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Filtration and Phototactic Behavior as Indices of Chronic Copper Stress in *Daphnia magna* Straus

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Abstract. Filtration rate and negative phototactic behavior of *Daphnia magna* were evaluated as potential predictors of the chronic no-effect copper concentration. The effects of copper on filtration and phototactic behavior were compared to the effects of chronic copper exposure on survivorship, number of juvenile molts, age at reproductive maturity, and neonate body length. Animals exposed to copper concentrations ≥ 20 μg/L exhibited reductions in filtration rate, negative phototaxis, body length of neonates and survival time. Animals exposed to 10 μg/L exhibited a reduction in filtration rate, negative phototaxis and body length of neonates, but not in survival time. Number of juvenile molts, age at reproductive maturity, and mean brood size each responded erratically to copper exposure and are poor indices of copper stress. Since phototactic behavior, filtration rate, and neonate body length were all reduced at copper concentrations which did not reduce longevity or reproduction, bioassays in which only the latter are examined may underestimate the toxicity of copper and other chemicals. This may occur if bioassays are conducted for the frequently chosen 21-day interval.

Although daphnid bioassays are widely used to predict the toxicity of chemicals to aquatic organisms, only two indices of toxic stress have been routinely utilized, *i.e.*, reductions in longevity, and/or in fecundity (Buikema *et al.* 1980). Acute lethality tests with *Daphnia* are simple and inexpensive but do not adequately predict chronic effects. Chronic toxicity tests which use survivorship or reproduction as indices of stress may require months for completion (Winner and Farrell 1976). Recent work suggests that more rapidly derived indices, such as body length (Geiger *et al.* 1980; Winner 1981) and juvenile molting (Leonhard 1979) may adequately predict chronic toxic stress in *Daphnia* for certain chemicals. Behavioral changes have been used successfully as rapid and sensitive indicators of toxic stress in fish (Sprague *et al.* 1965; Bengtsson 1974; Besch *et al.* 1977), but little has been done to develop behavioral indices with *Daphnia*. Two aspects of daphnid behavior, phototactic response and filtration rate, are easily quantified and widely reported. A decrease in light intensity from above will cause daphnids to ascend and an increase will cause them to descend (Ringelberg 1964). The speed of this vertical movement correlates directly with the rate of change in intensity, or with the magnitude of an instantaneous change (McNaught and Hasler 1964; Ringelberg 1964). Although this movement may be recorded by direct observation it has not been utilized as an index of toxic stress. The rate at which daphnids move water through the carapace is known to vary widely according to environmental conditions, but rarely has been used as an index of toxic stress (Cooley 1977). The objectives of the present study were to evaluate the utility of negative phototaxis and filtration rate as indices of chronic copper stress by comparing them to more conventional indices such as changes in longevity, growth rate, and reproduction.

Methods and Materials

Conditions of Culture: All tests were initiated with newborn *Daphnia magna* (<24 hr old). Test animals were maintained individually in 40 ml of pond water (total alkalinity varied between 90 and 109 mg/L and total hardness between 93 and 104 mg/L) at various concentrations of copper added as $CuSO_4$: $5H_2O$. Waters were renewed every three days. The daily food ration consisted of two drops of a concentrate of vitamin-enriched *Chlamydomonas reinhardtii*. Culture conditions and food preparation were the same as described by Winner and Farrell (1976), except that the daily algal ration was 1/3 less in the present study. Animals were maintained on a 15-hr photoperiod at a temperature of 21 ± 3.5°C.

Acute Toxicity: In six acute tests, at least nine daphnids were reared at each of seven or more copper concentrations. Mortality

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at 72 hr was analyzed by probit analysis (Helwig and Council 1979).

