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DEVELOPMENT AND VALIDATION OF A *DAPHNIA MAGNA* FOUR-DAY SURVIVAL AND GROWTH TEST METHOD

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Abstract—Zooplankton are an important part of the aquatic ecology of all lakes and streams. As a result, numerous methods have been developed to assess the quality of waterbodies using various zooplankton species. Included in these is the freshwater species *Daphnia magna*. Current test methods using *D. magna* involve acute lethality test methods ranging from 24 to 96 h in duration and chronic test methods with durations of 21 to 28 d. Whereas the current acute and chronic test methods are useful, a need exists for a shorter-duration test method that will provide a chronic or subchronic endpoint with this species. In the present study, a 4-d, static–renewal survival and growth test was developed for use with *D. magna*. The test results were compared to performance criteria and results from 7-d survival and reproduction tests with *Ceriodaphnia dubia* to determine the level of comparability between the two methods. Results from the 4-d *D. magna* survival and growth test method indicated that this method will produce consistent results with various reference toxicant materials and provide data that are both reproducible and useful for detecting potential toxicity in aquatic environments.

Keywords—*Daphnia magna* Test method Short-chronic Growth *Ceriodaphnia dubia*

INTRODUCTION

As part of the ongoing search for shorter-duration and more cost-effective methods of evaluating the toxicities of effluents, discharges, receiving waters, and sediments, an effort was undertaken to develop a short-term, subchronic toxicity test for use with the freshwater invertebrate *Daphnia magna* Straus. *Daphnia magna* has been used routinely to assess the effects of pollutants on aquatic life since the early 1940s [1]. The ease of laboratory culture was one of the initial reasons for the use of daphnids in aquatic and sediment toxicity testing, although the effect of stressors on daphnids also is important because of their role as a major food source for fish [2,3]. Testing conducted by various investigators shows that the results generated using various species of daphnids are comparable and that daphnids are more sensitive than fish to toxic materials [4].

Chronic survival and reproduction tests with *D. magna* typically require 21- to 28-d test durations, making them logistically difficult to conduct [4,5]. As early as 1980, researchers were discussing the logistical efforts of the 21- and 28-d *Daphnia* tests and looking at ways to shorten the method [6]. In 1988, Winner [7] evaluated the effectiveness of a 7-d, *D. magna* test compared to the standard 7-d *Ceriodaphnia dubia* Richards test. This *D. magna* method [7] used 4-d-old test animals to allow a reproduction endpoint, along with various growth endpoints, to be determined. In the early 1990s, researchers were even investigating methods that would shorten the 7-d duration of the *C. dubia* survival and reproduction test [8,9].

The goal for the present study was to develop a static–renewal method that used a 4-d test duration, a temperature of 25 ± 1°C (mean ± standard deviation throughout) and a test solution volume of less than 100 ml. These parameters made a reproduction endpoint unreasonable, but the growth rate of *D. magna* at 25°C made growth of the animals (as measured by mean dry wt), along with survival, a reasonable endpoint to use. When 21-d tests were conducted with *D. magna* at temperatures of 15, 20, and 24°C, no significant differences were observed in the toxicity of pentachlorophenol and 3,4-dichloroanaline [2]. These results indicated that the use of a 25°C test temperature would not adversely alter the results of the toxicity tests. It also meant that the results from 20 and 25°C tests could be compared using a reasonable level of confidence. In the studies conducted at 15, 20, and 24°C, the control mortality was elevated slightly in the 24°C tests; however, this could be caused by the use of nonoptimal control feeding. Efforts were made to address this issue with the development of this test method.

In the present study, the basic method was developed using standard reference toxicant materials (zinc sulfate, ammonium chloride, potassium chloride, and phenol). Once the 4-d *D. magna* growth and survival method was standardized, tests were conducted comparing the results from this method to results from the standardized 7-d *C. dubia* chronic survival and reproduction test method.

