

A Soil Toxicity Test Using the Nematode *Caenorhabditis elegans* and an Effective Method of Recovery

Steven G. Donkin and David B. Dusenbery

School of Biology, Georgia Institute of Technology, Atlanta, Georgia 30332-0230, USA

Abstract. A new method for recovering nematodes from soils in an efficient, reproducible, and non-destructive manner has been developed. It was used to conduct short-term soil toxicity tests using the soil-dwelling nematode *Caenorhabditis elegans* and several different soil types spiked with copper chloride. The recovery method, which involves centrifugation through a colloidal silica suspension, allows the nematodes to be extracted from the soil matrix so that lethality can be assessed. The nematodes are unharmed by the recovery procedure, and both live and dead individuals are recovered with high efficiency (well over 80%), allowing reproducible concentration-response curves to be made after a 24-h exposure. The LC₅₀s for copper were increased about tenfold by the presence of soil, and different soils had significantly different effects on toxicity. Toxicity of copper ion was also influenced by the concentration of sodium chloride and potassium chloride in the test solution, and the presence of bacteria increased the toxicity of copper ion in some soils. The LC₅₀s in soil were close to the LC₅₀ for the 2-week earthworm soil toxicity test, suggesting that a 24-h nematode toxicity test may be comparable to the 2-week earthworm test in terms of sensitivity.

In choosing a toxicological test organism, it is desirable to consider an animal that can provide a homogeneous test population to minimize random variations in response due to differences among individuals. Furthermore, the physiology of the test animal should be thoroughly understood so that mechanisms of toxic response can be interpreted. It is also important that the test procedure can be done quickly, inexpensively, and provide accurate and reproducible results (Giesy and Hoke 1989). The nematode *Caenorhabditis elegans* meets these and other requirements by virtue of its ease of culturing, short life cycle, invariant and completely mapped development, extensively mapped genome and nervous system, and ability to self-fertilize and produce mass cultures of genetically identical individuals, all of which have aided on its becoming one of the most completely characterized animal models available (Wood 1988).

C. elegans has shown promise as an indicator of toxicity when exposed to chemicals on agar plates (Williams and Dusenbery 1987; 1988) and in aquatic media (Williams and

Dusenbery 1990a). Research has been done on the toxic effects of metal exposure based on lethality (Williams and Dusenbery 1988), behavioral changes (Williams and Dusenbery 1990b), ultrastructural changes (Popham and Webster 1982), stress protein induction (Slice *et al.* 1990), and reproductive capacity (Popham and Webster 1979; Williams 1989), as well as genetic changes caused by various tumor promoting chemicals (Miwa *et al.* 1982; Lew *et al.* 1982). In the lethality studies, ranking among the LC₅₀s obtained for seven metals was well correlated with the ranking of LD₅₀s from mammalian tests, and *C. elegans* was as good as rats as a predictor of toxicity to other mammals (Williams and Dusenbery 1988).

A test for investigating environmental toxicity should, in addition to the above requirements, consider the natural habitat of the test organism, and how this relates to the test sample. Nematodes are one of the most ubiquitous groups of animals in soil, where they are important both in terms of their sheer abundance and their role in nutrient mobilization (Sohlenius 1980; Nicholas 1984). *C. elegans*, being a common soil-dwelling nematode, is thus especially well suited for use in assessment of contaminated soil.

The need for reliable soil toxicity tests stems from the increased awareness of the environmental and human health effects of contaminated soils, the difficulty in predicting the behavior of, and thus biological risk posed by, various contaminants in soils, and the current lack of well-established test animals that are native to soil habitats. Earthworms have generally been the animal of choice for such tests (Greene *et al.* 1989; Callahan *et al.* 1991). However, these tests usually require several weeks to perform and a larger sample volume than would the 1-mm long *C. elegans*. It is proposed here that development of a soil toxicity test for *C. elegans* could complement already existing tests and help fill the need many see for multispecies tests in ecotoxicological studies and site assessment (Porcella 1983; Dowd 1984).

