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THE USE OF A NEMATODE, CAENORHABDITIS ELEGANS, FOR BIOMONITORING HEAVY METALS IN PULP AND PAPER EFFLUENTS AND SLUDGE

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ABSTRACT

Current methods used by the pulp and paper industry for determining toxicity of effluents have been found to be expensive, time-consuming, and of uncertain reliability. A nematode, Caenorhabditis elegans, has been investigated as a test organism to evaluate pulp and paper sludges and effluents. In this research, heavy metals (Al, As, Cd, Cr, Ni, Zn, Cu, and Pb) in both an aquatic and a solid medium were used to evaluate the utility and applicability of this test method to the needs of the pulp and paper industry. Experimental results indicate good precision and reproducibility within laboratories, although inter-laboratory reproducibility was less successful. The use of C. elegans for toxicity testing has the potential to yield more highly reproducible results at less expense than those obtained with other test organisms such as Ceriodaphnia or fathead minnow.

The ability to use C. elegans to evaluate sludge toxicity is an added benefit. The sludge sample tested was not, itself, toxic to the nematodes. When the sludge was spiked with a heavy metal solution, the toxic effect on the nematodes was less than that seen in metal-spiked soils, indicating a large capacity on the part of sludge for binding metals and decreasing their bioavailibility.

INTRODUCTION

The environmental impact of potentially toxic chemicals found in pulp and paper mill effluents is the focus of increasing concern. Several methods are available for determining the toxicity of industrial effluents. Ceriodaphnia dubia (water flea) and Pimephales promelas (fathead minnow) are commonly used by the pulp and paper industry for internal monitoring of effluents and also by the EPA for regulatory compliance (USEPA 1989). Both methods are expensive, time-consuming, and of questionable reliability (Hall and Borton 1987; Kraus and Kornder 1987).

An important quality in any test method is the reliability of the method for producing consistent and reproducible results. In addition, an acceptable test method must also be rapid, economical, ecologically relevent, and capable of being performed routinely by technicians with little specialized training.

The free-living soil nematode, Caenorhabditis elegans, has recently been proposed as a suitable toxicity test organism for both aquatic (Williams and Dusenbery 1990) and soil (Donkin and Dusenbery 1993) samples, wherein it was found to be as sensitive to many toxic metals as other standard invertebrate test organisms. This 1-mm long roundworm is currently one of the most thoroughly characterized of all animals (Wood 1988; Kenyon 1990). It is the only organism which has its entire cell lineage mapped from fertilized egg to all 810 cells in the somatic tissue of the adult (Sulston and Horvitz 1977; Sulston et al. 1983). The connectivity of all 302 neurons has also been described (White et al. 1986). Thousands of C. elegans are easily cultured on agar petri plates using the bacterium Escherichia coli as a food source (Brenner 1974). Large populations can be stored in liquid buffer in the dauer larval stage, which is developmentally arrested, and induced to develop synchronously into adults as needed by transferring the dauer larvae to agar plates seeded with bacteria (Cassada and Russell 1975; Klass and Hirsh 1976; Riddle 1988). Additionally, the nematodes can be frozen in liquid nitrogen and thawed to start new cultures, which allows for long-term maintenance of stocks without genetic changes (Brenner 1974). Characteristics of C. elegans which make it attractive as a toxicity test organism include its ability to reproduce by self-fertilization and its 3.5-day life cycle, which make it possible to produce large cultures of genetically identical individuals within a short period of time.

Heavy metals can be found in pulp and paper mill discharges and are therefore potential environmental threats (NCASI 1990; Hamm and Gottsching 1989; McGovern et al. 1983). Metals are known to enter the system through raw material, additives, and ink attached to recycled fibers (Hamm and Gottsching 1989; Rusterholz and Smith 1989; Shapiro 1990). Due to recent concerns about chlorinated compounds, metals have been given a minimum amount of attention, but are still an area of environmental concern (McGovern et al. 1983).

This paper explores the potential of the C. elegans aquatic and soil toxicity test protocols to investigate the toxicities of pulp and paper mill discharges. Short-term exposures of the nematode to liquid and sludge samples spiked with known concentrations of metal ion were followed by assessment of nematode mortality, and the generation of comparative concentration-response curves.

