Buctril		
<24-h-old neonates	57 (45-70)	51 (40-62)
7-d-old adults	41 (34-52)	41 (34-52)
14-d-old adults	92 (71-117)	84 (62-106)
Bronate		
<24-h-old neonates	43 (35-56)	39 (32-49)
7-d-old adults	49 (38-60)	45 (37-59)
14-d-old adults	120 (91-175)	119 (97-152)
Blended hard		
Technical BO		
<24-h-old neonates	105 (82-136)	96 (77-119)
7-d-old adults	75 (59-102)	91 (72-111)
14-d-old adults	161 (125-206)	155 (112-204)
Buctril		
<24-h-old neonates	91 (72-111)	84 (65-102)
7-d-old adults	78 (57-99)	70 (55-93)
14-d-old adults	125 (102-162)	119 (97-152)
Bronate		
<24-h-old neonates	94 (75-116)	94 (75-116)
7-d-old adults	57 (46-70)	61 (49-75)
14-d-old adults	145 (114-177)	145 (114-177)

[a]95% C.I.s in parentheses.

bility, and delayed immobility among mobile adults was minimal during the postexposure period. In tests with 7-d-old adults exposed to BO in hard water, three of six immobile daphnids at 78 μg/L as technical BO and one of two immobile daphnids at 47 μg/L as Bronate recovered their mobility during the postexposure period, which resulted in a higher EC50 value at 96 h than at 48 h (Table 3).

Effect of feeding

Feeding had a pronounced effect on the acute toxicity of BO (as Buctril) to neonate daphnids in hard water (Table 4). In each set of tests, BO was significantly more toxic to daphnids in solutions without food than in those with food. The 48-h EC50 values of BO for fed and unfed daphnids differed by a factor of 1.9 in both sets of static tests (using neonates from different brood stocks) and 1.7 in the static-renewal tests. In tests with neonates from the same brood stock, differences in 48-h EC50s between static and static-renewal tests for a given feeding regime were not significantly different.

Effect of aging solutions

The toxicity of BO (as Buctril) solutions to neonates in hard water decreased progressively with the age of the solution (Table 5). Bromoxynil octanoate solutions aged for 0.5 d were 0.54 times as toxic to daphnids as were fresh solutions, whereas solutions aged for 16 d were 0.02 times as toxic as fresh solutions. Deactivation (i.e., the decrease in toxic activity) of BO in hard water was rapid, as indicated by the significant reduction in toxicity to daphnids of solutions aged for only 0.5 d compared with the fresh solutions. The estimated half-life of biological activity (i.e., time required to reduce toxicity by one-half) of BO (as Buctril) under these conditions was 0.55 d (13 h).

Effect of exposure duration

The acute toxicity of BO (as Buctril) to neonate daphnids in hard water was related to exposure duration (Table 6). The 48-h EC50s of BO ranged from >13,000 μg/L for exposures of 1.5 and 3 h, to 80 and 108 μg/L for exposures of 48 h. In short-duration exposures of 1.5, 3, and 6 h, 100% of the

Table 4. Effect of feeding on acute toxicity of bromoxynil octanoate (as Buctril) to neonate *Daphnia magna* from different brood stocks exposed for 48 h under static and static-renewal techniques in blended hard water at 20°C

Brood stock	Technique[a]	48-h EC50 (μg/L) Fed	48-h EC50 (μg/L) Unfed
F_{14}	S	279 (225-345)[b]	144 (108-181)[b]
F_{18}	S	171 (128-231)	91 (72-111)
	SR	125 (102-162)	75 (61-97)

[a]S = static exposure, SR = static-renewal exposure.
[b]95% C.I.s in parentheses.

Table 5. Acute toxicity of bromoxynil octanoate (as Buctril) in fresh and aged solutions to neonate *Daphnia magna* exposed for 48 h in blended hard water at 20°C

Test set and days aged	48-h EC50 (μg/L)	Deactivation index[a]
Set I		
0	91 (72–111)[b]	1.0
0.125	125 (99–171)	1.4
0.25	128 (102–177)	1.4
0.50	168 (136–207)	1.8
Set II		
0	121 (92–177)	1.0
1	775 (600–1,000)[c]	6.4
2	1,480 (1,200–1,900)	12.2
3	2,860 (2,070–3,610)	23.6
8	3,310 (2,630–4,070)	27.4
16	5,670 (4,540–6,950)	46.9

[a]48-h EC50 of aged solution/48-h EC50 of fresh solution.
[b]95% C.I.s in parentheses.
[c]No partial effects; 95% C.I.s: lower limit = highest test concentration with 0% effects; upper limit = lowest test concentration with 100% effects.

neonates were immobile in the highest BO concentration of 13,000 μg/L at the end of the exposure period. However, all recovered their mobility within 24 h after being transferred to clean water.