The main obstacle to designing such a toxicity test is the difficulty in extracting nematodes from soil in a way that yields efficient and reproducible recovery, and is not harmful to the nematodes. The two most common methods, the sucrose flotation and Baermann funnel methods (Niblack and Hussey 1985), are both unsatisfactory for this purpose. The sucrose flotation method relies on a separation by density in a sucrose solution, which can cause osmotic stress to the nematodes and result in

Table 1. Selected properties of soils used

Soil type ^a	Classification	Sand (%)	Silt (%)	Clay (%)	Organic matter (%)	pH _w ^b	pH _K ^c	Exchange capacity (cmol(+)/kg)	
								Cation	Al ³⁺
Cecil sl	Kaolinitic typic hapludults	66	18	16	1.7	6.2	5.2	5.0	0.1
Worsham sl	Mixed typic ochraquults	55	29	16	3.0	5.1	4.5	5.8	0.8
Davidson l	Kaolinitic rhodic paleaquults	51	29	20	3.4	6.1	5.2	8.0	0.2
Dyke cl	Oxidic typic rhodudults	28	33	39	2.2	6.2	5.4	10.3	0.0

^asl = sandy loam, l = loam, cl = clay loam

^bpH_w = pH determined in water with no background electrolytes, 1:2 soil to liquid ratio

^cpH_K = pH determined in 1.0 M KCl solution, 1:2 soil to liquid ratio
From Kim (1989)

many inactive individuals (Freckman *et al.* 1975). The Baermann funnel method, besides being a lengthy procedure, recovers only moving nematodes, and thus would exclude dead or moribund individuals. What is desired is a method which recovers both live and dead nematodes, but does not itself harm the animals, so that any observed adverse effects can be attributed to the chemical exposure and not the extraction procedure.

The method we have developed incorporates centrifugation through a colloidal silica suspension, which separates both live and dead nematodes from various types of soil by floating them on the surface while sedimenting the soil particles. The procedure is rapid, inexpensive, not harmful to nematodes, and consistently yields over 80% recovery in samples so far tested. This paper describes results from acute lethality studies in sand and four southeastern soils spiked with copper.

Materials and Methods

Nematode Culture

Caenorhabditis elegans, var. Bristol (strain N2), was maintained in stocks of dauer larvae (Cox *et al.* 1981) that were replenished every month and kept in a constant temperature 20°C incubator in a flask of M9 medium (Brenner 1974). Forty-eight h before the tests were to begin, several hundred dauers were placed on agar plates with established *Escherichia coli* strain OP50 (Brenner 1974) bacterial lawns as a food source, and cultured at 20°C. This allowed time for the dauers to develop into adults, at which time they were transferred to the test samples. The agar was a nutrient growth medium described by Brenner (1974), but with potassium phosphate buffer replaced by 0.03 M KCl. This medium, designated "K-agar," is described by Williams and Dusenbery (1988) for use in metal toxicity tests, and was used here in all tests except those for determining proper salt concentrations, in which the salt concentrations were varied, as detailed below.

Soils Used

Air-dried samples of four previously characterized soil types (described in Table 1) native to the southeast were kindly provided by Dr. W. P. Miller of the University of Georgia. The soil properties had been previously determined by Kim (1989), using methods described by Miller and Baharuddin (1986). The samples were pulverized and sieved through a 850 µm screen, and then stored at room temperature until use. Sea sand was obtained from Fisher Scientific and used unmodified.

Determination of Proper Salt Concentration for Solution Medium

The test samples consisted of 7 g sea sand in 60 × 15 mm Pyrex petri dishes with lids, with a 3 ml volume of test solution. The Pyrex dishes were cleaned between uses by overnight soaking in 1.5 N HNO₃ followed by copious rinsing in deionized water. Twenty-four h prior to beginning the test, a 50 ml volume of *E. coli* strain OP50 liquid culture, which had been previously grown to stationary phase, was centrifuged at 3,000 rpm in a swinging bucket rotor (16 cm radius) for 10 min, after which the bacterial pellet was resuspended in 25 ml 100% K-medium (0.05 M NaCl, 0.03 M KCl) (Williams and Dusenbery 1990a). The same procedure was simultaneously performed with 50% K-medium (0.025 M NaCl, 0.015 M KCl) and pure deionized water to obtain three solutions of bacteria and varying salt concentrations with optical densities of approximately 0.1 at 600 nm. These were termed "100% K-medium," "50% K-medium," and "water," and were used for making stock metal solutions and dilutions.

