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Short-term Toxicity Assay Based on Daphnid Feeding on the Microalga *Scenedesmus subspicatus*

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We developed and evaluated a method of short-term acute toxicity testing based on the feeding behavior of *Ceriodaphnia dubia*. In prior toxicity tests, neonates of *C. dubia* were hatched and cultivated with the addition of yeast only for the preparation of the transparent daphnid's gut. *Scenedesmus subspicatus* was supplied as food after 1 to 6 h of exposure to toxicants. The effects of 1-h and 6-h exposure time on test sensitivity did not significantly differ. A comparison of the short-term 1-h acute toxicity test developed in this study to the standard 48-h acute toxicity test using heavy metals, cyanide, and pentachlorophenol indicated that the 1-h test provided an acceptable sensitivity level in toxicity testing of *C. dubia*..

Key words: Acute toxicity test, *Ceriodaphnia dubia*, Feeding behavior, Sensitivity

Introduction

One of the most pressing issues in ecotoxicology is the need to evaluate the potential hazards of the many thousands of chemicals and countless industrial waste products introduced or released into our environment. An urgent demand therefore exists for simple, rapid, and cost-effective methods that can be applied routinely to screening the toxicity of these products.

The primary reason for using invertebrates in toxicity tests is that the effects of toxicants on aquatic invertebrates are essential to the productivity of aquatic ecosystems (Macriorwaski and Clarke, 1980). Because these organisms serve a crucial role in ecosystem food webs, the effects of toxicants on aquatic invertebrates can alter the structure and function of those ecosystems. There are also several advantages to using aquatic invertebrates in toxicity tests; because of their small size, aquatic invertebrates require little laboratory space. In addition, the life cycle of aquatic invertebrates is relatively short and a substantial supply of neonates can quickly be obtained for testing.

Acute toxicity tests are generally used to determine the concentration of a toxicant that produces a specific adverse effect on a specified percentage of test organisms in a short period of time. The most important data obtained from an acute toxicity test are the survival percentages of test organisms that are affected in a specific way by each of the treatments after specified lengths of exposure. Death is usually used as the effect criterion in acute toxicity tests because it is easily detected and is an obviously important adverse effect. Given that death is not easily determined for some invertebrates, immobilization or the lack of movement except for minor activity of appendages is used as the criterion of effect for daphnids (Buikema et al., 1980).

The water flea *Ceriodaphnia dubia* has been a popular test organism recently because of its sensitivity to toxic chemicals, short life cycle, ease of culture in the laboratory, and adaptability to toxicity test apparatus. These organisms are specified for biomonitoring in the discharge permits of many wastewater treatment facilities. The standard *C. dubia* bioassay is recommended in determining discharge permit compliance in the National Pollutant Discharge Elimination System (NPDES). This test provides a point estimate of effluent toxicity [median

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lethal concentration (LC_{50}) or median effective concentration (EC_{50})] or a no-observed-adverse-effect concentration (NOAEC) defined in terms of mortality [U.S. Environmental Protection Agency (US EPA), 1991]. Whereas chronic tests based on reproduction require 7 days. While such a test is appropriate for compliance monitoring, a short-term test based on the same indicator organism could be useful for routine toxicity screening or for toxicant fractionation studies. Bitton et al. (1995, 1996) introduced a short-term toxicity screening test based on suppression of *C. dubia* feeding activity. The test (CerioFast™) requires as little as 40 min for toxicant contact and can be completed within 1 h. The food source for the uptake measurement is yeast. To make the normally colorless yeast cells easy to detect in the daphnid gut, they are stained with the fluorescent dye, 5-(4,6-dichlorotriazin-2-yl) aminofluorescein. This allows the presence of yeast in the daphnid gut to be detected under an epifluorescence microscope. Absence of fluorescence in the daphnid gut indicates that no yeast has been ingested, which is a consequence of toxic inhibition. Bitton et al. (1995, 1996) showed that CerioFast™ was similar in sensitivity to the standard 48-h acute *C. dubia* bioassay. However, this test used an inexpensive bright field microscope rather than the expensive epifluorescence microscope used in the CerioFast™ method by Bitton et al. (1995, 1996). The purpose of this study was to develop and apply a short-term acute toxicity test based on the feeding behavior of *C. dubia* on *Scenedesmus subspicatus.*

Materials and Methods

Stock solutions of toxicant

The chemicals assessed for toxicity were cadmium ($CdCl_2 \cdot 1.5H_2O$), zinc ($ZnSO_4 \cdot 7H_2O$), copper ($CuSO_4 \cdot 5H_2O$), cyanide (KCN), and arsenic acid. Organic toxicants such as phenol and pentachlorophenol (PCP) were prepared for the study. Stock solutions of heavy metals and phenol were prepared in MilliQ (Millipore, Eugene, OR, USA) water. The stock solution of 500 mg/L PCP was prepared in 0.01 N NaOH. The pH levels of the test solutions containing PCP were in the same range as the pH levels of the other test solutions (pH 7.6-7.8).