Delayed immobility occurred in some of the neonates exposed to 7,800 and 13,000 μg/L of BO for 6 h. After 48 h (42 h in clean water), 10% of the neonates exposed to 7,800 μg/L and 70% to 13,000 μg/L of BO for 6 h had become immobile, and at 96 h (90 h in clean water), 40% of the neonates exposed to 7,800 μg/L and 80% to 13,000 μg/L were immobile. Immobility among daphnids exposed to BO concentrations ≤4,700 μg/L for up to 6 h did not exceed 10% after 96 h. The 48-h EC50 values for these short-duration exposures (≤6 h) were ≥98 times the continuous (reference) exposure 48-h EC50 value (Table 6).

Exposures of 18 and 24 h to BO produced 48-h EC50 values that were not significantly different from that of the reference 48-h exposure (Table 6). These results indicated that the 48-h EC50 values for continuous 48-h exposures approximated the incipient LC50 (i.e., concentration at which 50% of the population would survive for an indefinite time) of BO for daphnids. Moreover, the 96-h EC50s for these three exposure durations were nearly identical (100–101 μg/L). Some of the daphnids immobilized after 18 h of exposure to BO concentrations ≥216 μg/L initially recovered their mobility after being transferred to clean water. However, significant delayed immobility occurred in these daphnids during the remaining test period, and after 96 h (78 h in clean water) 100% of the daphnids exposed for 18 h to BO concentrations ≥130 μg/L had become immobile.

In the second set of tests (using neonates from

Table 6. Effect of exposure duration on acute toxicity of bromoxynil octanoate (BO, as Buctril) to neonate *Daphnia magna* exposed in blended hard water at 20°C

Test set and exposure duration (h)	EC50[a] (μg/L)				Relative toxic index[b]
	End of exposure	24 h	48 h	96 h	
Set I					
1.5	8,860 (7,800–13,000)[c]	>13,000	>13,000	>13,000	>120
3	8,200 (7,170–9,530)	>13,000	>13,000	>13,000	>120
6	8,120 (7,060–9,450)	>13,000	10,540 (8,710–15,690)	8,650 (6,780–12,650)	98
18	249 (100–439)	335 (234–593)	171 (128–231)	101 (78–130)[c]	1.6
24	–	168 (118–239)	159 (114–213)	100 (77–128)	1.5
48	–	172 (129–233)	108 (88–135)	101 (78–130)[c]	1.0
Set II					
12	241 (170–316)	224 (132–309)	160 (128–197)	114 (93–144)	2.0
12:12[d]	91 (72–111)	256 (167–365)	96 (77–118)	75 (61–97)	1.2
48	–	117 (96–149)	80 (62–98)	61 (49–75)	1.0

[a]95% C.I.s in parentheses.
[b]48-h EC50 of short-term exposure/48-h EC50 of 48-h exposure.
[c]No partial effects; 95% C.I.s: lower limit = highest test concentration with 0% effects; upper limit = lowest test concentration with 100% effects.
[d]Neonates were exposed to BO for 12 h, transferred to clean dilution water for 12 h, and reexposed for 12 h. EC50 (95% C.I.s) of BO at the end of the first 12-h exposure period = 202 (160–273) μg/L.

a different brood stock), the 48-h EC50s of BO for the double 12-h and reference 48-h exposures were similar, and both values were significantly lower than that for a single 12-h exposure (Table 6). The relative toxic index (ratio of 48-h EC50 of short-term exposure to 48-h EC50 of 48-h exposure) for the double 12-h exposure (1.2) was lower than that for the single 24-h exposure (1.5).