Reagent grade CuCl₂ · 2H₂O was obtained from Fisher Scientific. A stock solution based on the concentration of copper ion was prepared fresh for each test by dissolving the appropriate amount of metal salt in a small volume of the appropriate K-medium or water using a volumetric flask. Dilutions were made directly from the stock using K-medium or deionized water to get the desired concentrations of metal. A 3-ml volume of the test solution was added to the sand samples, which thoroughly wetted the sand and left little overlying liquid. The lids were put in place and the samples returned to the 20°C incubator where they were left for 24 h to allow the sand and liquid phases to equilibrate.

The agar plates on which the adult nematodes developed from the dauer stage contained either "100% K-agar," "50% K-agar," or "water agar," which varied only in their concentrations of NaCl and KCl. Nematodes were transferred from agar plate to a soil dish containing a solution of the same salt concentrations so as to minimize potential osmotic shock. Twenty adult nematodes were individually transferred to each dish using a platinum wire and a stereo dissecting microscope, through which their viability was confirmed. The dishes, with lids in place, were returned to 20°C for 24 h.

Each experiment contained single samples with 0, 100, or 200 mg/L Cu²⁺ in each of the three K-medium solutions, with or without sand, for a total of 18 sample conditions. This experiment was replicated five times, so that recovery and survival data were the means for 100 individuals.

Nematode Extraction and Scoring

Ludox HS-40, a 40% (w/w) colloidal silica suspension in distilled water, and Ludox AM, an aluminum-modified 30% (w/w) suspension, were obtained from E.I. Du Pont de Nemours & Co., Wilmington,

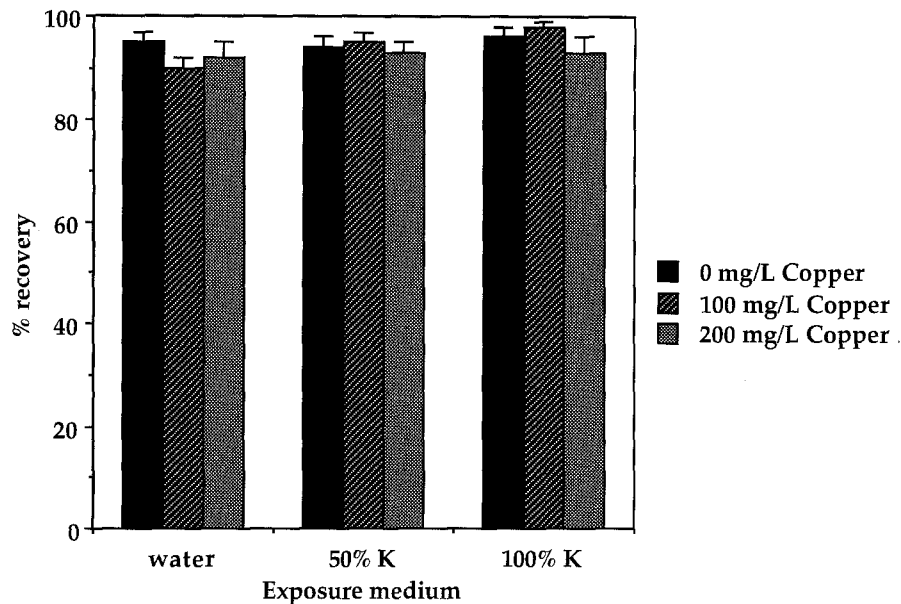


Fig. 1. Mean percent recoveries of nematodes from sand after 24-hour exposures. Nematodes were exposed to 0, 100, or 200 mg/L Cu^{2+} solutions (prepared with water, 50% K-medium, or 100% K-medium) in sand for 24 h and then recovered with the colloidal silica method. Bars represent mean percent recoveries (\pm S.E.) for five replicates with twenty nematodes per replicate. Both live and dead nematodes were recovered

DE. Ludox AM was neutralized with concentrated HCl, and Ludox HS-40, having a somewhat higher density than Ludox AM, was diluted 1:1 (v/v) with deionized water before being neutralized. Both solutions were kept in tightly capped polyethylene wash bottles at 20°C, and used interchangeably for extractions with no difference in results.