Organic pesticides were tested for methomyl [24.1% S-methyl N-(methyl carbamoyloxy)-thioacetimidate] and pyridaphenthion [30% O,O-dietyl-O-(3-oxo-1-phenyl-2H-pyrida-zine-6-yl) phosphorothioate]. The same set of stock solutions was used in all tests.

Cultivation of *C. dubia* and *S. subspicatus*

The *C. dubia* cultures were maintained in several aerated 1-L glass beakers that were partially submerged in a water bath incubator. The water bath, and therefore the *C. dubia* culture, was maintained at a constant temperature (20±2°C). Moderately hard reconstituted water was used as the culture medium and toxicity test dilution media for *C. dubia* (US EPA, 1985a, 1991). The reconstituted moderately hard water contained the following ingredients per liter of MillQ water: $NaHCO_3$, 96 mg; $CaSO_4 \cdot 2H_2O$, 60 mg; $MgSO_4$, 60 mg; and KCl, 4 mg (Peltier and Weber, 1985). The moderately hard water had a pH range of 7.4 to 7.8 and a total hardness range of 60 to 70 mg/L (US EPA, 1985a). The culture media were aerated before use.

The culture was maintained by replacing the medium weekly with fresh medium; *C. dubia* was fed 1.5 mL L^{-1} of yeast, trout chow, and alfalfa (YTCA) medium three times a week as recommended by the US EPA (1985a, 1991). The YTCA suspension was prepared by adding 3.9 g Fleischmann's® dry baker's yeast, 9.45 g trout chow, and 0.75 g dried alfalfa to 750 mL double-distilled deionized water; blending for 5 min followed, and then settlement for 1 h at 4°C. A supernatant volume of 300 mL was collected for use. A volume of 1.5 mL YTCA suspension was added per 4 L of *C. dubia* culture three times a week.

Axenic cultures of *S. subspicatus* were grown in 300-mL erlenmeyer flasks on a rotary shaker under fluorescent lighting. Chlorophyll *a* concentrations were analyzed according to the American Public Health Association Standard Methods (APHA et al., 1998).

Optimum feeding time

The optimum feeding time when measuring the rate of algae uptake by *C. dubia* was determined by exposing neonates (less than 24 h old) to selected chlorophyll *a* concentrations (1 or 3 μg/L) over a range of times (10-60 min). Each combination of chlorophyll *a* concentration and feeding time was tested using 30 neonates in a 50-mL beaker (25 mL liquid volume). Mean algal uptake by each daphnid was estimated by measuring chlorophyll *a* concentrations in the beaker liquid at the beginning and the end of the feeding period, multiplying this difference by the liquid volume (25 mL), and dividing by the number of daphnids (n=30). The test temperature was 25°C.

Table 1. Test conditions for the standard acute 48-h and the method used in the present study

	standard acute 48-h	present study
1. Test duration	48h	1h
2. Temperature (°C)	20±2	20±2
3. Light quality	Ambient laboratory illumination	Ambient laboratory illumination
4. Light intensity	50-100 footcandles ambient laboratory levels	50-100 footcandles ambient laboratory levels
5. Photoperiod	16-hour light/8-hour darkness	16-hour light/8-hour darkness
6. Test chamber size	30 mL plastic cup	50 mL plastic cup
7. Test solution volume	20 mL	25 mL
8. Age of test organisms	less than 24-h old	between 24-h and 48-h
9. No. of organisms per test chamber	10	5
10. No. of replicate chamber per concentration	3	3
11. Total number of Organisms per concentration	30	30
12. Feeding regime	Not fed during test; fed prior to use in the test	After 1 hour exposure, feed dapnids for 20 min
13. Aeration	None	None
14. Dilution water	Moderately-hard reconstituted water	Moderately-hard reconstituted water
15. Test concentration	5 concentrations and a control	5 concentrations and a control
16. Dilution factor	0.5	0.5
17. Endpoint	Mortality (LC_{50})	Suppression of food uptake
18. Obsevation	On the illuminated plate	naked eye
19. Test acceptability criterion	90% or greater survival in controls	90% or greater survival in controls