Comparison of sensitivity

Neonates from several brood stocks were used because acute toxicity tests were conducted over a six-month period. The relative sensitivity of each group of neonates to BO (as Buctril) was compared in Table 7. There was some variation in sensitivity to BO among neonates from different brood stocks and also between cohorts from the same brood stock, but no one brood was significantly more or less sensitive than all of the others. The high-to-low ratio of 48-h EC50s among different broods was 2.0 and between cohorts was 1.6. The geometric mean 48-h EC50 value for eight cohorts of neonate *D. magna* and BO (as Buctril) was 96 μg/L, and the range of 95% C.I.s was 58 to 181 μg/L.

DISCUSSION

Acute toxicity tests

Based on the acute toxicity rating scales of Passino and Smith [23], BO was found to be extremely toxic (48-h EC50s 10–100 μg/L) to highly toxic (48-h EC50s 100–1,000 μg/L) to daphnids. The 48-h EC50s obtained in the three water types bracketed the water solubility of BO (80 μg/L). The acute toxicity values obtained in this study for technical-grade BO tested with neonates in hard water (48-h EC50s 101–105 μg/L; Table 2) are close to the 48-h LC50 of 110 μg/L for *D. magna* exposed in fortified well water (hardness 165 mg/L $CaCO_3$, alkalinity 120 mg/L $CaCO_3$, and pH 7.9–8.3) at 21°C (Rhône-Poulenc Ag Company, unpublished data). Although EC50 and LC50 values are not directly comparable because the measured responses are different, the lack of recovery of immobilized daphnids during postexposure periods in this study indicated that the 48-h EC50s were acutely lethal to daphnids.

Table 7. Comparison of the relative sensitivity of neonates from different brood stocks of *Daphnia magna* to bromoxynil octanoate (as Buctril)[a]

Brood stock	48-h EC50[b] (μg/L)
F_{11}	73 A (58–99)
F_{12}[c]	108 AB (88–135)
	121 AB (92–177)
F_{14}[c]	91 AB (72–111)
	144 B (108–181)
F_{18}	91 AB (72–111)
F_{26}	82 A (60–103)
F_{27}	80 A (62–98)
	Geometric $\bar{x}$: 96 (58–181)[d]

Toxicity values sharing the same letter are not significantly different ($p = 0.05$).
[a]Tests conducted under static techniques in blended hard water at 20°C.
[b]95% C.I.s in parentheses.
[c]Two cohorts from the same brood stock were tested.
[d]Range of 95% C.I.s.

The acute sensitivity of daphnids to formulated bromoxynil in this study was similar to or greater than that of other freshwater invertebrates. Buhl and Faerber [24] obtained a 48-h EC50 of 1,900 μg/L for BO (as ME4 Brominal) and fourth instar larvae of *Chironomus riparius* tested in ASTM soft water. In a field study of small ponds treated with the bromoxynil formulation Torch DS, Muir et al. [3] reported that mortality of caged amphipods (*Hyalella azteca*, 0.05–0.1 g) at 50 h post-treatment was 60% at mean (and range) measured total bromoxynil concentrations of 52 (35–64) μg/L, 85% at 97 (86–110) μg/L, and 95% at 440 (290–650) μg/L. However, mortality of caged amphipods in their reference and lowest treatment (average and range 4.0 and 2.1–5.8 μg/L, respectively, of total bromoxynil) ponds was 30%. The range of acute toxicity values of BO (as Buctril and Bronate) for daphnids exposed under static conditions in hard water (57–145 μg/L; Table 3) obtained in our study overlaps the range of total bromoxynil (as Torch DS) concentrations that killed 60 to 85% of the amphipods (35–110 μg/L) in the study by Muir et al. [3]. The high mortality of amphipods in the reference and lowest bromoxynil treatments in the study by Muir et al. [3] precluded calculating a 50-h LC50 value for *H. azteca* for comparison with the acute toxicity values observed in our study.

The occurrence of floaters in the high BO concentrations of each herbicide tested in our study may have been related to the tendency of BO to persist at or near the water surface. Muir et al. [3] reported that in ponds treated with the bromoxynil formulation Torch DS by spray application, BO and bromoxynil butyrate (BB) were concentrated in the surface microlayer (0–0.1 mm thick) and formed a surface film. Daphnids that swam close to the surface may have become trapped in this film and retained at the surface. However, as was observed by Muir et al. [3], no slick on the water sur-

face was visible in any concentration tested. The lower incidences of floaters among adults compared with neonates may be partly due to the swimming ability of the animal. Presumably, the adults are stronger swimmers than neonates, and they may have been better able to swim out of the film compared with the neonates.