To process all the samples from a single 24-h assay typically required 4 h. Thus, to maintain the exposure periods as close to 24 h as possible, the extraction and counting procedure was timed to balance around the 24-h mark. In other words, processing was begun about 2 h prior to the 24-h mark, and ended about 2 h later. The order of processing for the samples was randomized for each test, so that results were not biased by the order of processing.

The soil and nematodes for a given sample were removed from the dish by vigorous washing with the Ludox suspension from a wash bottle into a funnel placed over a 50-ml, round-bottomed borosilicate centrifuge tube. Two samples were done simultaneously; the tubes were covered with Parafilm, shaken by hand for 10 sec to resuspend the soil, and then centrifuged at 2,200 rpm in a swinging bucket rotor (16 cm radius) for 1 min. This compacted the soil particles at the bottom of each tube, separating them from the nematodes, which floated on top. The supernatant and extracted nematodes were then poured off into a Pyrex flat-bottomed crystallizing dish for observation under the microscope. The soil pellet was immediately resuspended in Ludox, gently agitated, and allowed to sit while the first extraction was counted. Most of the 20 nematodes were recovered in the first extraction, but if they were not, a second spin was done on the resuspended soil, which usually recovered the missing ones.

Scoring of the nematodes was done by mouth pipetting all those found under the dissecting microscope with a drawn-out capillary tube, and transferring them to a small dish of K-medium. After all nematodes were collected from both Ludox extractions, they were inspected to determine mortality. Dead nematodes were counted as those which failed to move in response to gentle probing. Samples with no sand required no extraction, and were scored directly in the sample dish.

Determination of Effect of Bacteria and Soil Types

The four southeastern soils were each used with varying concentrations of copper in 100% K-medium, prepared as described above, with duplicate samples with and without bacteria run simultaneously. Samples with 7 g soil were used with 3 ml liquid, along with the appropriate controls, and each assay was replicated at least five times, so that

recovery and survival data are the means of at least 100 individuals. Controls with no soil and no copper were always included to assure the health of the nematode sample used. Extraction and scoring after a 24-h exposure were as described above. Tests were also performed using varying concentrations of Cu^{2+} with no soil present, but otherwise identical to the above assays, in order to determine the response to Cu^{2+} by aquatic exposure.

Statistical Methods

LC_{50} s and confidence intervals were determined using linear regression on the probit transformations of survival data (Finney 1971) and the SAS microcomputer software (SAS Institute, Cary, NC). No correction was made for control responses, since assays in which the control survival was less than 90% were not used in the analysis. Analysis of variance ($p = 0.05$) was also performed using SAS software.

Results

A first step in developing the test protocol was to determine the proper ionic composition of the medium for exposure. Since K-medium contains cations which probably compete with the copper ions for sorption sites on the soil particles, it was thought that this could have an effect on the resulting bioavailability of copper and thus the toxicity of the sample. For maximum simplicity, the test would be performed with no competing ions present, but this would not represent natural soil conditions. Typically, the major inorganic ions, such as Na^+ , K^+ , and Cl^- are present in the soil water at concentrations in the approximate range of 1–100 mM (Fried and Broeshart 1967). The presence of the ions may be necessary for nematode health, presumably by maintaining the proper osmotic balance.

Figure 1 shows the excellent recovery of both live and dead nematodes from sea sand after a 24-h exposure in various K-media and copper concentrations. This supports the colloidal silica recovery method and enhances the credibility of the survival data, which is shown in Figure 2. *C. elegans* appeared to

