Pergamon

Environmental Toxicology and Chemistry, Vol 14, No. 12, pp. 2139–2147, 1995
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Printed in the USA
0730-7268/95 $9.50 + .00

0730-7268(95)00168-9

INFLUENCE OF DEVELOPMENTAL STAGE, SALTS AND FOOD PRESENCE ON VARIOUS END POINTS USING *CAENORHABDITIS ELEGANS* FOR AQUATIC TOXICITY TESTING

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(*Received* 10 *January* 1995; *Accepted* 1 *June* 1995)

Abstract — This study used a randomized block design to investigate the importance of several variables in using the free-living soil nematode *Caenorhabditis elegans* for aquatic toxicity testing. Concentration–response data were obtained on nematodes of various developmental stages exposed to four metals (Cd, Pb, Cu, and Hg) and a water-soluble organic toxicant, sodium pentachlorophenate (PCP), under conditions of varied solvent medium (with or without salts and with or without a bacterial food source). The end points measured were 24- and 96-h mortality LC50 value, as well as development of larval stages to adulthood and evidence of reproduction. The results suggest that nematodes of various ages respond similarly to a given toxicant for all end points measured, although adults cultured from eggs appeared more sensitive than adults cultured from dauer larvae. The most important environmental variable in determining toxicity was the medium in which the tests were conducted. The presence of potassium and sodium salts in the medium significantly ($p < 0.05$) reduced the toxicity of many test samples. The presence of bacteria had little effect on 24-h tests with salts, but was important in 96-h survival and development. Based on sensitivity and ease of handling, adults cultured from eggs are recommended in both 24-h and 96-h tests.

Keywords — *Caenorhabditis elegans* Development Metals PCP Toxicity

INTRODUCTION

The free-living nematode *Caenorhabditis elegans* has previously been proposed as an aquatic toxicity test organism [1,2], the potential of which has since been investigated and discussed by others [3–5]. The organism is inexpensive and easy to maintain and holds a unique place among the invertebrates useful in aquatic ecotoxicological surveys for several reasons. First, it is a member of the phylum Nematoda, a ubiquitous, diverse, and ecologically important group of animals inhabiting both freshwater and marine environments, as well as sediments and soils [6–9]. Despite this, nematodes are unrepresented in standardized ecotoxicity testing protocols [10–15].

Second, although *C. elegans* is itself a soil inhabitant, it can be cultured and tested in aquatic media spanning a wide range of conditions such as pH and osmolarity. This makes it an ideal organism for investigating the effects of variable environmental conditions on the resulting toxicity of chemicals in aqueous solution. In addition, it is increasingly apparent that aquatic test data are applicable to the study of soil contaminants and their availability to indigenous invertebrates as the main route of exposure to soil toxicants is probably through the interstitial liquid [16–18].

Finally, *C. elegans* is one of the most extensively studied laboratory metazoans in existence, and the mechanisms underlying its biology are extremely well characterized [19,20]. As a result, it has the potential to allow not only quantification of responses to toxic stress but also elucidation of the molecular and genetic mechanisms behind those responses [4,5,21–23].

However, standard procedures and conditions for aquatic toxicity testing using *C. elegans* have yet to be established, and this lack of protocol may be responsible for differential results of toxicity tests performed by various laboratories [2–4]. Standard laboratory culture techniques and experimental methods for *C. elegans* have been established and are universally followed [24,25], but these methods may not be appropriate for assessing subtle toxic responses, which may be influenced by a variety of factors. For instance, standard *C. elegans* culture medium (M9) was found to precipitate some metals and thus was unsuitable as a vehicle for testing metals [2,26]. Likewise, some evidence indicates that salt concentrations in aquatic tests and bacterial presence in soil tests influence the toxic response of *C. elegans* to copper [27].

Both the presence of salts and a food source [28] have been found to influence the results of toxicity tests using other invertebrates. Likewise, a difference in sensitivity to toxicants has been observed among various developmental stages of some invertebrates [29]. These types of factors are considered in the design of existing aquatic toxicity tests with invertebrates. This study investigated the importance of such factors in determining the responses of *C. elegans* to various toxicants in aquatic tests. This is a first step toward the goal of identifying suitable test conditions that will be necessary for a standard *C. elegans* aquatic test protocol. Such a standardized test would allow for the use of a representative nematode species in aquatic toxicity testing.

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MATERIALS AND METHODS

Maintenance and age synchronization of nematodes

A wild-type N2 strain, var. Bristol, of *Caenorhabditis elegans* was maintained as dauer larvae stocks in M9 buffer, replenished monthly [30]. Development of dauer larvae into self-fertilizing adult hermaphrodites was initiated on K-agar plates [26] with lawns of *Escherichia coli* strain OP50 as a food source [24]. After several days, these plates contained many gravid adults from which fertile eggs were obtained by treating with a mild bleaching solution of 1% NaClO and 0.013 M NaOH [31].