Overall, formulation did not significantly affect the toxic activity of BO to daphnids in any of the water types tested (Tables 2 and 3). Depending on the formulation and herbicide, the inert ingredients in the formulation vehicle may or may not affect the toxicity of the active ingredient. For example, Buhl and Faerber [24] reported that for two of the four herbicides tested with larvae of *C. riparius*, the acute toxicity of the formulated herbicide differed significantly from the technical-grade compound; one was more toxic and the other less toxic than its respective technical compound. Moreover, in a comparison of the acute toxicities of technical-grade compounds with their formulated materials (based on percentage of active ingredient) for 48 pesticides, Mayer and Ellersieck [8] found that the toxicity of the active ingredient to freshwater animals was not affected by the formulation in 57% of the cases, increased in 32% of the cases, and decreased in 11% of the cases.

The acute toxicity of BO to neonate daphnids was significantly affected by the characteristics of the dilution water (Table 2). The greater toxicity of BO to neonates in soft water compared with that in hard water may be related to the physiological state of the neonates in a given dilution water type. Neonates tested in soft and oligosaline water were born in hard water and were only partially acclimated to these waters before testing, which may have placed a secondary stress on them. In hard-water tests, neonates were born and tested in the same water quality. Maki and Bishop [25] reported that the acute toxicity of the anionic surfactant $C_{11.8}$ linear alkylbenzene sulfonate (LAS) to neonate *D. magna* was related to the hardness of both the culture and the test water. In their soft-water tests (hardness 25 mg/L $CaCO_3$), LAS was significantly more toxic to daphnids cultured in hard water (hardness 225 and 350 mg/L $CaCO_3$) than to those cultured in soft water (hardness 50 mg/L $CaCO_3$). However, for their tests conducted in hard water (hardness 350 mg/L $CaCO_3$), LAS was equally toxic to daphnids cultured in soft and hard water. These results indicated that neonates may rapidly acclimate from soft to hard water but not from hard to soft water.

The reduction in BO toxicity to neonates in hard water compared to soft water in our study cannot be attributed to water hardness alone. The hard water was diluted well water, which contained all major ions at concentrations greater than those in soft water (Table 1) and included other ions (e.g., Fe and Mn) not added to the soft water. Therefore, it is likely that the total ionic composition of the test media rather than the components of hardness (i.e., Ca and Mg) alone were responsible for the differences in BO toxicity to neonates.

Adult daphnids (7 and 14 or 15 d old) in our study did not clearly exhibit increased sensitivity to BO in soft water, compared to their response in hard water (Table 3). For two of the three herbicides tested with a given age group, differences in acute toxicity values between soft and hard water were not significant. These results indicated that adults may have been better able to handle the rapid reduction in water hardness and other ions, compared to the neonates. Based on these findings, daphnids should be cultured and tested in the same water type to avoid stresses related to rapid ionic shifts in the media. Moreover, the chemical composition of the culture media for daphnids has been reported to affect their survival, reproduction, longevity, and sensitivity to chemicals [26–28].

Postexposure effects are rarely included in acute toxicity tests, and no comparable studies with cladocerans were found in the literature. In our study, daphnids that were immobilized by 48-h exposures to BO generally did not recover their mobility after being placed in clean water, and only a few of the mobile daphnids became immobile during the recovery period. However, in short-duration exposures of 1.5 to 6 h, immobilized daphnids recovered their mobility within 24 h after being transferred to clean water. Similarly, Kawatski et al. [29] and Parsons and Surgeoner [30] found that the ability of dipteran larvae to recover their mobility following acute exposures to pesticides decreased as exposure duration increased.