MATERIALS AND METHODS

Culture methods

The daphnids used in the present study were supplied from the U.S. Environmental Protection Agency (EPA) Culture Fa-

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Table 1. Summary of test conditions used in conducting 4-d survival and growth toxicity tests with *Daphnia magna*

Test parameter	Condition
Test type	Static–renewal (required)
Test duration	4 d (required)
Temperature	25 ± 1°C (required)
Photoperiod	16:8-h light:dark (suggested)
Test chamber size	60 ml (suggested)
Test solution volume	50 ml (required)
Renewal of test solution/chemical parameter analysis	Daily (required)
Age of test organisms	<24 h old (12-h age window) (required)
Organisms/test chamber	5 (required)
Replicate test chambers	4 (suggested)
Organisms/concentration	20 (suggested)
Feeding regime	0.3 ml of *Pseudokirchneriella subcapitata* and 0.2 ml of alfalfa extract (required)
Test solution aeration	None (suggested)
Dilution water	Moderately hard reconstituted water + selenium (suggested)
Test concentration	5 plus control (suggested)
Dilution series	0.5 (suggested)
Endpoint	Survival and growth (mean dry wt) (required)
Test acceptability	90% or greater control survival and control growth of 10-fold the initial dry weight (required)

cility (Cincinnati, OH). Cultures were maintained using a standard operating procedure based on the U.S. EPA Acute Testing Methods Manual [10]. The culture water was composed of a blend of well water, dechlorinated tap water, and Super-Q® (Millipore) deionized water mixed to a hardness of 90 to 120 mg/L as $CaCO_3$ and supplemented with sodium bicarbonate to achieve an alkalinity of 50 to 60 mg/L as $CaCO_3$. Cultures were maintained at 25 ± 1°C. In previous studies, *D. magna*, under optimal conditions, released their first brood within 10 d at 20°C and within 7 d at 25°C; successive broods were released every 3 to 4 d at 20°C and every 2 to 3 d at 25°C [4]. The use of a 25 ± 1°C culture temperature resulted in an increased brood development rate and the ability to generate more young in a shorter period of time. It also eliminated the need to condition the test animals to a different temperature, because the culture temperature was identical to the established test temperature.

Cultures were maintained in glass beakers containing 1 L of culture water and 15 animals per beaker. Water was changed and the young removed on Monday, Wednesday, Friday, and Sunday. Culture water also was changed between 6 and 7 PM the day before a test so that all young were removed. The cultures also were checked for young the following day between 6 and 7 AM; this provided young released within a 12-h time period. Daily culture feeding consisted of 4 ml of *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*; green algae, 100×10^6 cells/ml), 3 ml of a blended alfalfa extract, and 3 ml of digested flake food, alfalfa, and yeast. The *P. subcapitata* was cultured according to the procedures described for *C. dubia* culture and testing [10,11], and the digested flake food, alfalfa, and yeast (also known as YCT) was prepared according to procedures described for *C. dubia* and *D. magna* culture [11]. The alfalfa extract was prepared by blending 7.5 g of alfalfa in 1 L of Super-Q® deionized water for 5 min. This mixture was placed in the refrigerator overnight to settle. The next day, the top 500 to 600 ml of supernatant were collected and kept as the alfalfa extract. Studies have shown that maternal nutrition plays an important part in the sensitivity of neonates used in toxicity studies [12]. The use of standard culture methods reduces the chance of a spurious toxicant response because of poor test animal condition.

Test design

Test conditions for this method are summarized in Table 1. Moderately hard reconstituted water, supplemented with selenium (1 μg/L), was used in testing to minimize the effects caused by poor or inconsistent water quality [10] and to help alleviate concerns over water-quality effects with certain toxicants. Increases in the hardness and humic acid content of dilution water have been shown to increase significantly the median lethal concentration (LC50) of zinc to *D. magna* and the reproduction endpoint of 21-d *D. magna* survival and reproduction tests [13]. An increase in humic acid also has been shown to increase the LC50 of trivalent chromium to *D. magna* [14]. The relationship between toxicity, bioavailability, and chemical speciation of trace metals have been described extensively in the literature, particularly through the developments of the free-ion activity model and biotic ligand model [15–18].

Four reference toxicants and eight environmental samples were tested with this 4-d *D. magna* survival and growth method. Reference toxicants were tested using a five concentration dilution series (×0.5). The high concentrations for each reference toxicant were as follows: Zinc sulfate ($ZnSO_4 \cdot 7H_2O$), 500 μg/L; phenol (C_6H_5OH), 10 mg/L; ammonium chloride (NH_4Cl), 400 mg/L; and potassium chloride (KCl), 1,000 mg/L. The test solutions and control water for each test were allowed to reach 25 ± 1°C before being dispensed into labeled, 2-ounce (60-ml) polystyrene cups, similar to the 1-ounce (30-ml) cups recommended for use in the *C. dubia* acute and chronic tests [10,11]. Once the cups were filled, test animals were assigned randomly to each container. After all tests were set up, the animals were fed a combination of 0.3 ml of *P. subcapitata* and 0.2 ml of blended alfalfa extract, which was prepared following the same methods previously described for the cultures. At this time, four sets of 10 animals each were randomly selected for measurement of initial dry weight; this number of animals was selected to ensure an accurate dry-weight average. The drying and weighing procedure was based on that used for the 7-d *Pimephales promelas* Rafinesque survival and growth test [11]. The animals were weighed by using pretared weighing pans or by transferring all animals from each replicate onto a zeroed weighing pan. In the present study,