The eggs were washed by centrifugation in K-medium [2] and divided among three Pyrex® petri dishes containing K-medium with no food source. The dish designated to produce synchronized adult worms for the toxicity tests immediately received a food source in the form of *E. coli* strain OP50. This was done by centrifuging a volume of saturated L-broth (3.0 g beef extract, 5.0 g peptone, 5.0 g lactose per liter) culture equal to twice that of the volume of liquid in the petri dish, washing the pellet in K-medium, and adding it to the dish containing the eggs. All three dishes were incubated at 20°C overnight to allow the eggs to hatch. In the presence of food, *C. elegans* normally develops through four larval stages (L1–L4), separated by periods of molting during which the old cuticle is shed and the worm increases in size, resulting in the sexually mature adult hermaphrodite stage. Eggs hatched in the dishes without bacteria produced worms that remained arrested in the L1 stage until a food source was provided. Those in the dish with bacteria produced worms that followed normal and synchronous development through all four larval stages and into adulthood.

The remaining two dishes were designated to produce either L2–L3-stage worms or L4-stage worms, and bacteria were added to each of these at times calculated to produce synchronized populations of the age desired for the test. These times were determined by following the development of worm populations during trial runs, and using previously determined developmental timing schedules [32,33] as guidelines. These studies found that, when hatched in M9 buffer at 20°C in the presence of food, worms developed to the L3 stage after 22 to 25 h, to the L4 stage after 30 to 36 h, and to the adult stage after 42 h or more. We found similar results with eggs hatched in K-medium. Developmental stage was determined visually, and L2- and L3-stage worms were considered as one group because of the difficulty of visually distinguishing the two. In addition to the three developmental stages produced from eggs, a fourth group consisting of adults produced from the dauer larva stage was cultured by adding the larvae to K-medium with food about 30 h prior to beginning the test.

Experimental design and test conditions

A two-factor randomized complete block design was used in which the factors were developmental stage of the nematodes tested (L2–L3, L4, adults from egg, and adults from dauer) and test medium (K-medium, with and without bacteria, and deionized water, with and without bacteria). Monitored end points were 24-h and 96-h mortality, development of larval stage nematodes to adulthood after 96 h, and evidence of reproduction determined by the presence of offspring after 96 h. The health of the organisms was determined by the use of a vehicle (i.e., deionized water, K-medium) control.

Aquatic toxicity tests were performed in 24-well tissue culture plates (Falcon 3047). The metal salts $CdCl_2$, $Pb(NO_3)_2$, $CuCl_2 \cdot 2H_2O$, and $HgCl_2$ and deionized water were used to mix fresh stock solutions based on concentration of metal ion for each test. Sodium pentachlorophenate (PCP) was similarly prepared in stock solutions of 1,000 mg/L. Separate stock solutions and tenfold dilutions from these were mixed in either K-medium or deionized water, with and without bacteria. Simultaneous controls were prepared for each medium without toxicant.

For solutions containing bacteria, a 200-ml volume of OP50-saturated L-broth was centrifuged, and the pellet resuspended in K-medium or deionized water, centrifuged again, and resuspended in a volume of K-medium or deionized water equal to half that of the L-broth originally centrifuged. This solution of K-medium or water plus bacteria was then used for making stock solutions and dilutions. The pH of each solution was measured with a Corning model 10 pH meter (Corning Co., Corning, NY), calibrated with Fisher certified buffers (Fisher Scientific, Pittsburgh, PA), and was not adjusted. However, to control for possible toxic effects from pH extremes alone, test samples were run with the various media adjusted by addition of HCl or NaOH to reflect the range of pH encountered in the toxicant solutions.

Nematodes were transferred from the four synchronized populations to 500-μl volumes of the test solutions in the culture plate wells. Nematodes were first washed by centrifugation in K-medium to remove bacteria, then at least four nematodes were pipetted in 5-μl volumes into each well. Counts were made after transfer to verify the number of nematodes present. Samples were kept covered in the dark at 20°C for a total of 96 h. Visual inspections were made under a dissecting microscope after 24 and 96 h to assess the end points described above. Death was determined as a lack of response to gentle probing. Dead nematodes were not removed.

Nematodes that were in the larval stage were assessed for normal development after 96 h by noting whether they had progressed to adulthood. Reproduction was assessed by noting the presence of offspring without counting them. (It should be noted that by scoring only the presence or absence of offspring, we were only asking of the reproduction test that it indicate normal development to a reproducing adult. A goal of a future study should include the actual counting of the number of offspring.)