MATERIALS AND METHODS

Nematode Culture

Culture and general test methods for C. elegans used in the aquatic tests are described by Williams and Dusenbery (1990), and those for the soil/sludge tests are described by Donkin and Dusenbery (1993). C. elegans wild type 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 hours (for sludge evaluation) and twenty-four hours (for effluent evaluation) before the tests were to begin, several hundred dauer larvae were placed on K-agar plates (Williams and Dusenbery 1988) with established E. coli strain OP50 (Brenner 1974) bacterial lawns as a food source, and cultured at 20°C. This allowed time for the dauer larvae to develop into mature adults (after 48 hours) or young adults (after 24 hours) (Cassada and Russell 1975), at which time they were transferred individually to the test samples. Mature adults were used in the sludge tests because their larger size made extraction from the sludge easier, and this was also the nematode age used in the previous soil tests to which the sludge results were compared.

Evaluation of Aquatic Samples

The metal ions tested were nickel (Ni), aluminum (Al), chromium (Cr), arsenic (As), and cadmium (Cd). These tests were performed at the Institute of Paper Science and Technology (IPST), Atlanta, GA. Metals were chosen so that both inter- and intra-laboratory reproducibility could be evaluated. The results of these tests were compared with those obtained earlier by Williams and Dusenbery (1990) at the School of Biology, Georgia Tech, Atlanta, GA.

Reagent-grade metallic salts were obtained from Sigma (St. Louis, MO) as $NiCl_2$, $Al(NO_3)_3 \cdot H_2O$, $K_2Cr_2O_7$, $NaAsO_2$, and $CdCl_2$. Stock solutions based on metal ion concentration were prepared in K-medium (0.05 M NaCl, 0.03 M KCl) with E. coli bacteria (Williams and Dusenbery 1990). Dilutions from the stock solutions were made using K-medium to obtain the desired metal ion concentrations. No adjustment was made in the pH.

After the dauer larvae had been allowed to mature for 24 hours into young adults, ten nematodes were transferred, using a platinum wire, to each 60 X 15 mm Pyrex petri dish containing 3 mL test solution. The dishes containing test solution and nematodes were kept covered in a dark incubator maintained at 20°C throughout the test period. The dishes were removed every 24 hours to evaluate nematode survival using a dissecting microscope with transmitted light. Death was defined as a total lack of response to gentle probing with a small wire. Nematodes not found were assumed dead and totally decomposed.

A minimum of five concentrations of each metal ion were tested for toxicity, along with a control containing K-medium with no added metal. Each concentration and control was replicated with ten sample dishes and ten nematodes per sample dish. This allowed for 100 individuals to be exposed to each concentration. A complete randomized block design was used for each experiment to statistically eliminate time and operator effects.

Evaluation of Sludge Samples

Primary/secondary processed sludge was obtained from a pulp and paper mill and stored sealed at 4°C. Some physical and chemical characteristics were determined by the Soil and Plant Testing Lab in Athens, GA, and these included a 56.1% moisture content, 33.6% organic matter content, negligable amounts of trace metals, and a pH of 7.8. Samples were prepared by placing 4 g unmodified sludge in a 60 X 15 mm Pyrex petri dish with lid, followed by 5 mL K-medium, which was enough to thoroughly moisten the sludge, but left little overlying liquid. Initial toxicity tests with sludge samples moistened with pure K-medium revealed no lethal effects to nematodes exposed for 24 hours. Subsequently, pure K-medium plus sludge was used in control samples when the toxicities of added metal ions were tested.

Reagent-grade metal salts [$ZnCl_2$, $CdCl_2$, $Pb(NO_3)_2$, and $CuCl_2 \cdot H_2O$] were obtained from Fisher Scientific (Pittsburgh, PA), and used to test the effects of sludge on the bioavailability of toxic metals to the nematodes. A stock solution based on the concentration of metal ion to be tested was prepared fresh for each test by dissolving the appropriate amount of metal salt in a small volume of K-medium. Dilutions were made directly from the stock using K-medium to obtain the desired concentrations of metal ion. Sludge samples were then spiked with 5 mL of the metal solutions as described above, and allowed to equilibrate for 24 hours in the dark at 20°C with the lids in place. No bacterial food source was added to the sludge, and the pH was not adjusted.