all surviving animals from each replicate were placed into the same pan and weighed together to give a replicate weight. The animals were dried for 24 h at 60°C and placed in a desiccator for at least 1 h to cool. A Cahn C-32 balance (Cahn Instruments) capable of reading 1 μg (precision, ±0.0012%) was used to measure the weights. This provided an initial weight measurement that was used to determine the minimum acceptable growth factor, one of the test acceptability criteria.

Each day, the test animals were transferred into fresh solution, and the number of live and dead animals were counted and recorded. To do this, the test solution was poured from the cup into a Petri dish, the cup refilled with fresh test solution, and using a 4-mm bore glass or plastic pipette, the animals transferred into the fresh solution. Once all animals were transferred, food was added to each test container. Routine chemical parameters (pH, dissolved oxygen, conductivity, and temperature) were measured and recorded daily for both the fresh and old test solutions from each test concentration or control sample.

To end the test, the number of live and dead animals was counted and recorded. The measured survival of control animals was used to establish the control survival acceptability criteria for this method. The live animals were removed, sacrificed using carbonated water for 90 s (until fully immobile), and then placed into labeled, aluminum weigh pans for drying and weighing. The weights were determined using the procedure described above for drying and weighing the animals at the start of the test.

The test methodology used for 7-d *C. dubia* survival and reproduction tests followed those methods described in the U.S. EPA Short-Term Chronic Toxicity Testing Manual [11]. Animals used were from an in-house culture. Tests were conducted at 25°C in 15 ml of control or test dilution water. Animals were fed daily with 0.1 ml of *P. subcapitata* and 0.1 ml of digested flake food, alfalfa, and yeast. All foods were prepared according to recommendations in the U.S. EPA Short-Term Chronic Toxicity Testing Manual. Test solution was renewed daily, and the number of live or dead animals was counted and recorded. The number of young released by each female was counted daily. All *C. dubia* data were generated in-house.

Statistical analysis

For all tests conducted using both *D. magna* and *C. dubia*, the following endpoints were analyzed using TOXIS 2.4A (EcoAnalysis): The growth or reproduction no-observed-effect concentration (NOEC), the survival NOEC, the 25% inhibitory concentration (IC25), and the LC50. Data analysis followed the guidelines provided for analysis of this type of data in the U.S. EPA Short-Term Chronic Toxicity Testing Manual [11]. Survival data were arcsine square-root transformed before analysis with hypothesis tests. All hypothesis tests used a risk level (p-value) of 0.05. The IC25s were generated using the linear interpolation method recommended by U.S. EPA [19].

RESULTS

Five separate tests were conducted with three of the reference toxicant materials (zinc sulfate, ammonium chloride, and potassium chloride); six tests were conducted using the reference toxicant phenol. The results of the reference toxicant tests are reported as nominal concentrations. Concentrations for tests using ammonium chloride, phenol, and potassium chloride are reported in mg/L, and concentrations for tests using zinc sulfate are reported in μg/L of zinc (Zn^{2+}).

Tests also were conducted with environmental samples supplied by U.S. EPA Region VI (Big Springs, Texas, USA effluent; sample BS) and Region IX (Penn Mine, CA, USA site; sample PM). The BS and PM samples were both from remediation sites where the concerns were high total dissolved solids and water hardness. The BS sample had pH in the range of 7.8 to 8.2 and conductivity of greater than 4,000 μS/cm^2. The PM sample had pH in the range of 8.0 to 8.5 and conductivity of greater than 5,000 μS/cm^2. The tests conducted with these samples and those conducted with the reference toxicant tests were performed concurrently with 7-d *C. dubia* survival and reproduction tests to provide a measure of the reliability and sensitivity of this *D. magna* test method. Six additional environmental samples used in testing were from a site in eastern Ohio and were collected by U.S. EPA Office of Research and Development (Cincinnati, OH). This was an acid mine drainage site with concerns related to low pH and dissolved heavy metals.