Statistical analysis

All tests were replicated five times, with at least four nematodes per well and five wells per replicate test. The purpose of this study was not to determine LC50 values precisely, but rather to compare responses to defined toxicant concentrations among a variety of parameters. The LC50 estimates were located within a range defined by two neighboring toxicant concentrations whose responses bracketed the 50% survival concentration.

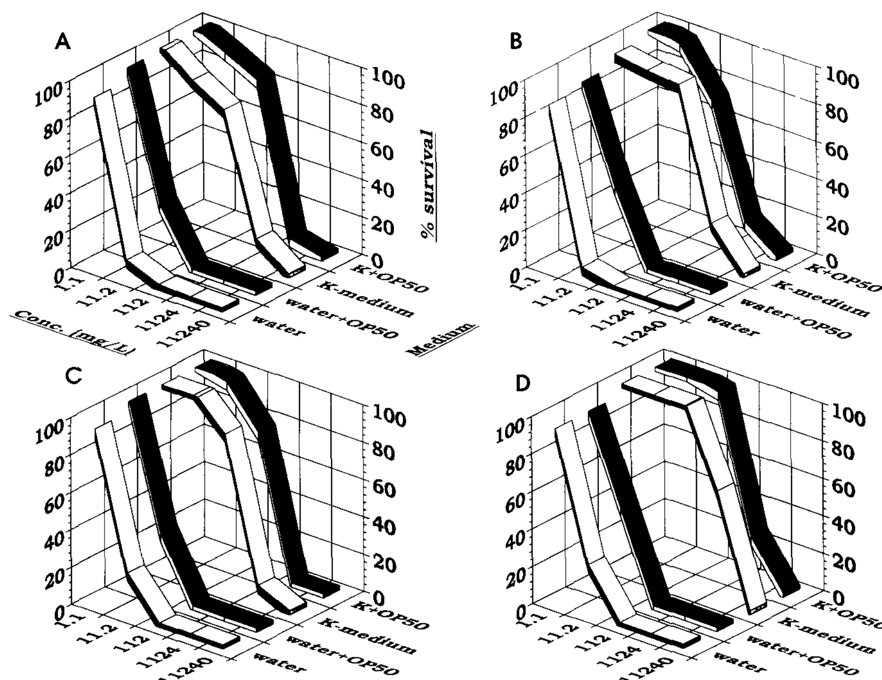

Fig. 1. Concentration-response for 24-h exposure to Cd. Bars are mean percent survival of five replicates for nematodes exposed to various concentrations of Cd (mg/L) in each of four media. (**A**) L2–L3 worms; (**B**) L4 worms; (**C**) adults cultured from eggs; (**D**) adults cultured from dauer larvae. All axes for **A**–**D** are the same as those labeled in **A**. Only data for which control survival was >90% are shown.

A two-way factorial analysis of variance (ANOVA) was performed by comparing the survival means within the entire range of concentrations producing lethality for each toxicant under the various conditions. Because the same concentrations were used for all tests with the same toxicant, it was possible to assess individual and interactive effects of test medium and nematode developmental stage on the lethality resulting from those concentrations. Differences among means were determined by Duncan's multiple range test. All procedures were performed with the SAS software [34].

RESULTS

Mortality (24 h)

The 24-h survival plots are shown in Figures 1 to 5 (control survival was >90%) for each toxicant–medium–developmental stage combination. All concurrent control tests had less than 10% mortality, in accordance with ASTM standards for invertebrate tests [10,11]. The K-medium generally had a pH between 5.5 and 6.0, and it was found that addition of metal salts to both K-medium and water resulted in a variety of pH changes between 4.0 and 10.0. The potential effect of these pH changes on mortality was tested in controls containing only K-medium or water with pH adjusted (between 4.0 and 10.0) to include this range. These samples exhibited less than 10% mortality after 24 h (not shown).

The LC50 values for Cd in K-medium with and without OP50 bacteria were generally between 112 and 1,124 mg/L, and LC50 values for Cd in water, with and without bacteria, were between 1.1 and 11 mg/L. Copper showed similar trends at 24 h, with the LC50 values between 6.4 and 63.5 mg/L in K-medium, and between 0.6 and 6.4 mg/L in water. Again, no great differences among developmental stages were apparent. The LC50 values for Pb were between 2.1 and 207 mg/L in K-medium, and consistently between 2.1 and 20.7 mg/L in water.

In contrast, the toxicities of Hg and PCP were not as greatly influenced by the medium. The LC50 value for Hg was generally between 2 and 20 mg/L, and for PCP between 100 and 1,000 mg/L.