Toxicity tests

The standard 48-h *C. dubia* acute bioassay

The 48-h acute bioassay was performed using standard methods (US EPA, 1991). *Ceriodaphnia dubia* first-instar neonates (less than 24 h old) were used for testing. The test temperature was 20±2°C. Ten daphnids were exposed to each toxicant concentration in plastic cups containing 20 mL of the toxicant dilution or a control (moderately hard reconstituted water). A solvent control containing the highest amount of solvent present in any treatment level was included when testing organic toxicant solutions. The number of live (motile) and dead (immobilized) daphnids was counted at each toxicant concentration and in the control after 48 h. This was easily performed by swirling the test solution in a circular motion, which propelled the daphnids to the middle of the test chamber. The live daphnids began to swim away from the middle as the solution came to a standstill. When several live daphnids were present, a pipette was useful for removing them one at a time while counting. Five toxicant concentrations and a water control (reconstituted moderately hard water) and a solvent control were tested in triplicate for each toxicity test. The test conditions for the standard 48-h *C. dubia* acute bioassay are summarized in Table 1.

Ceriodaphnia dubia feeding activity suppression test

First, the digestive tracts of the organisms were cleared. This was necessary because the digestive tracts of *C. dubia* grown on YTCA were green. Neonates less than 24 h old were transferred to 500 mL moderately hard water in a beaker to which 2.5 mL of yeast suspension (0.5 g dry yeast/50 mL double-distilled deionized water) were added. The neonates quickly began defecating, and within 40 min, their digestive tracts were cleared of green matter. All neonates used in the present study were initially treated with this method.

Suppression of algal uptake was determined in static tests using 50-mL beakers, each containing ten neonates, 20 mL of moderately hard water, and 5 mL of toxicant working solution. After exposing *C. dubia* to toxicants, 1 mL of *Scenedesmus* culture with an optical density of 1.0 (path length=10 mm, wavelength=665 nm) was added to each beaker. This resulted in a final chlorophyll *a* concentration of approximately 5 μg/L. After a 35-min feeding period, neonates were removed from the test mixture, placed on a plain microscope slide, and examined under bright-field illumination at ×100. The test end point was the presence or absence of green-colored matter in the daphnid gut (a slight green color in the gut was

counted as "absent"). The times at which toxicants and algae were added to the test samples were staggered to allow for subsequent examination with minimal (1-2 min) variation in the length of either the contact or feeding periods.

The 48-h static acute bioassay was conducted according to conventional methods [American Society for Testing and Materials (ASTM, 1988; US EPA, 1985a)]. Ten neonates were exposed to a selected toxicant concentration in a 50-mL glass beaker containing 20 mL of moderately hard water and 5 mL of a toxicant working solution. The number of active and immobilized daphnids was counted after a 48-h exposure period. Five toxicant concentrations plus a control were tested in triplicate for each toxicity test. All tests were performed at 25°C.

Statistical analysis

The median effective concentration (EC_{50}), or the dosed concentration providing 50% inhibition of algal uptake (or 50% immobilization), was calculated with the probit method using US EPA software (Finney, 1971; US EPA, 1994). An EC_{50} was computed for each replicate test. For each toxicant, three replicate tests were carried out; each replicate consisted of five toxicant concentrations plus the control. One-way analysis of variation (ANOVA) was used to test the hypothesis that mean EC_{50} values obtained at different times were equal. Differences between EC_{50} values of the 1-h test and the 48-h acute bioassay were tested using a two-tailed t-test.

Results and Discussion

Effect of feeding time on uptake of algal cells by *C. dubia*

Algal uptake by daphnids increased with feeding time and algal concentration (Fig. 1). Uptake began leveling off after approximately 35 min. Parallel microscopic observations indicated that the neonates' digestive tracts were filled with algae after 30 to 40 min of feeding. This was somewhat longer than the 20-min saturation period for uptake of fluorescently stained yeast by *C. dubia* neonates reported in Bitton et al. (1995).

Algal uptake by *C. dubia* was higher at the higher initial chlorophyll *a* concentration. The difference after 35 min of feeding was approximately 60% (0.05-0.08 g chlorophyll *a* per daphnid) based on the best-fit lines shown in Fig. 1. Bitton et al. (1995) showed that yeast uptake by *C. dubia* increased with yeast concentration. To maximize algal uptake in the shortest possible feeding time, a feeding time of 35 min and an initial chlorophyll *a* concentration of 5 µg/L were adopted.