Performance criteria

Survival and growth of *D. magna* was measured throughout these tests to establish single-laboratory performance criteria for the method. Survival of the animals exposed in the control groups exceeded 90% during testing; therefore, the intralaboratory control survival acceptance criterion was set at 90% or greater. When the initial dry weight of the control animals at the start of the test were compared to the final dry weight of the control animals at the end of the test, the weights in the control group at the end of the test were at least 10-fold those of the animals used to start the test. Based on these data, an intralaboratory minimum acceptable growth factor of 10-fold or greater the initial dry weight was established for control animals.

Developmental reference toxicant tests

Growth of *D. magna* in the reference toxicant tests used for method development was measured using mean dry weight for both the initial test animals and the animals surviving at the end of the test. Initial dry weight varied from test to test, with values ranging from 6.2 to 13.7 μg/individual and coefficients of variance (CVs) ranging from 4.2 to 13.1%. The average initial weight was 10.0 μg/individual (CV, 21.0%). Final dry weight also varied from test to test, with values ranging from 73 to 167 μg/individual and CVs ranging from 2.6 to 19.8%.

Survival of *D. magna* in the reference toxicant tests used for method development is shown in Table 2. The five tests conducted using ammonium chloride showed good consistency, as did the six tests conducted using phenol. When converted to reflect un-ionized ammonia (NH_3), the ammonium chloride tests showed nominal NH_3 LC50s in the range of 2.0 to 3.0 mg/L. The results from the potassium chloride tests were the most consistent of the four reference toxicant materials tested. The survival NOECs and growth NOECs were the same for all five tests, whereas the LC50s and IC25s ranged as shown in Table 2.

Four of the five tests conducted with zinc sulfate had consistent results (Table 2). The results of the fifth test were considerably higher than those of the other four, mainly because of the lack of mortality in the two highest test concentrations. No chemical analysis data were generated for these samples,

Table 2. Results for a series of reference toxicity tests conducted while developing the 4-d *Daphnia magna* survival and growth testing method[a]

Toxicant	No. of tests	Concn. units	Survival NOEC[b]	LC50[c]	Growth NOEC[d]	IC25[c]
Ammonium chloride	5	mg/L	100, 200 (15.2–34.1%)	223–278 (7.9%)	25, 50, 100 (2.1–4.1 μg)	120–148 (10.2%)
Phenol	6	mg/L	2.5, 5 (17.7–32.3%)	5.4–6.8 (9.5%)	0.625, 1.25, 2.5 (3.1–6.2 μg)	1.1–2.7 (34.9%)
Potassium chloride	5	mg/L	500 (22.1–36.8%)	652–707 (6.9%)	500 (0.5–2.2 μg)	593–625 (2.2%)
Zinc	4	μg/L	125 (15.5–28.2%)	154–177 (23.6%)	62.5, 125 (1.6–5.2 μg)	88.4–122 (13.2%)
Zinc	1	μg/L	500	500	500	444

[a] IC25 = 25% inhibitory concentration; LC50 = median lethal concentration; NOEC = no-observed-effect concentration.
[b] Values in parentheses represent the range of difference in survival necessary for a sample to be considered statistically different from the control.
[c] Values in parentheses represent the coefficient of variation.
[d] Values in parentheses represent the difference in growth necessary for a sample to be considered statistically different from the control.

so technician discrepancy in the preparation of the samples for this test is a possible cause for this error. Results from the fifth test were never repeated in any subsequent testing and, therefore, are classified as outliers.

Survival and reproduction NOECs, LC50s, and IC25s for the 7-d *C. dubia* tests conducted using the reference toxicants are shown in Table 3.

Environmental samples

Results from tests using the PM effluent (Table 4) showed a survival NOEC of 50% effluent for the 4-d *D. magna* test, versus a survival NOEC of 100% effluent for the 7-d *C. dubia* test. The *C. dubia* survival in the 100% PM effluent was 80%; thus, a LC50 could not be generated. The growth NOEC for the *D. magna* test and the reproduction NOEC for the *C. dubia* test were identical, and the IC25s for both test species were similar (Table 4).

