Effects of Diet on Seven-Day *Ceriodaphnia dubia* Toxicity Tests[1]

BLAS CERDA AND JOHN H. OLIVE, Department of Biology, The University of Akron, Akron, OH 44325-3908

ABSTRACT. **The purpose of this study was to evaluate the effects of four diets on the results of seven-day *Ceriodaphnia dubia* toxicity tests. Survival and reproduction were used as indices to detect the sensitivity of this species to acute and chronic copper stress. All toxicity tests were conducted using the moderately hard reconstituted water recommended in 1989 by the U.S. Environmental Protection Agency. Diet differentially affected the acute and chronic toxicity of copper. Daphnids fed *Selenastrum capricornutum* (alga) showed the greatest sensitivity, followed by those fed the alga *Chlamydomonas reinhardti,* then by animals fed a Yeast–Cerophyll™–Trout Food (YCTF) mixture plus *Selenastrum,* and finally by animals fed YCTF alone. These differences may result from the poor nutritional adequacy of *Selenastrum* when fed alone, the different caloric contents of the diets, the increased toxicant uptake by the organisms through ingestion of copper-laden algal cells, and/or copper ions sequestered by fats and insoluble substances in YCTF. We recognize that diet is an important variable in seven-day toxicity tests, and that the selection of a diet should not be based only on its effects on long-term culturing of *C. dubia*, but also on its possible effects on test results.**

OHIO J. SCI. 93 (3): 44–47, 1993

INTRODUCTION

Daphnids are frequently used as experimental organisms for evaluating the toxicity of complex effluents. Traditionally, bioassays using complete life cycles of *Daphnia magna* were widely used and, with few exceptions, are well standardized (APHA 1989). However, because of their time-consuming (3-4 weeks) and costly nature, these tests are not practical for routine monitoring. To circumvent these problems the U.S. Environmental Protection Agency (U.S. EPA) introduced, in 1984, the seven-day static renewal survival and reproduction test using *Ceriodaphnia dubia.*

The introduction of the seven-day toxicity test triggered an extensive use of *C. dubia* as an experimental animal (Mount and Norberg-King 1985, Mount et al. 1986, Knight and Waller 1987, Winner 1988). But unlike *D. magna, C. dubia* does not have a long history in routine testing. The first article on culture methods and description of the use of this species in toxicity testing was published in 1984 (Mount and Norberg 1984). As more researchers have adopted *C. dubia* as a test organism, periodic problems have been encountered in conducting valid toxicity tests, especially with regard to food type (DeGraeve and Cooney 1987). Consequently, test methods and approaches have been modified and investigators seem to show preferences for particular diets; therefore, many different diets have been proposed for *C. dubia.*

The initial food described was a simple suspension of yeast (Mount and Norberg 1984). Several authors (Knight and Waller 1987, Cowgill et al. 1985a, Winner 1989) have suggested various species of algae for maintaining cultures of this cladoceran. A diet commonly used is a Yeast–Cerophyll™–Trout Food (YCTF) mixture which was suggested by investigators at the U.S. EPA in 1985 (Horning and Weber 1985). At present the U.S. EPA recommends a feeding combination of YCTF and the alga *Selenastrum capricornutum* (U.S. EPA 1989).

Although efforts have been made to evaluate the adequacy of several diets for maintaining *C. dubia* cultures (Cowgill et al. 1985a, Winner 1989, Norberg and Mount 1985), more work is needed to evaluate the effects that diet might have on the seven-day *C. dubia* toxicity test results. This article presents the evaluation of the effects of four diets on the sensitivity of *C. dubia* to copper in seven-day static renewal survival and reproduction toxicity tests.