Twenty mature adult nematodes cultured from the dauer larvae stage 48 hours previously were then transferred from agar plate to sample dish using a sterile platinum wire and a stereo dissecting microscope, through which their viability was confirmed. If a nematode did not survive the transfer, it was removed and replaced with a new one. The dishes, with lids in place, were returned to 20°C for an additional 24 hours. All sample test were replicated five times, so that 100 individuals were tested at each concentration.

Ludox AM (E. I. Du Pont de Nemours & Co., Wilmington, DE), an aluminum modified 30% (w/w) colloidal silica suspension in distilled water, was used in extracting the nematodes from sludge. Its preparation was similar to that described by Donkin and Dusenbery (1993), except that the relatively low density of sludge required a decreased density in the Ludox AM suspension in order to separate the sludge from the nematodes during the centrifugation step described below.

This was obtained by diluting the Ludox AM 1:1 (v/v) with deionized water, followed by neutralization with concentrated HCl. The modified suspension was kept in a tightly capped polyethylene wash bottle at 20°C.

The sludge and nematodes in 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 such samples were done at one time. The tubes were then covered with Parafilm, shaken gently by hand for 10 seconds to resuspend the sludge, then centrifuged at 2200 rpm in a swinging bucket rotor (16 cm radius) for one minute. This compacted the sludge 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 sludge pellet was immediately resuspended in Ludox, gently agitated, then allowed to sit while the first extraction was counted. The centrifugation and extraction procedure was then repeated two more times in order to recover as many nematodes as possible.

Scoring of the nematodes was done by 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 the three Ludox extractions, they were inspected to determine mortality. Dead nematodes were counted as those which failed to move in response to gentle probing.

Statistical Methods

Means and standard errors were determined, and analysis of variance ($p = 0.01$) was performed, using SAS (SAS Institute, Cary, NC) or TOXstat (Univ. of Wyoming, Laramie, WY) microcomputer software. LC_{50}s and 95% confidence intervals were determined using linear regression on the probit transformations of survival data (Finney 1971, pp. 50-80). No correction was made for control responses, since tests with sludge in which the control survival was less than 90% were not used in the analysis. For the four-day aquatic survival tests, 80% was the accepted minimum survival for controls.

RESULTS

Aquatic

For the aquatic toxicity evaluation of metals, a total of 50 control dishes, with 10 nematodes per dish, were scored. The mean day one survival for controls was 100%. On day four the mean survival for control dishes was 96%, with 66% of the control dishes showing 100% survival for the fourth day, and no dish showing less than 80% survival. Since control survival was consistently high, control mortality was not factored into treatment LC_{50}s.

Results from the aquatic tests performed at IPST, along with those from Williams and Dusenbery (1990), are shown in Table I. LC_{50}s determined in the IPST study and those reported by Williams and Dusenbery (1990) were similar for arsenic throughout the four days of the test. Other metals produced some disparate results between labs for some days, but it is expected that this variability will be reduced as more replicates are done and the technique is refined.

The randomized block design of the experiments performed at IPST allowed for the use of standard analysis of variance techniques to determine significant variation caused by repetitions and treatments. Each metal and day were analyzed separately. Results indicate that there were significant effects among treatments, but there were no significant differences among repititions (data not shown).

In the case of nickel and aluminum, the experiment was separated into two sets of five repititions. Each subdivision was completed on separate occasions so as to determine potential time effects. No significant differences between subdivisions were observed (data not shown).

Sludge

Sludge without added metal ion was not lethal to nematodes in the 24-hour exposure test, thus toxicity tests were performed using sludge spiked with various concentrations of metal ion. Mean efficiencies of nematode recovery from sludge are shown for each metal ion concentration in Figure 1, along with mean percent survival at each concentration.

Previously obtained data for aquatic exposure and exposure in Worsham soil, a soil type common to the southeastern U.S., are included for comparison (Donkin and Dusenbery 1994). Recovery efficiencies from sludge were erratic and not always as good as those obtained from soils, which were consistently above 80% (Donkin and Dusenbery 1993; 1994). This was probably due to the high organic matter content of sludge, which had a tendency to cause clumping and subsequent difficulty in removing nematodes. In addition, sludge organic matter apparently has a density close to that of biological tissue, which also makes separation difficult. However, it was still possible to determine that the presence of sludge greatly increased the survival of nematodes when they were exposed to each of the four metals, presumably due to its binding of metal ions and thus the reduction in metal bioavailability.