Results from testing the BS effluent (Table 4) showed that *D. magna* survival and growth NOECs, LC50s, and IC25s were all 100% effluent. The only mortality in the 4-d *D. magna* test was in the 100% effluent sample, where survival was 80%. Growth in the *D. magna* control sample was 147 μg; growth in the effluent dilutions was not statistically different from the control, ranging from 136 to 160 μg. Survival and reproduction NOECs, LC50s, and IC25s for the 7-d *C. dubia* test are shown in Table 4.

The 4-d *D. magna* test was used as part of a monitoring program for an acid mine discharge site located in southeastern Ohio (USA), where six sites were sampled for a variety of indicators, including water-column toxicity. Samples were collected five times, beginning in July and ending in January. The results from these tests, which are summarized in Table 5, show a high degree of consistency. The survival NOEC for the samples collected from Snow Fork at Essex (SFE), Snow Fork at Buchtel (SFB), and Brush Fork (BF) ranged from 10 to 25% effluent over the seven-month period, although it should be noted that the 10% effluent result for these samples is a result of the dilutions used in the first test series, as is the 10% effluent growth NOEC result for site SFE. The toxicity tests were first conducted as range-finding tests, with dilutions of 1, 10, and 100% effluent. Once the level of toxicity was identified, a new dilution series was developed for each site. The growth NOEC data for these sites were not as consistent but still within reason. Because these were environmental samples, the level of variability in the growth NOEC could be caused by sample variability rather than test variability. Monday Creek was another section of the acid mine discharge site that was tested at the same time. It was determined that this sample was not as toxic as SFE, SFB, or BF, so it was tested using a dilution series of 25, 50, and 100% effluent. Over the seven-month testing period, the survival NOECs were either 50 or 100% effluent; the growth NOECs were 50% effluent for all tests. The Salt Run and Little Monday Creek sites were reference sites for the watershed in question in the monitoring program. Over the duration of testing, survival for both sites was 100%, and growth in these sites was never different from that of the control sample. All control growth for the tests conducted as part of the monitoring program in southeastern Ohio met or exceeded the criterion of 10-fold (×10) the initial weight. Initial weights ranged from 6.3 to 11.2 μg/individual, with control growth ranging from 77 to 148 μg/individual.

Additional reference toxicant tests

A series of additional reference toxicity tests were conducted using the 4-d *D. magna* survival and growth test method, after the initial test method development was completed,

Table 3. Results from a series of reference toxicity tests conducted using a 7-d *Ceriodaphnia dubia* survival and reproduction test method[a]

Toxicant	No. of tests	Concn. units	Survival NOEC[b]	LC50[c]	Reproduction NOEC[d]	IC25[c]
Ammonium chloride	5	mg/L	50–100 (12.2–18.6%)	100–200 (12.2%)	50–100 (1.1–3.6 μg)	75–125 (17.7%)
Phenol	5	mg/L	2.5 (18.1–32.6%)	NA (NA)	2.5 (2.1–6.3 μg)	3–4 (20.2%)
Potassium chloride	12	mg/L	250–500 (22.1–32.3%)	379–707 (11.3%)	125–250 (1.3–3.7 μg)	203–355 (19.0%)
Zinc	10	μg/L	250 (17.2–27.6%)	177–250 (11.8%)	62.5, 125 (2.6–5.4 μg)	94–194 (21.4%)

[a] The age of the test animals at the start of each test was less than 24 h, with an 8-h age window. For each test conducted, the animals exposed in the moderately hard reconstituted water control sample met or exceeded the minimum reproduction criterion of 20 young/female. IC25 = 25% inhibitory concentration; LC50 = median lethal concentration; NA = not applicable; NOEC = no-observed-effect concentration.
[b] Values in parentheses represent the range of difference in survival necessary for a sample to be considered statistically different from the control.
[c] Values in parentheses represent the coefficient of variation.
[d] Values in parentheses represent the difference in growth necessary for a sample to be considered statistically different from the control.