Chronic Toxicity: Chronic test 1 consisted of cohorts of 11 animals continually exposed to 0, 10, 20, 30 and 40 μg Cu/L and was terminated after 88 days. Chronic test 2, consisting of cohorts of 12 animals exposed to 0, 10, and 20 μg Cu/L, was terminated after 41 days. The second test was to confirm the no-effect concentration identified in test 1. Replication of concentrations obviously above the no-effect level was unnecessary. Animals were examined daily for mortality, the presence of eggs in the brood chamber, exuviae and young. Upon examination, any exuviae or young were counted and removed. During each chronic test, body sizes of newborn young (<24 hr old) were determined for the first brood from each adult and for a second group of young collected during the fourth week. Young were preserved in sugar-formalin (Haney and Hall 1973). Broods within each treatment group were combined and at least 20 were randomly selected from each treatment for measurement. Carapace length was measured from the anteriormost part of the head to the base of the caudal spine; spine lengths were also measured. Carapace lengths and (in most cases) caudal spine lengths of animals exposed to various copper concentrations were measured after testing in phototactic or filtration experiments at ages of four days and six days, respectively. Survivorship curves based on daily mortality data were compared by the log-rank chi-square test from the SAS "Survtest" procedure (Reinhardt 1980). All other chronic indices were analyzed by ANOVA and Duncan's New Multiple Range tests, by the SAS "GLM" procedure (Helwig and Council 1979). Statements in the text relative to statistical significance refer to the 0.05 probability level unless stated otherwise.

Phototactic Experiments: The testing apparatus (Figure 1) consisted of a vertical, pond-water-filled glass tube, 125 cm in length, 6.7 mm in inner diameter, and graduated in centimeters. Four-day-old daphnids, 1.5 to 2.8 mm in total length, were able to swim within the tube, but significant movement was restricted to the vertical dimension. Two glass fittings near the top permitted the insertion, by pipet A, of a test organism, and the outflow of water, respectively. A vertical light gradient was created by a microscope lamp (American Optical Co., GE bulb 1493, 7 volts) shining downward through the water column. Light energy decreased exponentially from approximately 67 to 3.8 microeinsteins $m^{-2}sec^{-1}$ over the length of the water column. Water from a 2-L reservoir circulated upward through the tube by means of an electric pump. Water leaving the tube flowed into a 50-ml receiving vessel, from which test animals could be recovered by pipet B. The materials contacted by the circulating pond water were glass, rubber, Tygon, nylon, stainless steel, and silicone sealant. Several changes of water were circulated through the system before it was employed for testing purposes. Before each test, the system was rinsed with distilled and pond water. Behavioral testing was carried out in a darkened room (0.2–0.3 microeinsteins $m^{-2}sec^{-1}$), from 1 to 6 hr after the beginning of the photoperiod. Temperature-controlled facilities were not available. The temperature of the water in the testing apparatus varied among tests from 23 to 27°C and as much as 2°C during a test. Since acclimation reportedly takes place in 15 to 20 min (Smith and Baylor 1953), animals to be tested were removed from the environmental chamber and allowed at least 25 min to acclimate to light conditions in the testing room. However, beakers often had not reached room temperature at the time of the animal tests. Thus, animals were often subjected to a sudden change of 1 to 2°C, occasionally as high as 4 to 5°C, upon insertion in the testing