Mortality (96 h)

Results of 96-h exposures were more erratic, and no clear trends among media or developmental stages were apparent. The only control samples having at least 90% survival were those with both K-medium and OP50 bacteria (not shown). The low survival discounts the other test media for use in chronic 96-h exposure tests, and only plots for the various toxicants in K-medium and OP50 (with control survival >90%) are shown (Fig. 6).

Influence of developmental stage and medium

Figures 1 through 6 allow a quick assessment of general trends in toxic responses to be made. An overall pattern of reduced toxicity in K-medium and K-medium with OP50 compared to water and water with OP50 is apparent with Cd, Cu, and Pb. The toxicities of Hg and PCP were not as strongly influenced by the medium. Likewise, developmental stage of the nematodes did not seem to matter greatly in the pattern of responses.

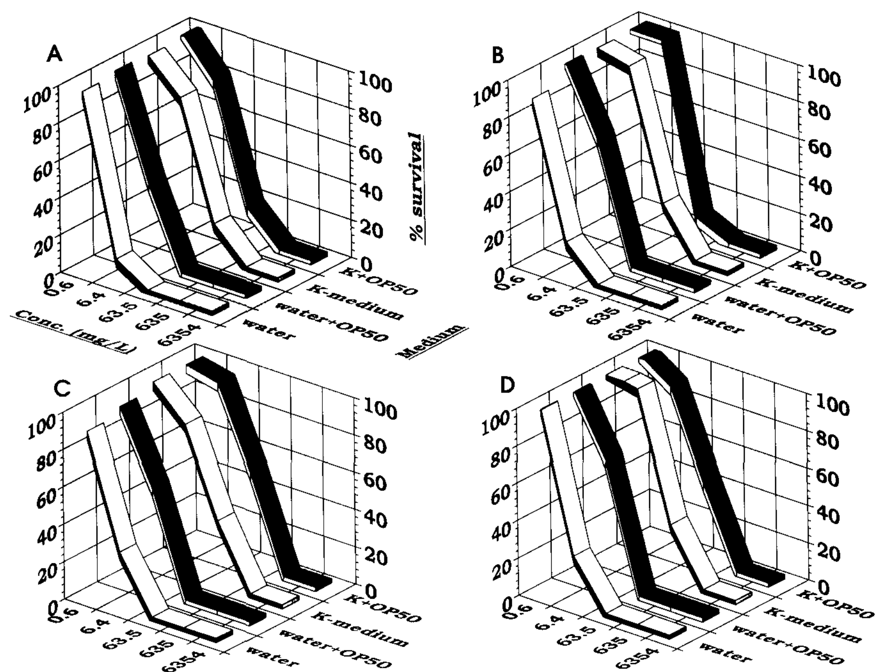

Fig. 2. Concentration-response for 24-h exposure to Cu. Bars are mean percent survival of five replicates for nematodes exposed to various concentrations of Cu (mg/L) in each of four media. (**A**) L2–L3 worms; (**B**) L4 worms; (**C**) adults cultured from eggs; (**D**) adults cultured from dauer larvae. All axes for **A**–**D** are the same as those labeled in **A**. Only data for which control survival was >90% are shown.