MATERIALS AND METHODS

Seven-day toxicity tests were run for each of the diets evaluated in this study. All tests were initiated with young (<24 h old) *C. dubia.* Test organisms were maintained individually in 30 ml disposable clear plastic cups containing 15 ml of test solution. Trays holding the plastic cups were covered with clear plastic sheets to retard evaporation. Organisms were maintained in an environmental chamber at 23± 1° C on a 16 h light, 8 h dark photoperiod at a light intensity of approximately 70 ft-c. Animals were transferred to fresh test solution daily. Each daphnid was fed the appropriate diet and examined every day until it died (i.e., showed no movement) or until the test ended. Offspring were counted and discarded at renewal time. Records of survival, young per female, number of broods, and age at reproductive maturity were kept and used to estimate the endpoints for acute and chronic toxicity reported in this study. All tests were conducted using the moderately hard reconstituted water recommended by the U.S. EPA (1989) as the dilution water. Total hardness, alkalinity, and pH ranged from 80 to 90 mg $CaCO_3$/L, 55 to 70 mg $CaCO_3$/L, and 7.4 to 7.8, respectively. The dilution water contained only four salts with the following ionic concentrations (mg/L): Ca^{2+} 14, Mg^{2+} 12, Na^{+} 26.3, K^{+} 2, CO_3^{2-} 70.6, Cl^{-} 1.9, and SO_4^{2-} 48.

Four diets were tested: 1) A mixture of Yeast-Cerophyll™-Trout Food (YCTF), 2) a combination of YCTF and the alga *Selenastrum,* 3) a feeding suspension of *Selenastrum*

[1]Manuscript received 22 June 1992 and in revised form 17 February 1993 (#92-18).

alone, and 4) a feeding concentrate of *Chlamydomonas* (alga). The YCTF mixture was prepared following the U.S. EPA guidelines (U.S. EPA 1989). Both algae were cultured in the algal medium described by the U.S. EPA (1989), to which a vitamin supplement (Murphy 1970) was added. Algal cultures were maintained at room temperature on a 16 h light, 8 h dark photoperiod under fluorescent and Gro-lux™ lights. Algae were allowed to grow for seven to ten days before being fed to daphnids. For feeding purposes, algae were concentrated by centrifugation. The algal density in the concentrates was determined by optical density using a spectrophotometer. To achieve the desired cell density, the concentrated algae were resuspended in the supernatants (or further concentrated) until a spectrophotometric absorbance of 1.5 at 665 nm was reached. This method of concentration provided a cell count of 3.0 to 3.5 x 10^7 cells/ml in the concentrates used to feed the test animals. Algal feeding suspensions and cultures were routinely inspected for viability and foreign algal contamination. Each test organism was fed 0.1 ml of the appropriate diet every day. For the combination YCTF + *Selenastrum*, the daily ration consisted of 0.05 ml YCTF and 0.05 ml *Selenastrum*.

Each toxicity test consisted of ten young (<24 h old) *C. dubia* at each of the following copper concentrations: 0 (control), 5, 10, 20, 30, 40, 50, 60,70, 80, 90, and 100 µg/L. Each test was repeated until two valid toxicity tests (a minimum of 80% survival and 15 young/female in controls) were obtained for each of the diets evaluated. In the case of YCTF + *Selenastrum*, two of two tests were valid, for YCTF alone two of three were valid, and for *Chlamydomonas* two of five were valid. For *Selenastrum* alone eight tests were run, all of which met the survival requirement but failed to meet the reproduction requirement. However, two of those tests had a mean young production by the controls that was not significantly lower than 15 young/female (12.9 and 13.3), based on hypothesis testing (P <0.001).

Copper was added as reagent grade $CuSO_4 \cdot 5H_2O$. A stock solution of 100 mg Cu/L was prepared daily in glass distilled water. Test concentrations were obtained by diluting appropriate aliquots of the stock in the dilution water. Test concentrations were prepared daily during the

TABLE 1

Effects of diet on seven-day Ceriodaphnia dubia *survival and reproduction toxicity tests*

Diet	Test	Survival			Reproduction		
		LC50[a] (µg/L)	NOEC[b] (µg/L)	LOEC[c] (µg/L)	IC50[d] (µg/L)	NOEC[e] (µg/L)	LOEC[f] (µg/L)
Selenastrum	1	8.5a (3.9-13.1)	5e	10i	5.0n (2.9-8.2)	5s	10v
	2	8.5a (4.0-12.5)	5e	10i	4.7n (3.6-6.8)	5s	10v
Chlamydomonas	1	10.8b (6.5-16.5)	10f	20j	8.2p (6.4-11.4)	5s	10v
	2	10.8b (7.3-14.1)	10f	20j	8.1p (6.2-8.9)	5s	10v
YCTF* + *Selenastrum*	1	39.6c (33.5-45.1)	20g	30k	17.7q (16.3-18.5)	10t	20w
	2	39.0c (33.5-43.8)	20g	30k	17.6q (16.5-22.2)	10t	20w
YCTF*	1	46.9d (42.0-50.0)	40h	50m	26.3r (16.3-31.3)	20u	30x
	2	46.3d (42.2-49.2)	40h	50m	28.7r (17.7-44.1)	20u	30x