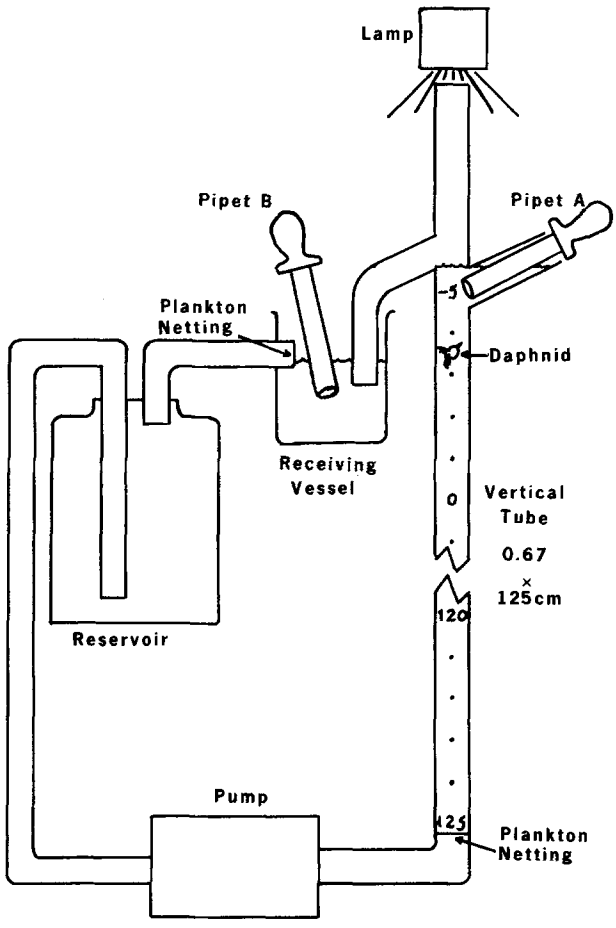

Fig. 1. Phototactic testing apparatus

apparatus. With the pump off, an acclimated daphnid was placed in the tube at a depth of '−5 cm' (Figure 1). Descent began once the animal became oriented to the light gradient within the tube (usually within 10 sec). Timing was started as the animal crossed a depth of '0 cm'. Thereafter, at intervals, depth in the tube was recorded to the nearest centimeter. Once recording was completed (no more than 90 sec), the pump was turned on to return the animal to the top of the tube for removal, and to circulate the water before testing the next individual. Less than three min was required to test each animal. Daphnids of different treatment groups were tested in alternation, to compensate for any changes in conditions. Six phototactic experiments were conducted with groups of from 13 to 25 four-day-old daphnids, including a control group and one or two groups which had been reared at concentrations of copper from 10 to 40 μg/L. Mean depth descended by copper-stressed animals was compared to depth of control animals by a t-test, at each timing interval.

Filtration Rate Measurement: Filtration rate was measured by the technique of Burky and Benjamin (1979), in which animals were allowed to clear a suspension of polyvinyl toluene (PVT) beads (Dow Diagnostics, Dow Chemical Co., Indianapolis, IN). Settling rates are very low because of the small diameter of the beads (2.02 μm) and their nearly neutral buoyancy (specific gravity of 1.027 at 25°C). Prior to each experiment, a 10 mg/L suspension of PVT was prepared with pond water which had been aerated for 24 hr at experimental temperatures and prefiltered through a Millipore filter (pore diameter 0.45 μm). Six ex-

periments were conducted with groups of six-day-old *Daphnia magna* reared at various concentrations of copper. From each group, 10 or 12 pairs of animals were transferred to 50-ml beakers containing 20 ml of filtered pond water, and allowed to acclimate to experimental conditions for 30 min. Twenty ml of the PVT suspension were added to each chamber resulting in a 40 ml, 5 mg PVT/L suspension; included were several control chambers without animals and two blank chambers containing only pond water. Daphnids were allowed to filter for 6 to 8 hr. At the end of each experiment, a 20-ml aliquot from each experimental, control, and blank chamber was dried at 95°C in a 40-ml Kimax glass centrifuge tube loosely covered with aluminum foil. PVT-laden feces were avoided by drawing each experimental aliquot from the top of the chamber. PVT in each tube was dissolved by agitating with 1.0 ml of dioxane, and optical density of the solution was determined at 267 nm with a spectrophotometer. Optical density of dioxane solutions of residues from the blank aliquots was used to correct for dissolved materials in the pond water. Filtration rates were calculated from the equation of Coughlan (1969):

$$F = \frac{V[\log_{10}(E_C - E_B) - \log_{10}(E_S - E_B)]}{\log_{10}(e \cdot t \cdot n)}$$

where:

F = filtration rate (ml animal^{-1}hr^{-1}), V = volume of suspension (ml), E_C = average absorption of the control chambers, E_S = average absorption of the experimental chambers, E_B = average absorption of the blank chambers, t = experimental time period (hr), e = the natural base of logarithms, n = number of animals per experimental chamber.