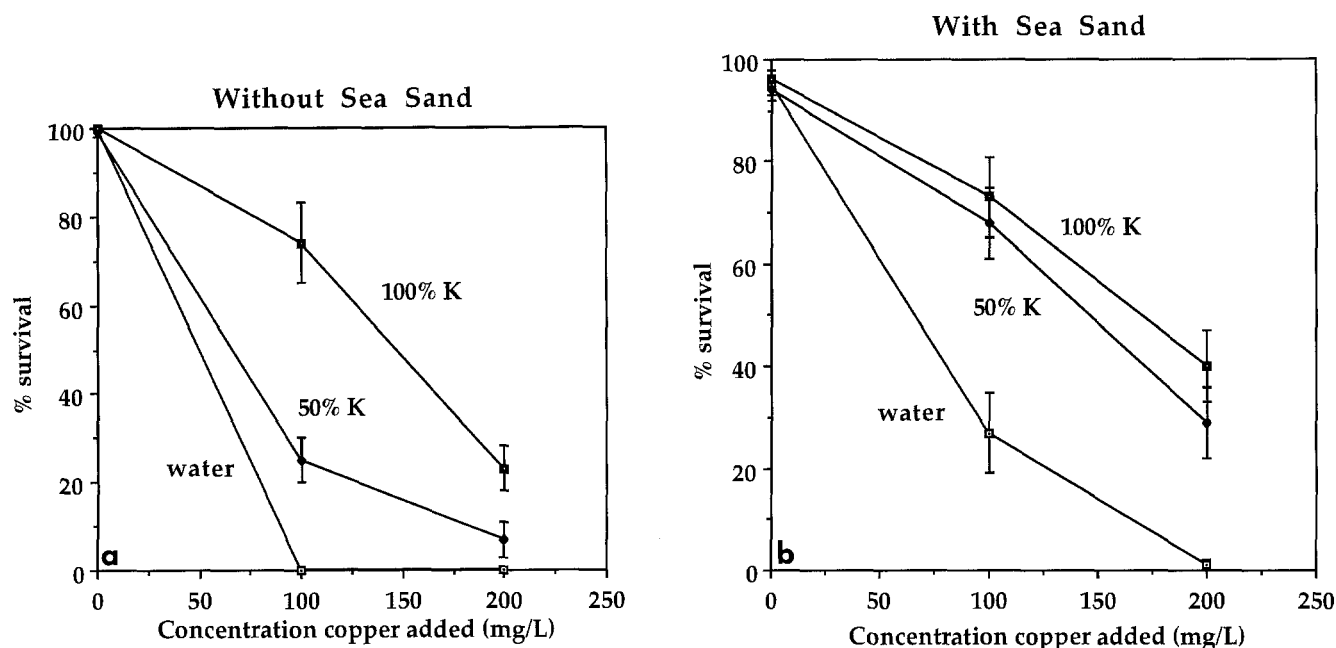


Fig. 2. Concentration-response curves for Cu^{2+} prepared in various concentrations of K-medium. Points are means (\pm S.E.) of five replicates with twenty nematodes per replicate. (a) Exposure with no sand present (aquatic); (b) exposure with sand present

be tolerant of a wide range of salt concentrations when no copper was present, as shown by the near 100% survival after a 24-h exposure in water, 50% K-medium, or 100% K-medium. This was true both with and without the presence of sand.

However, when toxic concentrations of copper were added, the degree of lethality was dependent upon the salt concentration of the solution medium. The 100% K-medium exhibited the greatest percent survival at both 100 and 200 mg/L Cu^{2+} , and water exhibited the lowest survival. This is presumably because the osmotic stress caused by low salt concentrations, while not lethal when no copper was present, became lethal when the stress of copper toxicity was added. This effect was also observed in samples with no sand present; thus, it was not due exclusively to sorption processes between copper ions and sand particle surfaces, but rather it suggested a physiological effect on the nematodes.

Another observation is that the presence of sand had the effect of decreasing the lethality of copper in water and 50% K-medium. This may be due to the sorption of copper ions on the surfaces of the sand particles, which decreased the amount of bioavailable copper. However, this effect was not seen in the 100% K-medium, perhaps due to the increased concentration of solution ions competing for surface sorption sites on the sand particles.

It was established from this study that 100% K-medium is desirable to assure that the measured stress is due primarily to the test chemical rather than osmotic stress caused by a low salt concentration in the surrounding medium. It is also representative of typical soil solutions. All subsequent studies were performed using 100% K-medium (hereafter referred to as "K-medium") for making solutions.

The next step was to determine the effect of bacteria in the medium. For 24-h exposures, a food source is not essential for nematode survival (Williams 1989), although for longer term chronic exposures it is probably necessary. Therefore, we wished to see whether the presence of *E. coli* affected the bioavailability of copper to a large enough degree to show a

difference in nematode survival between samples with *E. coli* and those without. It was hypothesized that sequestration of copper by the bacteria may decrease the concentration of copper sorbed to the soil particles. If these bacteria are then ingested by the nematodes, the effective dose received by the nematodes may increase, thus shifting the concentration-response survival curve to lower concentrations.