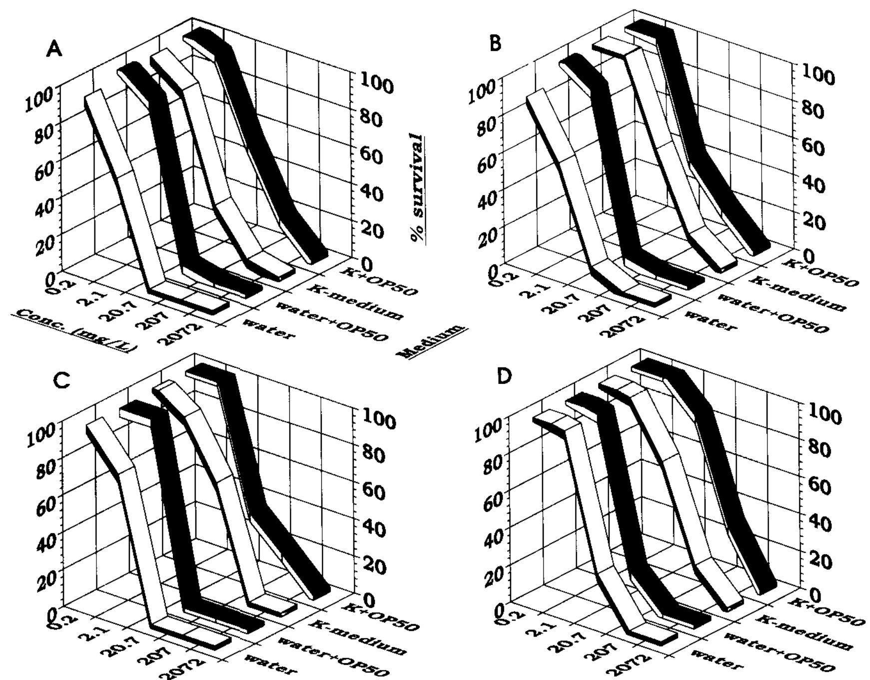

Fig. 3. Concentration-response for 24-h exposure to Pb. Bars are mean percent survival of five replicates for nematodes exposed to various concentrations of Pb (mg/L) in each of four media. (**A**) L2–L3 worms; (**B**) L4 worms; (**C**) adults cultured from eggs; (**D**) adults cultured from dauer larvae. All axes for **A**–**D** are the same as those labeled in **A**. Only data for which control survival was >90% are shown.

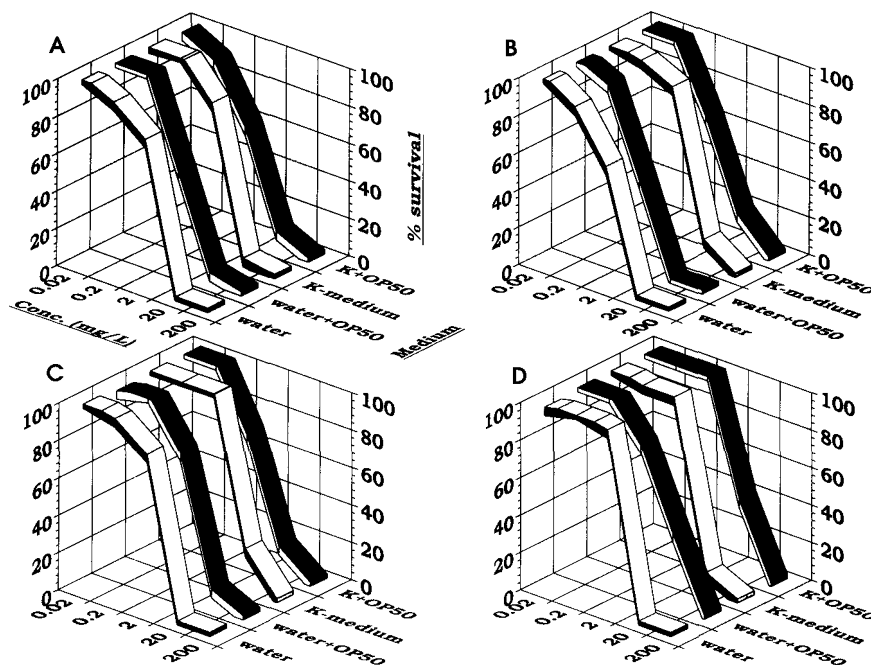

Fig. 4. Concentration-response for 24-h exposure to Hg. Bars are mean percent survival of five replicates for nematodes exposed to various concentrations of Hg (mg/L) in each of four media. (**A**) L2–L3 worms; (**B**) L4 worms; (**C**) adults cultured from eggs; (**D**) adults cultured from dauer larvae. All axes for **A–D** are the same as those labeled in **A** Only data for which control survival was >90% are shown.