[a]Median lethal concentration (CI), based on Probit analysis.
[b]No-observed-effect-concentration, based on Fisher's Exact test (P <0.05).
[c]Lowest-observed-effect-concentration, based on Fisher's Exact test (P <0.05).
[d]Inhibition concentration (CI), based on linear interpolation method.
[e,f]Based on Dunnet's test (P <0.05).
*Yeast–Cerophyll™–Trout Food mixture. LC50s and IC50s not followed by a common letter are significantly different (Duncan multiple range test, P <0.05). NOECs and LOECs not followed by a common letter are significantly different (Kruskal-Wallis test, P <0.05).

duration of each test. Copper concentrations were measured for the freshly prepared test solutions before animals were introduced into them. Stock cultures of *C. dubia* were maintained and monitored on each of the diets studied for at least seven consecutive generations prior to being used as a source of test animals.

Acute copper toxicity was evaluated by calculating a 48 h median lethal concentration (LC50) (U.S. EPA 1989) for each bioassay using the Probit Analysis (Finney 1971). The effect of chronic copper stress on survival was determined by estimating the no-observed-effect-concentration (NOEC) and lowest-observed-effect-concentration (LOEC) (U.S. EPA 1989) for each test using the Fisher's Exact test (Finney 1948). Effects of chronic toxicity on reproduction were determined by calculating inhibition-concentrations (IC50s) (U.S. EPA 1989) based on the Linear Interpolation Method using the "BOOTSTRP" program developed by Teresa Norberg-King (U.S. EPA 1989), and by estimating NOECs and LOECs using Dunnet's test (Dunnet 1985). Comparisons of the individual LC50 and IC50 values were made by Duncan's Multiple Range Test (Duncan 1955). Comparisons of NOEC and LOEC values for survival and reproduction were based on Kruskal-Wallis test (Ott 1988).

RESULTS

Acute Toxicity

Comparisons of the individual LC50s (Table 1) indicated no significant differences between pairs of test repetitions ($P<0.05$), but significant differences were found between diets ($P<0.05$). *Selenastrum* alone produced the highest sensitivity, followed by *Chlamydomonas,* then by YCTF + *Selenastrum,* and finally by YCTF alone.

Chronic Toxicity

Individual NOEC and LOEC values for survival and IC50 values for reproduction were not significantly different between test replicates ($P<0.05$), but were significantly different between diets ($P<0.05$) in the same order of decreased sensitivity indicated for acute toxicity (Table 1).

In NOEC and LOEC values for reproduction (Table 1), test replicates were not significantly different ($P<0.05$), and again there were significant differences among diets ($P<0.05$). However, in this case no significant differences were found between the two algal diets ($P<0.05$). Test organisms fed the algal diets were the most sensitive, followed by those fed YCTF + *Selenastrum,* and then by animals fed YCTF alone which were the least sensitive to copper stress.

DISCUSSION

The results of the present study indicate that the four diets evaluated *(Selenastrum, Chlamydomonas,* YCTF + *Selenastrum,* and YCTF alone) differentially affect the sensitivity of *C. dubia* to acute and chronic copper toxicity. Survival and reproduction data are consistent to show that algal-fed organisms are more sensitive to copper than those whose diets included YCTF. These differences can be attributed to the nutritional properties of each diet. Winner et al. (1977) indicate that foods of poor nutritional value impose a nutritional stress in addition to the copper stress. Of the four diets tested in the present study, *Selenastrum* has frequently been regarded as nutritionally inadequate for *C. dubia* in long-term culturing (Cowgill et al. 1985a, Winner 1989, Norberg and Mount 1985). The fact that *Selenastrum*-fed animals showed the greatest sensitivity to copper supports the argument that nutritionally inadequate diets increase the sensitivity of test organisms to toxicants. These observations, of course, should not be extended to suggest that all algal diets impose a nutritional stress that would increase the sensitivity of test animals. In the case of *Chlamydomonas,* a diet considered adequate for *C. dubia* (Winner 1989), the sensitivity of test organisms was less than those of *Selenastrum*-fed daphnids.