In order to simultaneously test the efficiency of the colloidal silica method for recovering nematodes from soils other than pure sand, which allowed fairly easy extraction due to the large size of the particles, the studies with and without bacteria were performed with four common southeastern soils of varying properties. The recovery efficiencies, shown in Figure 3, were again very good. The consistently high recoveries average at or above 90%, except in some samples with high copper concentration, which may have caused early death and decomposition of the nematodes. For this reason, any non-recovered nematodes were automatically scored as dead. All control samples with soil and no copper showed at least 90% survival.

Figure 3 also shows the concentration-response survival curves for copper in each of these four soils, with and without bacteria present. Analysis of variance was performed between the two survival means (with and without bacteria) at each concentration of copper. The Dyke soil showed a significant difference between the two means at all concentrations, while the Davidson soil showed no significant difference at any concentration. The Cecil and Worsham soils showed slight shifts in the curves, with only one concentration of copper giving a significant difference in response means for each. In all cases, the significant shifts that occurred were in the direction expected if bacteria do in fact increase the nematode exposure to copper, with the presence of bacteria lowering the percent survival.

Table 2 shows the LC_{50} s for copper ion in the four soils, sea sand, and K-medium alone, all with bacteria added, as determined by linear regression on the probit transformations of survival data. LC_{50} s obtained from soil samples without added