In our study, 7-d-old adults were either as sensitive or more sensitive than neonates to BO, whereas 14- or 15-d-old adults were generally less sensitive than either age group to BO (Table 3). These findings are in agreement with those of Adema [31], who compared the relative sensitivities of 1- and 7-d-old *D. magna* to organic toxicants. In his study, 7-d-old adults were as sensitive or slightly less sensitive as 1-d-old daphnids to 1,1,2-trichloroethane and pentachlorophenol (PCP). Similarly, Nebeker et al. [32] reported that 1- and 5-d-old *D. magna* had very similar sensitivities to cadmium, copper, and cyanazine. In 72-h tests with

pure and technical PCP, Stephenson et al. [33] found that 2-, 6-, and 19-d-old *D. magna* were equally sensitive to pure PCP; but for technical PCP, 2- and 6-d-old daphnids were more sensitive than 19-d-old adults.

The sensitivity of different-aged daphnids to BO may be related to the number and timing of molts that occur during the exposure period [32]. It has been suggested that daphnids are more sensitive to toxicants during molting than during intermolt periods [34]. In our study, no observations were made on the number of molts that occurred during the tests. However, both 7- and 14- or 15-d-old adults produced young during the exposure period (i.e., neonates were present in the test solutions), which occurs simultaneously with molting [12]. Also, cast exuvia were observed in tests with neonates and adults, which indicated that each age group molted at least once during the 48-h exposure period.

Reduced BO toxicity to daphnids in solutions with food compared to those without food (Table 4) may have resulted from one or both of the following factors: the adsorption of BO by the food particles, thus reducing its effective concentration, or the improved condition of fed neonates compared to unfed neonates, which enhanced their tolerance to BO. Without residue analyses to determine if BO was associated with the food, the role of each of these factors in decreasing BO toxicity is not known. However, considering that BO is highly lipophilic (octanol/water partition coefficient, K_{ow} 288,000; Rhône-Poulenc Ag Company, unpublished data), it is likely that some of the compound was sorbed to the food and other organic matter present. Similar differences in acute sensitivity to inorganics between fed and unfed daphnids has been reported by other investigators [32,35,36].

In our study, the observed decrease in toxicity of aged BO (as Buctril) solutions to daphnids (Table 5) was probably due to the rapid hydrolysis of BO to bromoxynil phenol (BP). In storage stability studies of bromoxynil herbicides in runoff water and purified tap water at room temperature, Brown et al. [37] found that after 24 and 72 h of storage about 42 and 88%, respectively, of the BO had degraded to BP. Muir et al. [3] observed that 42% of the BO and BB added to pond water (average pH 8.2) at 25°C hydrolyzed to BP after 4 h of storage. In our study, the reduction in toxicity of BO solutions aged for 3, 24, and 72 h was 27, 84, and 96%, respectively, compared to the fresh solutions (Table 5). Moreover, the estimated half-life of biological activity of BO obtained in our study (13 h) was within the range of pseudo-first-order half-lives of 7 to 24 h reported by Muir et al. [3] for BO in subsurface water of ponds treated with the bromoxynil formulation Torch DS.

Bromoxynil phenol is markedly less toxic to aquatic organisms than BO. The acute values for BP range from a 96-h LC50 of 2,000 μg/L for rainbow trout (*Oncorhynchus mykiss*) to a 48-h EC50 of 12,500 μg/L for *D. magna*, which are 20 and 114 times higher, respectively, than those reported for technical BO (96-h LC50 100 μg/L for rainbow trout and 48-h LC50 110 μg/L for *D. magna*; Rhône-Poulenc Ag Company, unpublished data). The 48-h EC50s of BO (as Buctril) solutions aged for ≥3 d in our study are within the range of those for BP, which provided further evidence that BO degrades rapidly in alkaline water to the less toxic phenolic form.

The results of our study showed that brief exposures of ≤24 h to BO concentrations found to be toxic in 48-h exposures can cause adverse effects in daphnids and that the exposure duration required to elicit these effects was between 12 and 18 h (Table 6). Exposures to BO concentrations as high as 4,700 μg/L (about 44 times the reference exposure 48-h EC50) for 1.5, 3, and 6 h immobilized ≤10% of the daphnids. Exposures to toxic BO concentrations for 12 h produced 48- and 96-h EC50s that were about twice as high as those for daphnids continuously exposed for 48 h, whereas exposures to the same BO concentrations for 18 and 24 h produced 48- and 96-h EC50s that were similar to those for daphnids continuously exposed for 48 h. The relatively small increase in BO toxicity to daphnids between 18 and 48 h of exposure may be partly due to the hydrolysis of BO. These findings indicated that the level of damage in daphnids produced during the first 12 to 18 h of exposure (even though the concentrations of BO were decreasing) was similar to that produced during 48-h exposures; however, the overt toxic effect may not be manifested until 1 to 3 d after the short-term exposure. The duration of exposure to toxic BO concentrations that caused irreversible damage in daphnids could not be accurately predicted from these data because the median effective times (ET50s; exposure time required to immobilize 50% of the daphnids at a given BO concentration) were not determined.