Although *Chlamydomonas,* YCTF, and YCTF + *Selenastrum* are all diets considered adequate for the culturing of this cladoceran (U.S. EPA 1989, Winner 1989, Horning and Weber 1985), *Chlamydomonas*-fed animals were more sensitive than those whose diet included YCTF. These differences suggest that there may be other mechanisms besides nutritional value by which the sensitivity of *C. dubia* to toxicants is affected. One of these mechanisms can be the caloric content of the diet. It has been indicated (Buikema et al. 1980) that increasing the caloric intake of daphnids improves their ability to withstand experimental stress. The lesser caloric content of *Chlamydomonas* (5,259 g-cal/g) compared to that of YCTF (6,799 g-cal/g) (Winner et al. 1977) can explain, at least in part, the greater sensitivity of the animals fed this diet.

Another way in which diet can affect the sensitivity of test organisms is by altering the availability of the toxicant in the test solution (Buikema et al. 1980). It is our contention that by including algae in the diet the toxicity of copper is increased, while the addition of YCTF has a detoxifying effect. Chandini (1989) noticed that when algae are fed to test organisms, the algal cells incorporate the toxicant, therefore the uptake of toxicant into the organism may be through the ingestion of toxicant-laden algal cells as well as by direct absorption. This effect can increase organismal sensitivity, because toxicant uptake is enhanced by feeding. The fact that animals fed YCTF alone were less sensitive to copper than those fed YCTF + *Selenastrum* in the present study supports this idea. Conversely, fats and insoluble substances present in YCTF (Cowgill et al. 1985b) may detoxify the test solution by sequestering copper ions at the surface and bottom of the test chamber, places where daphnids are less likely to be found. The lesser sensitivity of animals fed YCTF + *Selenastrum* compared to those fed *Selenastrum* alone may then be the result of the detoxifying effect of YCTF. Buikema et al. (1980) have indicated that food can detoxify test solutions, especially if it is fed *ad libitum.* In the present study food was available *ad libitum* because the amount of food offered to test animals in their daily rations was more than they could ingest in 24 h.

Two additional lines of evidence suggest that the different sensitivities observed in the present study resulted from the varied nutritional value of the diets and their interactions with the test solution. One is that reproduction

and survival in the control organisms and in our stock cultures increased in the following order: organisms fed *Selenastrum* alone, then those fed *Chlamydomonas,* followed by animals fed YCTF alone, and finally by those fed YCTF + *Selenastrum*. These differences indicate that the four diets do have different nutritional values and can potentially make test organisms more or less sensitive to toxic stress. The fact that *Selenastrum*-fed organisms in which survival and reproduction were the lowest were also the most sensitive to copper, and that the inverse situation was observed in animals whose diet included YCTF suggests that this potential was realized. Second is the fact that in the absence of copper stress, organisms fed YCTF + *Selenastrum* showed better reproduction and survival than those fed YCTF alone, but in the presence of copper, animals fed YCTF + *Selenastrum* were more sensitive than those fed YCTF by itself. These findings indicate that by combining *Selenastrum* and YCTF the nutritional value of the diet increases, therefore survival and reproduction are enhanced, but at the same time the sensitivity of the organisms is increased. If the nutritional quality of this diet is increased because of the combination of the alga and YCTF, then the increased sensitivity cannot be the result of a nutritional deficiency, therefore other mechanisms, such as increased toxicant uptake by test animals through the ingestion of copper-laden algal cells must be in effect. These observations highlight the effects that the interactions between diet and test solution can have on test results.

Finally, it is recognized that diet is an important variable that affects the sensitivity of C. *dubia* to toxicants through a series of mechanisms that should be taken into consideration when running seven-day toxicity tests. It is of considerable importance that several diets that have been considered adequate for *C. dubia* produce quite different levels of sensitivity. Therefore, the selection of a diet should be a matter of extreme caution, and it should be made based not only on its effects on long-term culturing, but also on its possible effects on test results.

ACKNOWLEDGEMENTS. We extend our appreciation to Xochitl Tejeda-Cerda for her assistance in the laboratory.

LITERATURE CITED

APHA, American Water Works Association and Water Pollution Control Federation 1989 Standard Methods for the Examination of Water and Waste Water, 17th ed. American Public Health Association, Washington, DC.