For each test, filtration rates were compared by a one-way analysis of covariance (ANCOVA) in which copper treatment was the factor having discrete levels. Since the body length of individuals varied, this was included as a covariate to control for its possible effects on filtration rates. When the ANCOVA f-statistic corresponding to 'treatment' was significant, filtration rates, adjusted for covariate, were compared with pairwise t-tests.

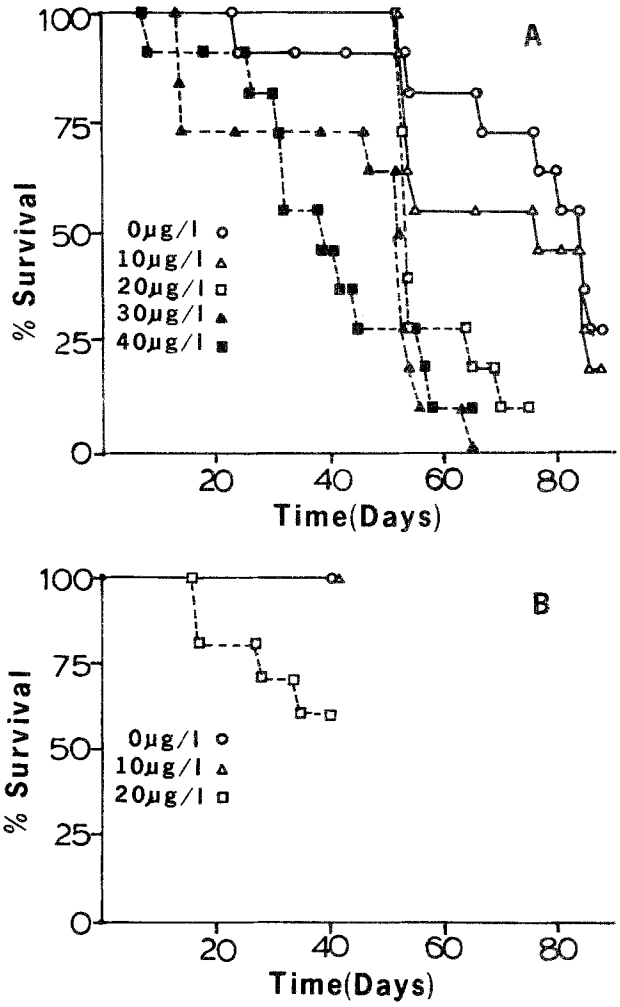

Fig. 2. Effect of copper exposure on survival in *Daphnia magna* in two chronic tests. Dashed line indicates that curve is significantly ($P \leq 0.05$) different from the control curve, by the logrank chi-square test

Results

Acute Toxicity: In six acute tests, performed over a period of five months, the 72-hrLC_{50} for copper varied from 44.5 to 68.7 µg/L.

Chronic Survivorship: Chronic test 1 was stopped on day 88, when control survival was 27% (Figure 2A). Chronic test 2 was stopped on day 41, before the occurrence of any control mortality (Figure 2B). In both chronic tests, chi-square analyses showed that survivorship curves for animals reared at 10 µg Cu/L were not significantly different from those of the controls, but curves for all groups reared at copper concentrations ≥20 µg/L were different from control curves.

Juvenile Molting and Reproductive Effects: In chronic test 1, daphnids exposed to 20, 30, or 40 µg Cu/L underwent a significantly greater number of molts before becoming reproductively mature, and showed a significant delay in maturation. In test 2, however, the number of juvenile molts was unaffected at 10 and 20 µg/L, and maturation was significantly accelerated at 20 µg/L (Table 1). Mean brood size was not affected by copper exposure in chronic test 1, but was significantly reduced at 20 µg/L in chronic test 2 (Table 2).