Table 4. Results from side-by-side tests with 4-d *Daphnia magna* and 7-d *Ceriodaphnia dubia* toxicity tests[a]

Sample site	Species	Concn. units	Survival NOEC[b]	Survival LC50[c]	NOEC[bd]	IC25[cd]
PM	*D. magna*	%	50 (25.1)	100 (NA)	50 (3.6)	66.4 (45.2–71.5)
PM	*C. dubia*	%	100 (13.5)	NA	50 (11.1)	68.3 (48.7–72.4)
BS	*D. magna*	%	100 (17.2)	100 (NA)	100 (4.8)	100 (NA)
BS	*C. dubia*	%	42.2 (22.3)	48.3 (33.2–56.3)	42.4 (15.3)	37.9 (31.9–45.6)

[a] Tests were conducted using water column samples collected from two sites characterized by high total dissolved solids and water hardness: The Penn Mine (PM) site (U.S. Environmental Protection Agency [EPA] Region IX), and the Big Springs (BS) effluent (U.S. EPA Region VI). IC25 = 25% inhibitory concentration; LC50 = median lethal concentration; NA = not applicable; NOEC = no-observed-effect concentration.
[b] Values in parentheses represent the minimum significant difference.
[c] Values in parentheses represent upper and lower confidence intervals.
[d] Values for *D. magna* are for growth; values for *C. dubia* are for reproduction.

as part of the laboratory quality-assurance/quality-control (QA/QC) program. Results of this testing, conducted over a period of four to six years, are presented in Table 6. No ammonium chloride tests were conducted during this phase.

DISCUSSION

In developing and validating this test method, the first concern was the feasibility of getting a consistent growth endpoint at the end of the 4-d test duration. The data indicate that the initial weight of *D. magna* was consistent and easy to measure using an ultra balance. In 21 sets of measurements, initial dry weight in the developmental reference toxicant tests ranged from 6.2 to 13.7 μg/individual, with CVs ranging from 4.2 to 13.1%. The average initial weight was 10.0 μg/individual (CV, 21.0%). These ranges show consistency in both the size of the animals used from test to test and in the variation of the animals in each set. The same level of consistency was found in the final dry weight. The final dry weight ranged from 73 to 167 μg/individual, with CVs ranging from 2.6 to 19.8%. The smallest final dry weight in the control animals was generated from the smallest set of animals used to start a test, and likewise, the largest final dry weight in the control animals was generated from the largest set of animals used to start a test. This trend was seen throughout the remaining testing as well. Other researchers have attempted to use *D. magna* growth as an endpoint, with mixed levels of success. At the end of the 21-d *D. magna* test, Pereira et al. [20] found that growth measurements did not correspond well to the differences found using reproduction as the endpoint. They believed this was caused by the energy expended in reproduction. The 4-d *D. magna* test avoids that issue by measuring growth before the release of young. This way, any energy put into the production of young is still measured, because any eggs produced are present in the female and are measured as part of the total animal weight at the end of the test. Others have shown that in a related cladoceran species (*Daphnia pulex* Leydig), a strong correlation exists between 21-d growth and 7-d prereproductive body length [6]. Baillieul et al. [21] found the growth endpoint to be a viable indicator of sublethal effects resulting from contaminants based on measurement of physiological energetics (scope for growth) [21]. Billoir et al. [22] came to a similar conclusion concerning growth using dynamic energy budget in toxicology analyses. Based on our test data and the information available from the literature, the growth of *D. magna* in a 4-d test appears to be a reasonable endpoint. The test acceptability criteria for control animals were determined based on the method development tests conducted and the available literature data. The control survival criterion of 90% or greater was based, in part, on the control survival for acute testing [10]. The control survival criterion for a 48-h *D. magna* acute test is 90% or greater. Results from these 96-h tests found that less than 5% of tests conducted failed to meet this criterion. The ×10 control growth criterion was based strictly on methods development testing. In reviewing the data, it was found that less than 12% of the tests failed to meet the criterion. Results from testing conducted since development was completed have seen this level drop to less than 10%.

Table 5. Survival and growth no-observed-effect concentrations (NOECs) from a series of 4-d *Daphnia magna* toxicity tests run using water column samples collected from an acid mine discharge site in southeastern Ohio, USA[a]

Sample site	No. of tests	Concn. units	NOEC	
			Survival[b]	Growth[c]
SFE	5	%	10–25 (21.2–33.3)	10–25 (1.3–3.7 μg)
SFB	5	%	10–25 (16.3–21.7)	1–12.5 (2.2–4.1 μg)
BF	5	%	10–25 (22.7–35.4)	1–6.25 (2.5–4.6 μg)
MCD	4[d]	%	50, 100 (12.1–15.3)	50 (3.1–5.2 μg)
SR	5	%	100 (NA)	100 (NA)
LMC	5	%	100 (NA)	100 (NA)

[a] Water was collected from the sites five times over a six-month period, beginning in July and ending in January. BF = Brush Fork; LMC = Little Monday Creek; MCD = Monday Creek; NA = not applicable; SFB = Snow Fork at Buchtel; SFE = Snow Fork at Essex; SR = Salt Run.
[b] Values in parentheses represent the range of difference in survival necessary for a sample to be considered statistically different from the control.
[c] Values in parentheses represent the difference in growth necessary for a sample to be considered statistically different from the control.
[d] Sample not collected in January because of access issues.