Buikema, A. L., Jr., J. G. Geiger, and D. R. Lee 1980 *Daphnia* toxicity tests. *In*: A. L. Buikema, Jr. and J. Cairns, Jr. (eds.), Aquatic Invertebrate Bioassays, ATSM STP 715. American Society for Testing and Materials. pp. 48-69.

Chandini, T. 1989 Survival, growth and reproduction of *Daphnia carinata* (Crustacea: Cladocera) exposed to chronic cadmium stress at different food (*Chlorella*) levels. Environ. Pollut. 60: 29-45.

Cowgill, U. M., K. I. Keating, and I. T. Takahashi 1985a Fecundity and longevity of *Ceriodaphnia dubia/affinis* in relation to diet at two different temperatures. J. Crust. Biol. 5: 420-429.

____, H. M. Emmel, and I. T. Takahashi 1985b Inorganic chemical composition of trout food pellets and alfalfa used to sustain *Daphnia magna* straus. Bull. Environ. Contam. Toxicol. 43: 890-896.

DeGraeve, G. M. and J. D. Cooney 1987 *Ceriodaphnia*: An update on effluent toxicity testing and research needs. Environ. Toxicol. Chem. 6: 331-333.

Duncan, D. B. 1955 Multiple range and multiple F test. Biometrics 11: 1-44.

Dunnet, C. W. 1985 Multiple comparison procedure for comparing several treatments with a control. J. Amer. Statist. Assoc. 50: 1096-1121.

Finney, D. J. 1948 The Fisher-Yates test of significance in 2x2 contingency tables. Biometrika 35: 145-156.

____ 1971 Probit Analysis. Cambridge Univ. Press, London.

Horning, W. B., II and C. I. Weber, eds. 1985 Short-term methods for estimating the chronic toxicity of effluents and receiving waters to fresh water organisms. EPA/600/3-76/051. U.S. Environmental Protection Agency, Cincinnati, OH.

Knight, J. T. and W. T. Waller 1987 Incorporating *Daphnia magna* into the seven-day *Ceriodaphnia* effluent toxicity test method. Environ. Toxicol. Chem. 6: 635-645.

Mount, D. I. and T. J. Norberg 1984 A seven-day life cycle cladoceran toxicity test. Environ. Toxicol. Chem. 3: 425-434.

____ and T. J. Norberg-King, eds. 1985 Validity of effluent and ambient toxicity tests for predicting biological impact, Scippo Creek, Circleville, Ohio. EPA/600/3-85/044. U.S. Environmental Protection Agency, Duluth, MN.

____, A. E. Steen, and T. J. Norberg-King, eds. 1986 Validity of effluent and ambient toxicity tests for predicting biological impact, Back River Baltimore Harbor, Maryland. EPA/600/8-86/001. U.S. Environmental Protection Agency, Duluth, MN.

Murphy, J. S. 1970 A general method for the monoxenic cultivation of the Daphniidae. Biol. Bull. 139: 321-337.

Norberg, T. J. and D. I. Mount 1985 Diets for *Ceriodaphnia reticulata* life cycle tests. *In*: R. D. Cardwell, R. Purdy, and R. C. Bahner (eds.), Aquatic Toxicology and Hazard Assessment: 7th Symposium, ASTM STP 854. American Society for Testing and Materials, Philadelphia. pp. 42-52.

Ott, L. 1988 An Introduction to Statistical Methods and Data Analysis. Plus-Kent Publishing Co., Boston, MA.

U.S. EPA (U.S. Environmental Protection Agency) 1989 Short-term methods for estimating the chronic toxicity of effluents and receiving waters to fresh water organisms. EPA/600/4-89/001, Cincinnati, OH.

Winner, R. W. 1988 Evaluation of the relative sensitivities of 7-d *Daphnia magna* and *Ceriodaphnia dubia* toxicity tests for cadmium and sodium pentachlorophenate. Environ. Toxicol. Chem. 7: 153-159.

____ 1989 Multigenerational life-span tests of the nutritional adequacy of several diets and culture waters for *Ceriodaphnia dubia*. Environ. Toxicol. Chem. 8: 513-520.

____, T. Keeling, R. Yeager, and M. P. Farrell 1977 Effect of food type on the acute and chronic toxicity of copper to *Daphnia magna*. Freshwater Biol. 7: 343-349.