Growth Effects: When body sizes in animals reared for phototactic experiments were measured after four days of copper exposure, reduction in carapace length and increase in the ratio of caudal-spine length to carapace length (the 'spine ratio') were significant at copper concentrations of 30 to 40 µg/L, but neither effect occurred consistently at lower concentrations. With six-day-old animals reared for filtration experiments, body lengths and

Table 1. Mean age at reproductive maturity and mean number of juvenile molts for *Daphnia magna* exposed to selected copper concentrations. Mean ages and number of molts within a test not sharing the same letter are significantly different ($P \leq 0.05$) as determined by Duncan's new multiple range test. Sample size appears in parentheses below each mean, ± standard deviation

Copper (μg/L)	Age at reproductive maturity (days) ± SD		Number of juvenile molts ± SD	
	Test 1	Test 2	Test 1	Test 2
0	A 7.27 ± .90 (11)	A 7.21 ± .84 (12)	A 4.82 ± .60 (11)	A 4.42 ± .51 (12)
10	A 8.18 ± .75 (11)	A 7.54 ± .75 (12)	AB 5.18 ± .60 (11)	A 4.58 ± .51 (12)
20	B 9.91 ± 1.45 (11)	B 6.50 ± .71 (12)	B 5.73 ± .65 (11)	A 4.33 ± .49 (12)
30	C 13.88 ± 2.17 (8)		C 6.65 ± 1.51 (8)	
40	C 15.10 ± 2.60 (10)		D 7.70 ± .95 (10)	

Table 2. Mean brood sizes of *Daphnia magna* chronically exposed to selected copper concentrations. Mean brood sizes within a test not sharing the same letter are significantly different ($P \leq .05$) as determined by Duncan's new multiple range test. Sample size appears in parentheses below each mean, ± standard deviation

Copper (μg/L)	Chronic test 1	Chronic test 2
0	A 5.74 ± 2.41 (194)	A 5.77 ± 2.09 (106)
10	A 6.03 ± 2.57 (184)	AB 5.30 ± 2.29 (104)
20	A 5.77 ± 2.30 (142)	B 4.91 ± 2.39 (82)
30	A 6.03 ± 2.50 (93)	
40	A 5.43 ± 2.52 (79)	

spine ratios were always significantly different from controls at concentrations of 20 μg/L or greater (Table 3). Spine ratios of newborn young in chronic tests did not show any consistent relationship to copper exposure level. However, copper concentrations $\geq$10 μg/L caused a highly significant ($P \leq .01$) reduction in carapace length of newborn from the first brood, and of newborn taken during the fourth week of chronic tests (Table 4). The reduction in size of first-brood young could not be attributed entirely to age differences of adults at the time of first reproduction, since the fourth-week broods were collected from adults of the same ages at all concentrations, and since reproduction was not significantly delayed at 20 μg/L in test 2, nor at 10 μg/L in either test (Table 1).

Phototactic Experiments: In six experiments, exposure to copper concentrations varying from 10 μg/L to 40 μg/L tended to reduce the distance descended

Table 3. The effect of chronic copper toxicity on mean carapace length and the caudal spine:carapace length ratio of six-day-old *Daphnia magna* used in filtration experiments. Mean carapace lengths and ratios within a test not sharing the same letter are significantly different ($P \leq 0.05$) as determined by Duncan's new multiple range test