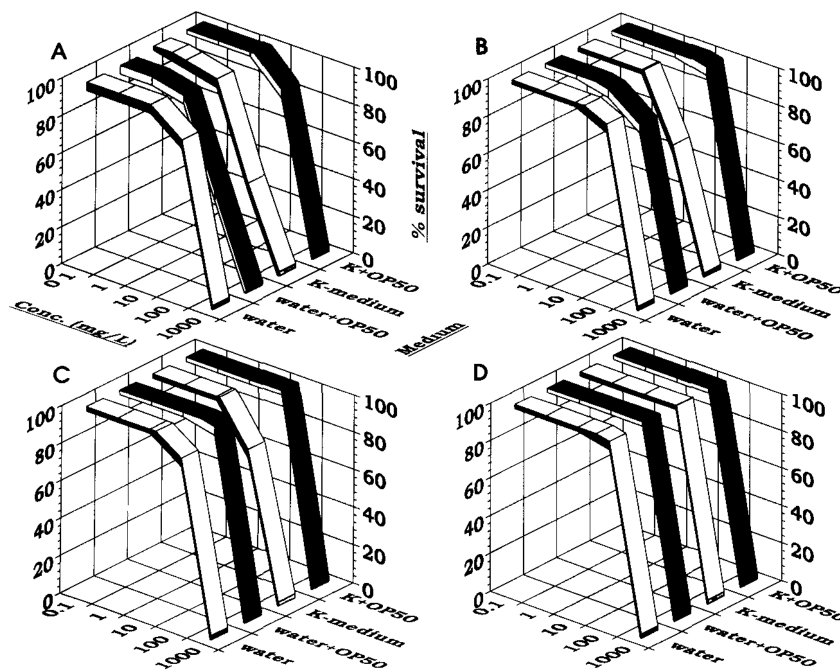

Fig. 5. Concentration-response for 24-h exposure to PCP. Bars are mean percent survival of five replicates for nematodes exposed to various concentrations of PCP (mg/L) in each of four media. (**A**) L2–L3 worms; (**B**) L4 worms; (**C**) adults cultured from eggs; (**D**) adults cultured from dauer larvae. All axes for **A–D** are the same as those labeled in **A**. Only data for which control survival was >90% are shown.

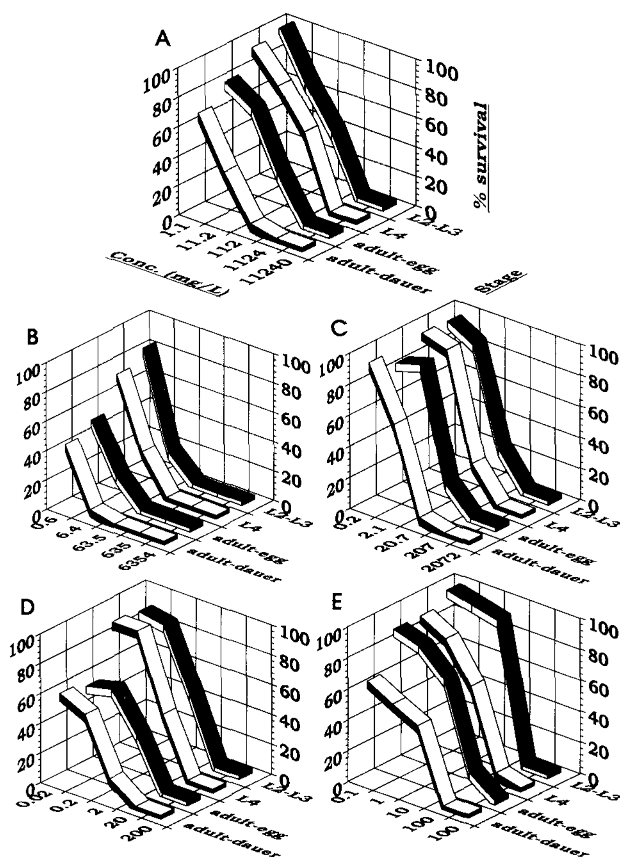

Fig. 6. Concentration-response for 96-h exposure to various toxicants in K-medium with OP50 bacteria. Bars are mean percent survival of five replicates for nematodes exposed to various concentrations of toxicant (mg/L) at each of four developmental stages. (**A**) Cd; (**B**) Cu; (**C**) Pb; (**D**) Hg; (**E**) PCP. All axes for **A–E** are the same as those labeled in **A**. Only data for which control survival was >90% are shown.

Table 1 shows the differences among means of percent survival for 24-h exposures as determined by two-way ANOVA and Duncan's multiple-range test. A similar analysis was done to test for differences among replicates, and no significant variation among means was found. The exposure medium was a significant factor ($p < 0.05$ for all) in determining the toxicity in tests with Cd, Cu, and Pb. With regard to the sensitivities to these toxicants, nematode developmental stage tended to be a less important factor, with larval stages exhibiting responses similar to adults cultured from eggs. However, adults cultured from dauer larvae were significantly ($p < 0.05$) less sensitive than were the other stages in all 24-h lethality tests. A possible reason for this is discussed below.

The toxicity of Hg was less dependent on the exposure medium (Table 1), although K-medium did provide a slight increase in survival in 24-h tests (Fig. 4). Nematode developmental stages appear to have an effect on survival, with larval stages exhibiting a lower tolerance in 24-h tests (Table 1). These same parameters showed no consistent pattern of effect on the toxicity of PCP.