Data from the developmental reference toxicant tests indicate that results from the 4-d *D. magna* survival and growth test method are consistent and reproducible. The results in Table 2 show, in most cases, that the difference between the high and low NOECs for survival or growth is two dilutions. The variation in the LC50 and IC25 point estimates is somewhat greater, but the difference between the high and low value for these point estimates is still approximately 20% or less, with two minor exceptions: The difference in the high and low IC25s for zinc is approximately 28% when the outlying result from the fifth test is not considered; however, the greatest difference is in the high and low IC25s for phenol (1.6 mg/L, a 59% difference).

Data from the developmental reference toxicant tests also were compared to published literature values to determine the comparability of the test results. The results of this method

Table 6. Results from a series of additional reference toxicity tests conducted using the 4-d *Daphnia magna* survival and growth testing method after the initial stage of test method development was complete[a]

Toxicant	No. of tests	Concn. units	Survival NOEC[b]	LC50[c]	Growth NOEC[d]	IC25[c]
Phenol	4	mg/L	2.5 (17.3–37.2%)	4.9–5.1 (2.8%)	1.25 (2.9–4.7 μg)	1.6–2.2 (22.3%)
Potassium chloride	13	mg/L	250–500 (25.7–41.1%)	517–707 (21.9%)	250–500 (1.1–3.4 μg)	440–625 (24.6%)
Zinc	11	μg/L	31.25, 62.5, 125 (18.9–31.3%)	77–270 (78.6%)	31.25–62.5 (2.6–9.3 μg)	37.2–86 (56.1%)

[a] No ammonium chloride tests were conducted during this phase. IC25 = 25% inhibitory concentration; LC50 = median lethal concentration; NA = not applicable; NOEC = no-observed-effect concentration.
[b] Values in parentheses represent the range of difference in survival necessary for a sample to be considered statistically different from the control.
[c] Values in parentheses represent the coefficient of variation.
[d] Values in parentheses represent the difference in growth necessary for a sample to be considered statistically different from the control.

compared reasonably well to the published values. The 4-d *D. magna* toxicity tests conducted with zinc sulfate (Table 2) showed survival LC50s ranging from 154 to 177 μg/L (with one value of 500 μg/L) and growth IC25s ranging from 88.4 to 122 μg/L (with one value of 444 μg/L). Standard methods report a mean acute value of 355 μg/L and a chronic value of 140 μg/L for *D. magna* in soft water; literature values for *D. magna* in moderately hard water include a mean acute value of 525 μg/L and a chronic (reproduction) value of 48 μg/L [23].

The ammonium chloride results from the present study are similar to published results, which include NH_3 LC50s of 2.0 to 2.6 mg/L at a pH range of 7.9 to 8.1 and a temperature range of 22 to 25°C [24]. The pH ranges noted in the literature are within the ranges of these tests, and when converted to reflect un-ionized ammonia (NH_3), these tests show nominal NH_3 LC50s in the range of 2.0 to 3.0 mg/L.

The available data for phenol show acute LC50s in the 9 to 10 mg/L range [25], compared to LC50s in these tests of 5.4 to 6.8 mg/L (Table 2). Using these data, it appears that the 4-d *D. magna* test method described here is more sensitive to phenol. This might be expected, because the majority of toxicity work with *D. magna* has been conducted using a test duration of 48 h whereas the LC50s generated in the present study are based on 96-h fed tests.

Data from the potassium chloride tests were compared to the available literature values for chloride [26]. The published values for *D. magna* in moderately hard water indicate a mean acute value of 1,500 mg/L and a mean chronic value of 370 mg/L. When converted to chloride levels, the results from the potassium chloride tests conducted with *D. magna* indicate a LC50 range of 309.9 to 335.8 mg/L and an IC25 range of 281.6 to 296.9 mg/L. As with phenol, the LC50 differences may be caused by the increased test duration in the present study.