The results of the double 12-h exposure, which produced 48- and 96-h EC50s that were 0.6 to 0.7 times lower than that for the single 12-h exposure (Table 6), indicated that the damage caused by repeated brief exposures to BO accumulated over time with no significant recovery during the BO-free periods. Also, the relative toxic index for the double

12-h exposure was similar to that for the 24-h exposure. These findings are consistent with the damage-repair model of Breck [38], which predicts that the effects of multiple short-term exposures to toxicants are additive if the rate of detoxification and/or repair between exposures is minimal.

Based on these findings, 48-h EC50 values derived from standard continuous exposure tests do not adequately define the acutely toxic concentrations of BO to daphnids. In standard acute tests, exposure time and toxicant concentration are held constant; thus, the effect of concentration and exposure duration cannot be measured independently of each other. For a given toxicant concentration, the exposure time required to elicit a toxic effect is less than or equal to the time it takes for the effect to be manifested [39,40]. Consequently, BO concentrations predicted to immobilize 50% of the daphnids after 48 h of exposure in a standard acute test may cause irreversible damage in 50% of the daphnids within a shorter exposure period; thus, its toxicity to daphnids would be underestimated by the standard acute test.

Other investigators have also reported that the effects on freshwater organisms of brief exposures to acutely toxic concentrations of pesticides may not be accurately predicted by standard toxicity tests. Anderson and Shubat [40] exposed the amphipod *Gammarus lacustris* to flucythrinate for periods of 1 to 48 h. They reported that brief exposures of about 29 h to a concentration close to the continuous-exposure 48-h LC50, and 38 h to a concentration of about one-half the 48-h LC50, would be expected to kill 50% of the animals within 48 h. Kleiner et al. [41] made a similar observation for short-term exposures of fathead minnows (*Pimephales promelas*), in which the LT50 for short-term exposures to an endosulfan concentration close to the continuous-exposure 96-h LC50 was about 38 h. Jarvinen et al. [42] exposed fathead minnows to chlorpyrifos, endrin, and fenvalerate for periods of 1 to 96 h and calculated both EC50 (based on deformities in fish) and LC50 values for each exposure period, as was done with EC50 values in this study. For all three pesticides they tested, the exposure times required to cause 50% deformities in fish at the continuous-exposure 96-h LC50s were, as expected, <96 h. The effective exposure times varied for each chemical in their study, ranging from 15 h for chlorpyrifos to 80 h for endrin. All of the investigators cited above have suggested that short-term exposure tests should be included in the environmental hazard evaluation process of pesticides because their effects on biota could be underestimated in standard acute tests, as was observed in our study. Moreover, Clark et al. [10,11] reported that laboratory toxicity tests that employed pulse exposure regimes similar to those in the field provided reasonable predictions of the acute toxicity of fenthion to nontarget estuarine animals under actual-use conditions.

The results of short-term exposure tests may help explain the seemingly incongruent results between tests with aged BO (as Buctril) solutions and the static-renewal tests. Tests with aged BO solutions demonstrated that BO degrades rapidly in hard alkaline water to a less toxic form (Table 5). Bromoxynil octanoate solutions that were aged for only 1 d were about 0.16 times as toxic to daphnids as the fresh solutions. Thus, static-renewal exposures should be more toxic to daphnids than static exposures due to the degradation of BO. However, static and static-renewal tests produced similar 48-h EC50s for each herbicide in a given water type (Table 2). The lack of difference in BO toxicity between static and static-renewal tests may be partly explained by the results of the short-term exposures, which indicated that the toxic effects of BO in daphnids occurred within the first 24 h of exposure, even though BO degraded rapidly during this period. Consequently, if the threshold level of damage in daphnids exposed to toxic BO concentrations was reached during the first 24 h of exposure, continued exposure to the same concentrations would not enhance the toxic effect. Daphnids exposed under static-renewal conditions to nontoxic concentrations of BO may have incurred higher levels of damage, compared to those exposed under static conditions, but the accumulated level of damage did not exceed their threshold level.