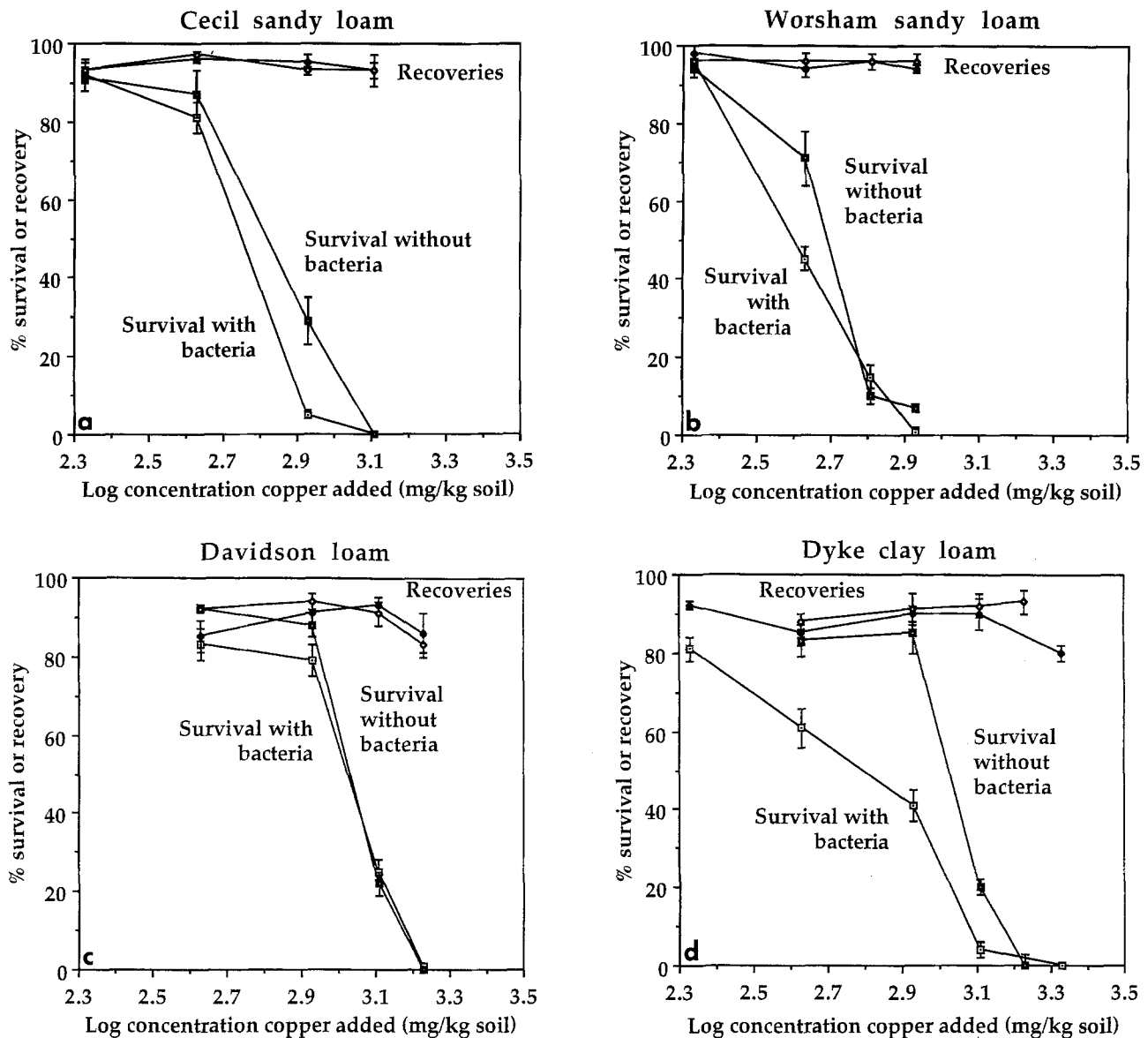


Fig. 3. Concentration-response curves for Cu^{2+} (in 100% K-medium) in four soil types. Points are means (\pm S.E.) of at least five replicates with twenty nematodes per replicate. Top two lines indicate percent recoveries corresponding to survival curves: (a) soil type = Cecil sandy loam; (b) soil type = Worsham sandy loam; (c) soil type = Davidson loam; (d) soil type = Dyke clay loam

bacteria are excluded because the absence of bacteria either had no effect or resulted in an increased LC_{50} (Figure 3); the presence of bacteria is more representative of the actual situation in natural soils. The copper ion LC_{50} for the earthworm *Eisenia fetida*, determined by a 2-week assay in an artificial soil (10% peat, 20% clay, 69% sand, 1% calcium carbonate) (Neuhauser *et al.* 1985), is included for comparison. The numbers in the Table are given in both mg/L, for comparison to the aquatic data, and in mg/kg soil, for comparison among soils and with the earthworm test.

Discussion

The success of the colloidal silica recovery method relies primarily on the fact that Ludox is a suspension rather than a true solution. Therefore, it has the density necessary for separating nematodes from soil particles without creating a stressful os-

motomic gradient across the nematode cuticle. Such a gradient can result in lethal desiccation of the nematode, as is often seen with the sucrose flotation method (Frechman *et al.* 1975). Both Ludox AM and Ludox HS-40 were used interchangeably with no difference in recovery or stress to nematodes. The combination of efficient, reproducible recovery, and benign impact on the nematodes makes the colloidal silica protocol a promising method for performing soil toxicity tests.

The results of the test for determining proper salt concentration indicate that, while salt concentration in the medium may not be important for nematode survival when no other stresses are present, it is important when a toxic stress such as copper is added (Figure 2). Therefore, 100% K-medium is recommended for test solutions.

The use of this protocol with the four southeastern soils produced several interesting results. Copper binds tightly to soil organic matter (McLaren and Crawford 1973), and this is supported by the observation here that Davidson soil, having the

Table 2. Toxicity of copper ion in different media

Exposure medium	Copper LC ₅₀ (mg/L) ^a	Copper LC ₅₀ (mg/kg soil) ^a	Test organism
No soil (aquatic)	105 (60–160)	—	<i>C. elegans</i>
Sand	163 (141–199)	70 (60–85)	<i>C. elegans</i>
Cecil (sandy loam) ^b	1246 (1202–1304)	534 (515–559)	<i>C. elegans</i>
Worsham (sandy loam) ^b	964 (905–1022)	413 (388–438)	<i>C. elegans</i>
Davidson (loam) ^b	2476 (2373–2581)	1061 (1017–1106)	<i>C. elegans</i>
Dyke (clay loam) ^b	1468 (1216–1927)	629 (521–826)	<i>C. elegans</i>
Artificial soil	—	643 (549–753) ^c	<i>E. fetida</i>