Test	Copper (μg/L)	N[a]	Mean carapace length (mm) ± S.D.	Mean spine:carapace length ratio ± S.D.
1	0	20	A 2.395 ± .191	—
	40	20	B 2.161 ± .210	—
3	0	20	A 1.850 ± .208	A 0.253 ± .034
	20	20	B 1.650 ± .230	B 0.308 ± .055
4	0	24	A 2.037 ± .161	A 0.240 ± .033
	20	24	B 1.879 ± .159	B 0.276 ± .024
	30	24	C 1.675 ± .112	C 0.297 ± .024
	40	24	D 1.463 ± .114	D 0.324 ± .031
5	0	24	A 2.016 ± .152	A 0.247 ± .045
	20	24	B 1.895 ± .114	B 0.272 ± .031
	30	24	C 1.714 ± .159	B 0.287 ± .045
	40	24	D 1.579 ± .131	C 0.320 ± .016
6	0	20	A 2.083 ± .083	A 0.266 ± .032
	10	20	A 2.050 ± .154	A 0.278 ± .032
	20	20	B 1.930 ± .052	B 0.292 ± .012

[a] Number of animals tested

by four-day-old *D. magna* exposed to overhead illumination (Figure 3). In most tests, the reduction in phototactic response was significant at some, but not all of the times at which depth was recorded. In each test in which a significant reduction was found, the effect was shown to be significant within the first 45 sec of the test, which indicates that the duration of the test could have been less. Therefore, data collected during the first 45 sec of each test were analyzed by two-factor repeated-measures analyses of variance. Each copper-stressed group was separately compared to controls. In all cases but one, copper exposure significantly ($P \geq 0.0001$) reduced phototactic response. Behavior of the 20 μg/L group

Table 4. Mean body length of newborn from the first and subsequent broods of *Daphnia magna* chronically exposed to selected copper concentrations. Mean body lengths within a test not sharing the same letter are highly significantly different ($P \leq 0.01$) as determined by Duncan's new multiple range test. Sample size appears in parentheses below each mean, ± standard deviation

Copper (μg/L)	Mean body length of newborn Daphnia (mm)			
	First brood of females		Fourth-week brood of females	
	Chronic test 1	Chronic test 2	Chronic test 1	Chronic test 2
0	A 0.974 ± .045 (32)	A 1.011 ± .059 (30)	A 1.155 ± .030 (36)	A 1.148 ± .054 (24)
10	B 0.905 ± .040 (36)	B 0.972 ± .056 (36)	B 1.121 ± .049 (51)	B 1.078 ± .056 (25)
20	BC 0.880 ± .043 (23)	B 0.945 ± .040 (33)	B 1.111 ± .037 (36)	C 1.035 ± .029 (23)
30	C 0.866 ± .061 (27)		C 1.048 ± .055 (36)	
40	BC 0.877 ± .065 (29)		D 0.988 ± .039 (41)	