Two chronic end points – development of larvae into adults and reproduction as evidenced by the presence of offspring – are shown in Table 2. This table shows the results for both larval stages, in which the end point was development to adulthood and reproduction (which always occurred together), and adult stages, where reproduction was the only measured end point. Only the results from tests in K-medium with OP50 are shown, since other media had high control mortality. Reproduction in adults cultured from dauer larvae appeared to be uninhibited up to very high toxicant concentrations as compared with adults cultured from eggs. It should be noted, however, that mortality in adults cultured from dauer larvae was usually higher by 96 h (Fig. 6); consequently, much of the reproduction of this group was in samples in which the adults had long since died. It should be noted that fertilized eggs within dead adults may continue to develop and hatch internally, thus producing live offspring that can burrow their way out of the carcass.

DISCUSSION

Toxicity end points in *C. elegans*, measured in response to various chemical stressors, include mortality assessed on agar plates [1,26,35,36], in aqueous solution (1–5,27], and in soils [27,37,38], as well as changes in ultrastructure [39], genome [40], rate and direction of movement [1,35,36,38, 41,42], feeding rate [23], stress protein production [4,5,21,22], fecundity [1,36,43,44], and development [44,45]. Such versatility allows for the simultaneous study of several actions of toxic chemicals on aquatic invertebrates. Another free-living nematode, *Panagrellus redivivus*, has been proposed as an aquatic toxicity test organism [46,47]. However, *C. elegans* offers the advantage of being extremely well-characterized at all levels of morphology, physiology, and genetics. Thus, the potential exists for elucidating the mechanisms of subtle toxicity responses in conjunction with performing rapid bioassessments in the field.

Results from this study are compared to results from similar studies using *C. elegans*. Williams and Dusenbery [2] found LC50 values of 904, 22, 129, and 10 mg/L for Cd, Cu, Pb, and Hg, respectively, for 24-h exposures in K-medium with bacteria, using adults cultured from dauer larvae. These values fall within the ranges we obtained using similar conditions (Figs. 1–4). The range of lethality for PCP in Figure 5 is consistent with the study by Kammenga et al. [3], who tested pentachlorophenol with *C. elegans* only up to a concentration of about 10 mg/L in a low-salt medium and found no lethality.

Stringham and Candido [4] reported 24-h LC50 values of about 21 mg/L for Cd, 13.5 mg/L for Cu, and 13 mg/L for Hg. These were based on an assay in which nematodes in the L2 or L3 stage were exposed in solutions made with distilled water and a bacterial food source. The LC50 value for Cd varied dramatically from that in Williams and Dusenbery [2], and the value for Cu to a lesser degree, but these values were close to the LC50 values we obtained for L2 or L3 larvae in water with OP50 (Figs. 1,2, and 4). Stringham and Candido

Table 1. Results of Duncan's multiple-range test for mean 24-h percent survival (determined over the same concentration range within each toxicant grouping) by test medium and nematode developmental stage

	Cd	Cu	Pb	Hg	PCP
Medium					
K-medium	61.0 A	41.9 A	50.0 A	59.9 A	73.3 A
K-medium + OP50	57.5 A	43.5 A	53.5 A	57.4 A,B	79.0 B
Water	20.8 B	22.7 B	34.6 B	54.5 B	77.3 B,C
Water + OP50	26.1 C	30.6 C	39.3 C	53.8 B	75.2 A,C
Nematode stage					
L2–L3	38.9 A	31.6 A	40.3 A	52.1 A	70.6 A
L4	39.4 A	34.5 A,B	43.0 A	53.2 A	76.5 B
Adult from egg	40.4 A	35.4 B	42.9 A	57.9 B	77.9 B,C
Adult from dauer	46.7 B	37.3 B	51.2 B	62.3 C	79.8 C

Numbers in the same column with different letters are significantly different ($p < 0.05$). Those with the same letters are not significantly different. Only data for which control survival was >90% are shown.

[4] proposed the difference in developmental stages tested as an explanation for the discrepancies between the two studies, but our data suggest that a more important factor may be the difference in ionic concentration between the medium. It is likely that water with no background ions causes osmotic stress on the nematodes, which reduces their resistance to toxicants.

It is interesting that Hg and PCP did not display the strong differences in 24-h lethal concentration ranges between K-medium and water that were exhibited by Cd, Cu, and Pb. This may be caused by different mechanisms of bioavailability, as controlled by the speciation of these chemicals. Both Hg and PCP have characteristics that differentiate their speciation behavior from the other toxicants tested here, and further study would be necessary to elucidate these properties and their influence on toxicity test results.

This study points out a number of important features of *C. elegans* that should be considered in designing aquatic toxicity tests with it. First, *C. elegans* apparently exhibits variable tolerance to chemical stressors depending on the ionic concentration of the surrounding medium. Low ionic concentrations may stress the nematode to the point that its tolerance of toxic chemicals is decreased. A similar conclusion was reached by Donkin and Dusenbery [27] based on studies with copper. The behavioral attraction of *C. elegans* to certain concentrations of NaCl similar to that of K-medium [48] may be partly due to an avoidance of stressful ionic concentrations. Thus, to increase sensitivity, and more closely mimic conditions that the nematode is likely to encounter in nature, a low ionic concentration in the test medium may be desired. However, long-term exposure requires higher ionic concentrations if lethal osmotic stress is to be avoided, and K-medium is a well-defined standard for this.