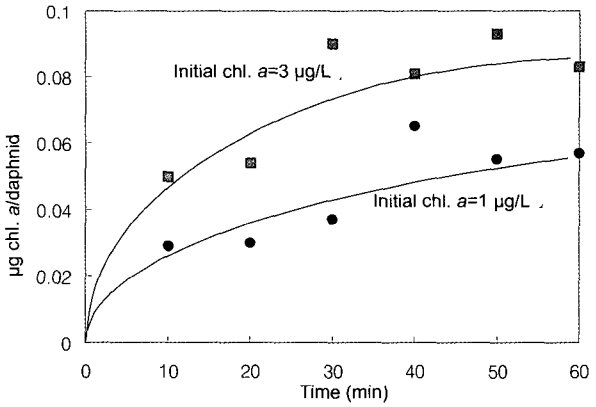

Fig. 1. Temporal variation of *Scenedesmus subspicatus* uptaken by *Ceriodaphnia dubia* in relation to chlorophyll *a* concentration.

Algal uptake by *C. dubia* in the presence of toxicants

Ceriodaphnia dubia neonates did not uptake algae under relatively high toxicant concentrations. However, the controls took up the algae and the digestive tracts of the neonates were invariably full of algae. At intermediate toxicant concentrations, some of the neonates took up some algae and had green digestive tracts. These two groups could be readily distinguished under the bright-field microscope. In relatively few instances (fewer than 10% of neonates examined), part of the digestive tract had a faint green color; to facilitate counting, those individuals were grouped with those that did not take up algae.

An example of a dose-response curve for cadmium, copper, and zinc is given in Fig. 2. The response was most variable at the intermediate toxicant concentrations. The EC_{50} values for Cd, Cu, and Zn were much lower than those reported for the 1-h *Daphnia magna* bioassay based on enzyme activity (EC_{50} values for Cd, Cu, and Zn were 0.41 mg/L; Janssen and Persoone, 1993). However, similar results were obtained using *C. dubia* as a test organism (Janssen et al., 1993). The EC_{50} values for CN and PCP were 820 µg/L and 700 µg/L, respectively, and were comparable to previous values (Janssen and Persoone, 1993, Janssen et al., 1993).

There was no consistent trend in the effect of contact time on sensitivity (Fig. 3). The EC_{50} values of cadmium, copper, and zinc did not differ significantly between 1 h and 6 h of contact time ($P > 0.05$). Similarly, Bitton et al. (1996) found that Cerio-Fast™ EC_{50} values were not significantly affected by

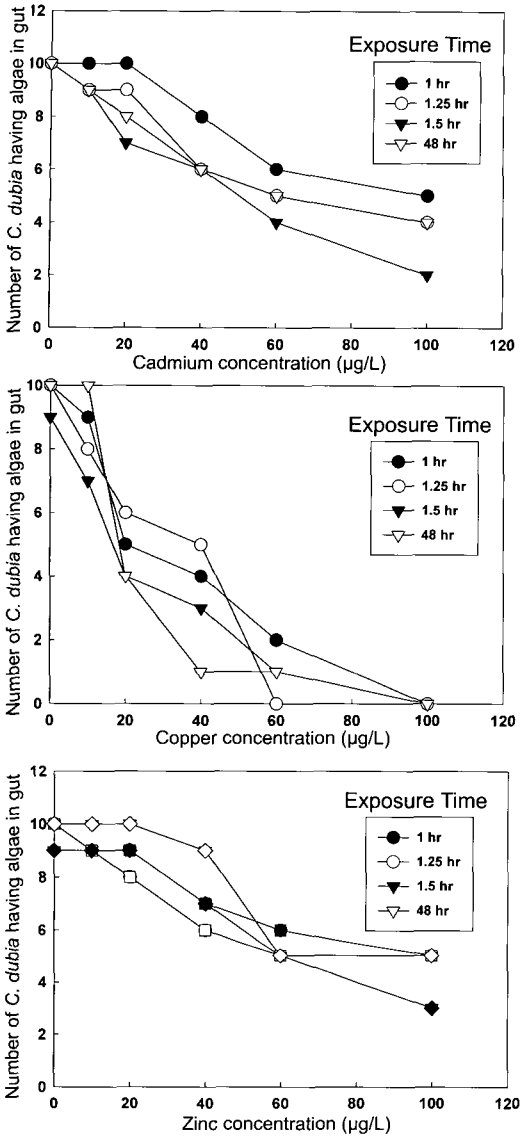

Fig. 2. Effect of exposure time and concentration of some toxicants on *Ceriodaphnia dubia* feeding behavior.