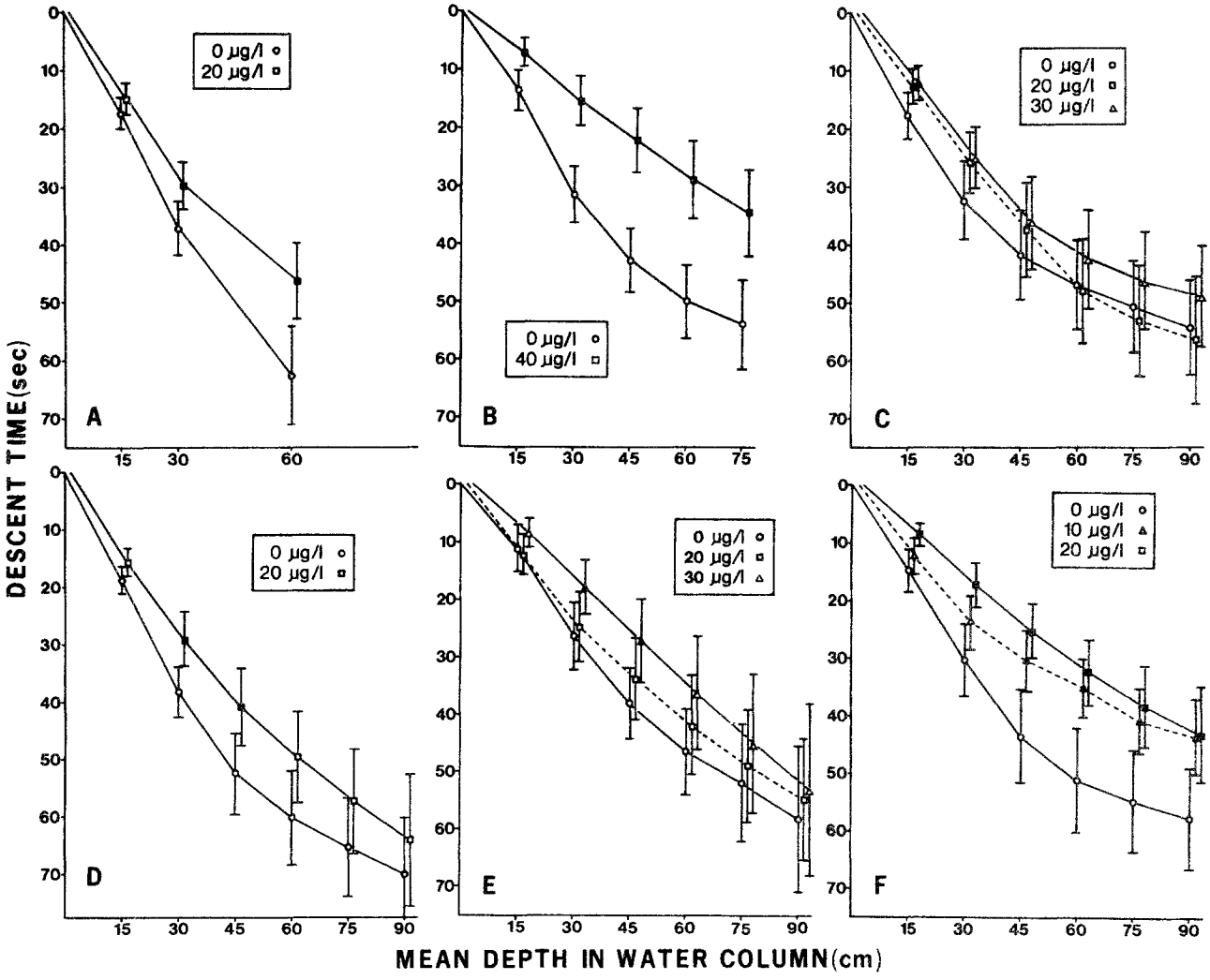

Fig. 3. Effect of copper exposure on phototactic locomotion in four-day-old *Daphnia magna*. Mean depth in water column at each of 3 to 6 descent times. Ninety-five % confidence limits around each mean are shown. Shaded symbols at each time point indicate significant difference from the controls as determined by *t*-test. Copper means are offset to the right for clarity. A. (n = 25); B. (n = 18); C. (n = 13); D. (n = 18); E. (n = 25); F. (n = 25)

Table 5. The effect of chronic copper exposure on the filtration behavior of six-day-old *Daphnia magna*. Filtration rate means within a test not sharing the same letter are significantly different ($P \leq .05$) as determined by Duncan's new multiple range test. Filtration rate means adjusted for body length were generated by an analysis of covariance. Adjusted means within a test not sharing the same letter are significantly different as determined by *t*-test analysis

Test	Copper (μg/L)	N[a]	Mean filtration rate ml animal^{-1} hr^{-1} ± S.D.	Filtration rate adjusted means
1	0	10	A 1.324 ± 0.184	A 1.163
	40	10	A 1.080 ± 0.373	A 1.224
2	0	10	A 1.027 ± 0.114	—
	40	10	A 0.735 ± 0.326	—
3	0	10	A 0.968 ± 0.388	A 0.959
	20	10	B 0.553 ± 0.258	B 0.563
4	0	12	A 3.814 ± 1.300	A 3.272
	20	12	B 0.933 ± 0.539	B 0.729
	30	12	B 0.335 ± 0.200	B 0.567
	40	12	B 0.322 ± 0.308	B 1.007
5	0	12	A 6.718 ± 0.309	A 6.329
	20	12	B 5.340 ± 0.714	B 5.214
	30	12	BC 5.232 ± 0.405	B 5.349
	40	12	C 4.736 ± 0.929	B 5.035
6	0	10	A 3.335 ± 0.658	A 3.450
	10	10	B 2.627 ± 0.699	B 2.682
	20	10	B 2.450 ± 0.774	B 2.287