The variability of the eight daphnid acute tests with Buctril conducted in hard water at different times was small, as evidenced by a high-to-low ratio of 48-h EC50s of two (Table 7). Canton and Adema [43] studied the variability of acute toxicity tests with *D. magna*, *D. pulex*, and *D. cucullata* and concluded that differences in LC50s of up to twofold can be expected for tests repeated at different times in the same laboratory. Schimmel [44] reported that acute LC50 values from repeated tests conducted within the same laboratory should not differ by more than a factor of two, whereas those obtained in different laboratories should be within a factor of four. Moreover, for seven of the eight tests (88%) the 95% C.I.s of the 48-h EC50s encompassed the geometric mean 48-h EC50 value (96 μg/L). The success rate of the individual 95% C.I.s for inclusion of the geometric mean acute value was

close to the expected rate of 95% (i.e., 95% of these C.I.s should include the "true" 48-h EC50 value for the test population), which indicated good precision among the eight tests conducted at different times.

In our study, there was no relation between daphnid sensitivity to BO and filial generation (Table 7). The two lowest 48-h EC50 values were obtained for neonates produced by the first and last generation of brood stock used in tests with hard water. Apparently, neither the toxic activity of BO (as Buctril) nor the sensitivity of daphnids changed significantly during the course of this study.

Comparison to field conditions

There are very few data on the concentrations of bromoxynil in wetlands or in runoff water from treated fields. Brown et al. [45] reported that BP residues in runoff water from winter wheat plots (in fall and early winter) 6 d after a BO treatment ranged from 71 μg/L for a conventionally tilled plot to 108 and 164 μg/L for no-tilled plots. Bromoxynil octanoate residues in these same runoff waters were below detection (5 μg/L). These researchers attributed the lack of detectable BO residues in runoff water to its rapid hydrolysis to the phenolic form. Therefore, aquatic organisms in wetlands receiving runoff from fields treated with BO would probably be exposed primarily to BP.

Direct comparisons of the results of toxicity tests in our study with those of Brown et al. [45] are not possible because the daphnids in our study were initially exposed to BO (until it degraded), and residues of BP in the exposure water were not measured. However, BO (as Buctril) solutions that were aged for 1 and 2 d in our study produced 48-h EC50 values that were ≥4.7 and ≥9.0 times higher, respectively, than the concentrations of BP in runoff water reported by Brown et al. [45]. Bromoxynil octanoate solutions aged for 3 d or longer in our study produced 48-h EC50s that were ≥17 times higher than their measured concentrations of BP in runoff water. The EPA [46] presumes that a pesticide poses an acute risk to nontarget aquatic organisms when the ratio of 48-h EC50 or 96-h LC50 to environmental concentration is ≤10. Muir and Grift [47] found that bromoxynil residues (as the free phenol) in two rivers draining agricultural watersheds in Manitoba, Canada, were detectable only after a major storm event. The highest concentration of BP they reported (about 0.125 μg/L) is considerably lower (≤0.001) than the 48-h EC50s of BO for daphnids tested in hard water in our study.

Because of the rapid degradation of BO in soil and water and the low toxicity of the phenolic form to daphnids, runoff water from rainstorm events occurring at least 3 d after a bromoxynil application would not be expected to adversely affect daphnids and other aquatic organisms with similar or lesser sensitivities. Although bromoxynil herbicides are not registered for use in or around surface waters (E. Mihaich, Rhône-Poulenc Ag Company, personal communication), direct application of bromoxynil to wetlands via overspray or runoff events occurring within 1 d after an application (before BO degrades to BP) may produce potentially toxic conditions for wetland invertebrate populations. Considering that wetlands act as repositories for sediments and dissolved nutrients carried in runoff, additional studies are needed on the effects of particulates and dissolved organic matter on the bioavailability and toxicity of BO to aquatic invertebrates to improve laboratory-to-field comparisons. Moreover, because BO is phytotoxic, its effects on macrophyte and algal communities should be investigated.

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