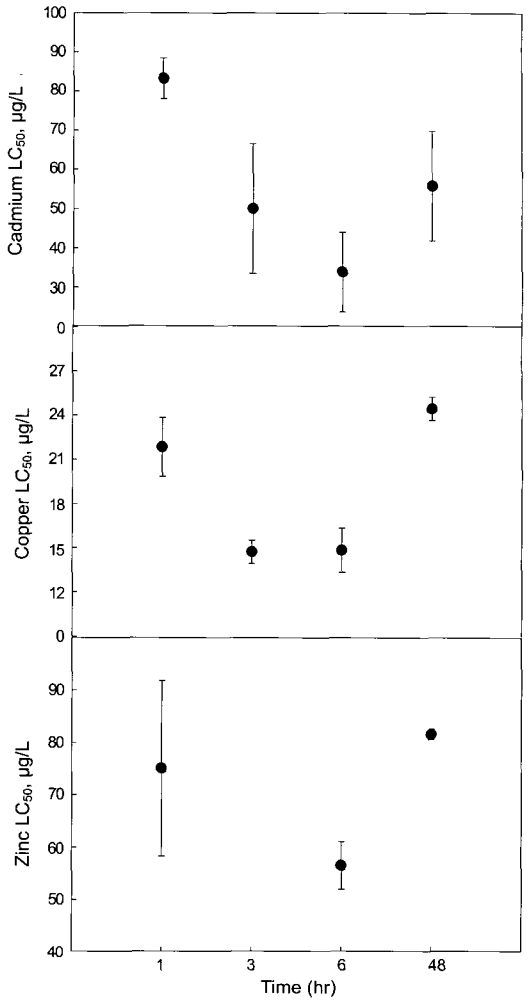

Fig. 3. Effect of toxicants exposure time on EC_{50} values (Bar indicates 95% confidence interval).

contact times in the 1- to 6-h range.

EC_{50} values for all the chemicals at 1 h of contact time in this study were compared with those of the 48-h bioassay. No significant differences were found between the two different contact times with regard to the EC_{50} values for all the chemicals tested ($P>0.05$).

Comparison to the standard 48-h bioassay

The assay based on the green algal feeding behavior of *C. dubia* was compared to the standard acute 48-h *C. dubia* bioassay for pure compounds, which included heavy metals and organic compounds. The resulting EC_{50} values of Cd, Cu, Zn, CN, and pentachlorophenol (PCP) are shown in Table 2. This study obtained slightly, but consistently, less sensitive EC_{50} values than CerioFast™.. The short-term acute toxicity test in this study was similar in principle to the feeding activity suppression test of Bitton et al. (1995, 1996) but differed in that bright-field microscopy was used instead of epifluorescence microscopy to determine the test end point. As seen in Table 2, the EC_{50} values of organic compounds for the standard 48-h *C. dubia* bioassay were lower than those based on green algal feeding behavior of *C. dubia*. Similar results have been observed using organic compounds (Mazidji, 1992; Rhodes, 1992; Lee et al., 1993). The EC_{50} of PCP decreased to between 150 and 1000 μg/L for the 1-h *D. magna* test and 160-1,040 μg/L for the 48-h test. However, considering the time spent on preparations and measurements for the test, the method used in the present study was more rapid and easier than CerioFast™.

Table 2. Comparison of EC_{50} among acute toxicity bioassay methods

Toxicant	Toxicity test (unit: μg/L)								
	Ceriodaphnia dubia					*D. magna*[b]	β-gal. syc.[c]	INT[d]	Microtox[e]
	1 hr[a)]	3 hr[a)]	6 hr[a)]	48 hr[a)]	48 hr[b]				
Cd	76.2	50.1	33.9	55.9	51-110	14-118	70	240	27,000
Zn	75.0		51.8	81.6	50-93	68-1,200	100		
Cu	21.8	14.8	13.5		9.7-27	10-200	29	140	380
CN	820	1,300	1,286	2,517			1,200	4,500	2,800
PCP[f]	700	650	881		160-1,040	150-1,000			

[a]Result obtained in this research
[b]Adapted from Rhodes (1992)
[c] β-galactosidase biosynthesis inhibition bioassay from Lee et al. (1993)
[d]INT-dehydrogenase bioassay from Lee et al. (1993)
[e]Data from Mazidji (1992)
[f]Pentachlorophenol

The new test bioassay is a promising and economically viable tool to screen and evaluate toxicity for a variety of wastewater sources.

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