[a] Number of pairs of animals at each treatment level

in test 3 was not significantly different ($P > 0.05$) from the control, perhaps due to the small sample size.

Since copper exposure was found to reduce body size of animals in several of the tests, it might be argued that reductions in rate of descent may result from this size reduction, rather than from a physiologically based alteration in behavior. This hypothesis was tested by repeating the two-factor ANOVA's, including individual measurements of carapace length and spine ratio as covariates. In test 2, addition of these covariates resulted in a loss of significance of the behavioral difference at 40 μg Cu/L. Therefore, the difference observed (Figure 3B) could indeed be explained as a result of size differences, which were extreme at this concentration (mean carapace length was reduced 44%, and mean spine ratio was increased 45% with respect to control). In all other tests, however, lower copper concentrations were used, and these size effects either were slight (<12% at 30 μg Cu/L) or were not significant (as at 10 and 20 μg Cu/L). In these tests, addition of the covariates did not reduce significance of the behavioral difference.

Filtration Experiments: Copper-induced alterations in filtration rate of six-day-old *D. magna* were not observed in tests 1 or 2 (Table 5). In the remainder of the experiments, a significant depression of filtration rate occurred at all treatment levels (Table 5). While there was a trend toward depressed filtration rates at increased toxicant concentrations, the rate observed at any given treatment level was highly variable from test to test. The lack of any measurable effect on filtration rate in the first two tests at copper concentrations considerably above those which had an effect in subsequent tests cannot be explained with the available data. Since a significant decrease in body size was observed in all tests and at all treatment levels except 10 μg Cu/L (Table 3), the possibility that the differences in filtration rate were due to differences in body size was tested using an ANCOVA with carapace length as the covariate. Mean filtration rates 'adjusted' for the effect of body length were compared using pair-wise *t*-tests. Adjusted means were not calculated for test 2 since body lengths were not measured in that test. The results (Table 5) indicate that, as true for negative phototaxis, the effects of copper on filtration rate are not due to an indirect effect of copper on body size.

Discussion

Daphnid toxicity tests usually rely on reductions in longevity and/or in reproduction as indices of harmful concentrations of toxic chemicals. However, in our experiments, reproduction was not reduced at copper concentrations which had a significant effect on negative phototaxis, body length of newborn offspring, and usually on filtration rate. Furthermore, concentrations which affected survival only after an exposure of 20 to 50 days, had an

effect on body length, filtration rate and negative phototaxis which could be detected within four to six days. Finally, in the few experiments conducted at 10 μg/L, body length of newborn, filtration rate and negative phototaxis were all significantly reduced even though there was no effect on longevity or reproduction.

Since significant changes in filtration rate, phototactic behavior, or growth would likely reduce a species ability to cope with the abiotic and biotic complexities of aquatic ecosystems, bioassays which evaluate only survival and/or reproductive responses may underestimate the toxic effects of some chemicals. This is particularly probable if the test duration is too short to detect significant reductions in life span. For copper, our data and those of Winner and Farrell (1976) show that the conventional 21-day chronic test will not detect concentrations which significantly reduce longevity. Winner (1981) has presented comparable evidence for zinc. It is suggested that, if the goal of toxicity evaluations is to predict concentrations of chemicals which will not adversely affect species in a complex, natural environment, toxicologists should reconsider the common practice of using only reductions in survival and reproduction over a 21-day interval as indices of significant toxic stress.

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