Table 2. Lowest-observed-effect concentrations (mg/L), the lowest toxicant concentrations to which nematodes were exposed and which inhibited normal development and reproduction after 96 h

Developmental stage	LOEC (mg/L)				
	Cd	Cu	Pb	Hg	PCP
L2–L3	11.2	6.4	20.7	2.0	100
L4	11.2	6.4	20.7	2.0	100
Adult from egg	112	6.4	20.7	2.0	100
Adult from dauer	11,240	6,354	207	201	100

All tests were done in K-medium with OP50 bacteria.
Only data for which control survival was >90% are shown. The study was not successful with 96-h exposures in water or water with OP50 bacteria (i.e., control survival was <90%) and no data are shown from that testing.

Second, *C. elegans* apparently has a wide range of pH tolerance, but, again, only in the short term. This could be helpful in studies examining the physicochemical effects of pH on the bioavailability of chemicals, as pH extremes alone may not be very toxic to the nematodes. A study that more thoroughly investigated pH as a potential factor influencing toxicity results with *C. elegans* would be worthwhile.

Third, the presence of food is not necessary for short-term tests, and thus it may be desirable to omit it to avoid complicating factors caused by bacterial adsorption of toxicants. However, this did not seem to occur to a large degree with the chemicals tested here in 24-h tests, for there was seldom a difference between lethality in medium with bacteria and in medium without bacteria. This also suggests that the primary route of toxicant uptake by the nematode may be the cuticle rather than the gut, since the presence of toxicant-binding bacteria that is subsequently ingested would be expected to increase the dosage and hence lethality. It should be noted, however, that the wide ranges of toxicant concentrations tested would not have allowed detection of subtle differences in responses, so a definitive statement about the effects of bacteria cannot be made at this point. Regardless of whether bacteria is added in short-term tests, it is essential for long-term tests, as indicated by the results of the 96-h lethality, development, and reproduction assays.

Finally, there does not seem to be much difference in tolerance to toxicants among different developmental stages of

C elegans Evidence for *Daphnia* spp indicates that organisms less than 24-h old are the most sensitive, but that this may simply reflect the fact that ecdysis, which is a particularly vulnerable period, occurs more often in juveniles [49] Similarly, in cases where *C elegans* larvae appeared more susceptible to toxicants than adults (e g , Hg), the increased rate of molting in larvae may be a possible explanation, although this requires further investigation However, the ease of working with *C elegans* adults as opposed to larval stages suggests their use in short-term tests, although chronic tests for development and reproduction would require larvae

An important observation in our studies was the increased tolerance in short term tests of adults that had been cultured from dauer larvae as compared to those that had been cultured from eggs Because the dauer stage is induced in response to stress, it is not surprising that an adult emerging from this stage should be more tolerant than one not so conditioned Anderson found dauer larvae to be more tolerant to thermal stress and oxygen deprivation than adults from eggs [50] However, in our 96-h tests, adults from dauer larvae displayed high mortality, probably due to natural aging

It thus appears that the presence of bacteria and the developmental stage are not crucial to a successful 24-h mortality test However, to maximize sensitivity, adult nematodes cultured from eggs should be used rather than adults cultured from dauer larvae, and a bacterial food source can be omitted Larval stages do not appear to differ greatly from adults in their response For 96-h tests, K-medium with bacteria should be used, and the preparation of bacteria should be performed as described above to ensure a rich enough food supply for preventing starvation effects For these tests, it again appears that, using reproduction as an end point, adults cultured from eggs are more sensitive than are adults cultured from dauer larvae, and are similar in sensitivity to larvae in which development to reproductive age is followed Given this similarity in response, the ease of manipulating adults as compared to larvae suggests the use of adults when possible For 96-h mortality tests, adults cultured from dauer larvae should probably not be used, as death from natural aging may complicate results

We believe that *C elegans* should be considered for use as a standard aquatic test organism However, before such testing can be routinely performed, much further standardization of the test method is needed This work should include effects of pH, the response of the organism in media commonly used in aquatic toxicity testing, and a comparison of responses to the commonly used aquatic reference toxicants

Acknowledgement—The authors would like to thank Tara Roth for her technical assistance This project was supported by the University of Georgia's Agricultural Experiment Station and the Georgia Power Company

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