Table 6 contains values for reference toxicant tests conducted as part of the laboratory QA/QC program after the completion of validation testing. These data show more variability in the endpoints for zinc and potassium chloride over a time period of four to six years, whereas the phenol data show the same level of variability in both the LC50 and IC25 endpoints over the same time period. The increased variation in the zinc and potassium chloride endpoints may result, in part, from conducting some of these tests in an entirely new laboratory facility, using a new water source, and completely different laboratory environmental conditions. After the first few months of testing, the test results in the new facility returned to those endpoints seen in the developmental tests (Table 2). Subsequent reference toxicant testing conducted in support of the laboratory QA/QC program produced results that reflect those generated during the developmental testing phase as well. For example, reference toxicant testing conducted using potassium chloride produced survival NOECs of 250 to 500 mg/L, growth NOECs of 250 to 500 mg/L, and growth IC25s ranging from 475 to 610 mg/L. These values all correspond well to the values generated during the developmental phase of this test method (Table 2).

Sensitivity is an issue with the test method. This 4-d *D. magna* survival and growth test is not intended as a replacement for the 7-d *C. dubia* survival and reproduction test but, rather, as a companion method. The 4-d *D. magna* test could be used when it is believed the results from a 7-d *C. dubia* test might be influenced more by physical factors than by chemical or toxicant factors. Such a case would be in tests with samples containing excessive levels of total dissolved solids. *Ceriodaphnia dubia* is more sensitive than *D. magna* to total dissolved solids [27], so the results from a *C. dubia* test in a high total dissolved solids sample may be caused by total dissolved solids, not by a chemical contaminant. The use of a 4-d *D. magna* test in conjunction with a 7-d *C. dubia* test would provide results that could either confirm the presence of a chemical contaminant or show that total dissolved solids were responsible for the *C. dubia* results. A more complete description of issues associated with ion imbalance can be found in Goodfellow et al. [28].

Results from tests comparing the 4-d *D. magna* test to the 7-d *C. dubia* test show that the level of sensitivity between the two species can vary, depending on the toxicant and sample being tested (Tables 2 and 3). Results from reference toxicant tests with potassium chloride show that the *C. dubia* reproduction endpoint is more sensitive than the *D. magna* growth endpoint, with NOECs and IC25 ranges that are approximately 50% those of *D. magna*. The potassium chloride survival endpoints are similar for both species. The results from reference toxicant tests with zinc indicate that the two species have a similar level of sensitivity, with complete overlap of the LC50 and IC25 ranges in most cases. The only variation was in the *D. magna* test that had an IC25 of 444 μg/L; as mentioned earlier, this test was classified as an outlier. It should be noted that the *D. magna* growth response was measured in only 4 d, versus 7 d for the *C. dubia* growth response.

Results from side-by-side tests with natural water samples show the same type of variability (Table 4). The results from the tests conducted with the PM effluent sample show similar NOECs and IC25s for both species. This indicates that *D. magna* was as sensitive as *C. dubia* to the toxicant in this

sample. The results from the tests with the BS effluent sample indicate that *C. dubia* was significantly more sensitive to this sample. Survival and reproduction in the *C. dubia* test was reduced to the point that both a LC50 and an IC25 could be generated. Neither endpoint could be generated with the data from the *D. magna* test, indicating that this sample had no effect on the *D. magna*.

Results from a series of tests conducted with mine discharge samples from southeastern Ohio show that the 4-d *D. magna* test can monitor for temporal changes in toxicity. The growth endpoint was able to detect changes in the various discharges that altered the growth of the organisms at different times. At the same time, the method also was able to validate the consistency of the two selected reference sites.

This method provides a consistent, logistically simple means to measure both survival and subchronic growth endpoints with a relatively sensitive invertebrate species. It requires no special equipment except for an ultra balance, which a facility should have if they conduct the 7-d *P. promelas* survival and growth test. The test species used is a common toxicity test organism in the United States, Europe, and Asia and is easy to culture in the laboratory. To be acceptable, a test conducted using the 4-d *D. magna* survival and growth test method must have 90% or greater control survival (single-laboratory precision data), and the weight of the control animals must be at least 10-fold that of the animals used to start the test (single-laboratory precision data). The 4-d *D. magna* method can function as a companion to the 7-d *C. dubia* and *P. promelas* tests currently in use. It provides an endpoint that is as sensitive as the *C. dubia* endpoint in some cases, and it provides a method for use in cases where data from the 7-d *C. dubia* test may be suspect because of physical influences rather than contaminant stressors.

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