^aLC₅₀s are in mg/L water added and mg/kg soil. 95% confidence intervals are in parentheses

^bLC₅₀s for soil samples with bacteria

^cNumbers for the artificial soil test with earthworm are from Neuhauser *et al.* (1985)

highest organic matter content of the four soils, also had the highest LC₅₀ (Table 2), presumably because the organic matter fraction made the copper unavailable to the nematodes. The effect of bacteria on the resulting concentration–response curves varied with the soil used (Figure 3), and this may also be a reflection of the relative amounts of organic matter present in the soils. Dyke soil, which has relatively little organic matter, exhibited a significant shift of the concentration–response survival curve when bacteria were present, whereas Davidson soil exhibited no such shift when bacteria were present (Figure 3). In Dyke soil, when no bacteria are present, most of the adsorbed copper is probably bound to the substantially large clay fraction, which, like organic matter, can effectively sequester the copper away from the bulk solution. The bacteria, when present, may compete more successfully for copper, binding it in large amounts but then becoming ingested by the nematodes and thus increasing the dose to the nematodes.

In Davidson soil, the bacteria may not compete as successfully with the more abundant organic matter, which is probably the primary copper adsorbent, so most of the bound copper is in the soil organic matter unavailable to the nematodes. When allowed a choice between living bacteria and dead or no bacteria, *C. elegans* prefer living bacteria (Andrew and Nicholas 1976), and it may be that in soils they preferentially ingest living bacteria rather than non-living organic matter. This bio-magnification effect of bacteria has been observed elsewhere in soils (Loutit *et al.* 1967) and among bacterial feeding tubificid worms in river sediments (Patrick and Loutit 1976). Since any effect of bacteria in this study was always to increase toxicity, future studies should probably routinely include bacteria in the test sample so as to minimize the potential for underestimating the toxicity.

When bacteria were present, the LC₅₀ for copper in Davidson soil was roughly twice that of the Dyke, Cecil, and Worsham soils, yet these in turn were an order of magnitude greater than the LC₅₀ when no soil was present (Table 2). Pure sand produced an LC₅₀ much closer to that for aquatic exposure, indicating that copper sorption by sand is relatively minor.

Table 2 also shows that nematode sensitivity to copper ion is comparable to that of the earthworm. The LC₅₀ values for *C. elegans* in sand and Worsham sandy loam were lower than the LC₅₀ value for *E. fetida* in artificial soil, while that for Davidson loam was higher, and those for Cecil sandy loam and Dyke clay loam were similar to the value for *E. fetida*. It is also important to consider that the *C. elegans* test involves only a 24-h exposure compared to 2 weeks for the earthworm test, and more individuals can be easily assayed in a short time with the

C. elegans test, increasing the power of the statistical analysis. The smaller size of *C. elegans* also allows for less soil to be used per sample. While the earthworm is cited as a good toxicity test subject for soil because it lives within and actually ingests the soil (Callahan *et al.* 1991), visual observation of the *C. elegans* gut, which is transparent, revealed a similar tendency to ingest soil particles. Live nematodes as well as dead ones contained soil particles in their guts, so the ingestion of soil by itself is not harmful.

Because only four soil types were used, correlations between LC₅₀s and soil characteristics are of limited statistical significance. There was no single soil characteristic that was strongly correlated with the LC₅₀s observed, suggesting that the toxicity of a metal in a particular soil cannot be accurately predicted without considering several characteristics at a time. However, more varied soil types will have to be tested before a definitive statement about how soil characteristics affect metal bioavailability can be made. The main objective of this study was simply to develop the protocol for the nematode test, rather than to make correlations between soil properties and metal toxicities.

The *C. elegans* soil toxicity test shows promise as a rapid and reproducible method for routine testing. Based on responses reported here to copper in soil, as well as to several other metals in various soils (Donkin and Dusenbery, in preparation), *C. elegans* appears to be comparable to the earthworm in its sensitivity. Due to the importance of nematodes to soil ecosystems, it would be wise to include them in any multispecies bioassessment protocol. Although more soils need to be tested to provide correlations of statistical significance, the *C. elegans* test may also be useful for inferring possible correlations between sorption phenomena occurring in characterized soils and the resultant effect on bioavailability.

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