DISCUSSION

In addition to the previously cited advantages of sensitivity, low cost, and rapidity, we found the C. elegans toxicity test method to be acceptable for routine effluent monitoring in terms of its intra-laboratory reproducibility. Inter-laboratory reproducibility was less acceptible, but this may be due to slight differences in experimental technique which need to be improved upon, and also the fact that only two laboratories were compared. The method also exhibited a much better

control survival than that of the standard Ceriodaphnia test, which has suffered from inconsistent survival in control control solutions (Hall and Borton 1987; Kraus and Kornder 1987; NCASI 1990). As an aquatic test organism, C. elegans may be considered ecologically relevent since all free-living nematodes require a micro-environment of water in which to live and move, and hence can be considered essentially aquatic organisms (Nicholas 1984, p. 163).

C. elegans is commonly found in organic soils, and so it was expected to be suitable for testing a solid organic medium like pulp and paper mill sludge. As was seen in previous soil tests, the colloidal silica method for efficiently and harmlessly extracting nematodes from sludge was successful enough to generate concentration-response curves for nematode survival in metal-spiked sludge samples. The recoveries were not always as high as they were in soil extractions, but they were sufficient to demonstrate that sludge itself has no apparent toxicity. In addition, sludge provides protection against the toxic effects of added metal ions, and to a greater degree than the four soils previously tested.

These results lend support to the suggestion that C. elegans can be used for toxicity testing of both liquid and solid effluents from the industry, and that these tests may be more reproducible than the tests currently employed. The greatest advantage, however, is that the tests with C. elegans would be much less expensive, requiring minimal and inexpensive laboratory equipment, and less than 35 hours of technician labor to complete a four-day survivability test. This will allow many more samples to be tested, providing a more comprehensive understanding and earlier warning of any toxicity problems.

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Figure Caption

Figure 1. Mean percent responses of nematodes to different concentrations of zinc, cadmium, copper, and lead in various media. Each point is the mean of five replicates (20 nematodes per replicate). Error bars indicate standard error of the mean. Shown are percent recovery and percent survival in pulp and paper mill sludge. Unrecovered nematodes were scored as dead. Included for comparison are previously obtained data for percent survival in metal-spiked Worsham sandy loam soil and water with no soil (aquatic) (Donkin and Dusenbery 1994).

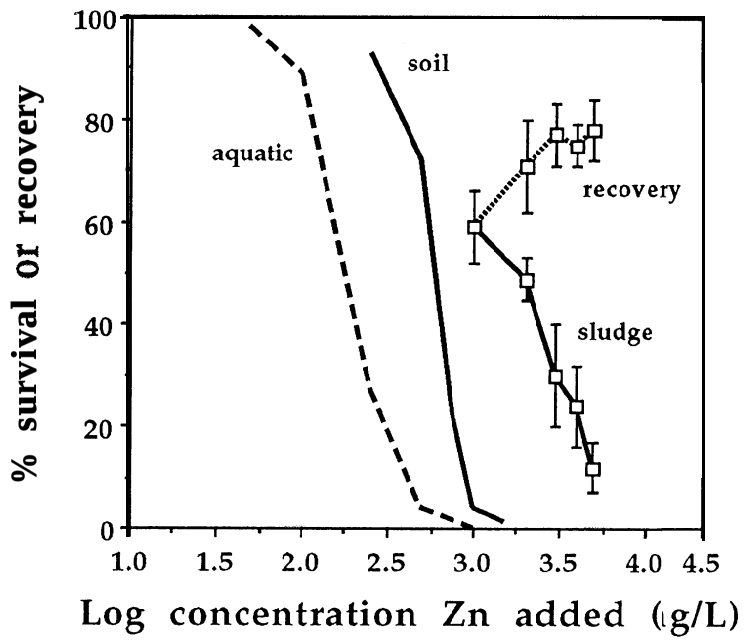
Table I. Inter-laboratory comparisons of LC_{50}s obtained for the nematode aquatic test using five metals.

	Day 1		Day 2		Day 3		Day 4	
Metal	IPST	GT	IPST	GT	IPST	GT	IPST	GT
Al	34	79	12	2	6	2	2	2
As	195	182	181	179	175	176	176	173
Cd	295	904	246	22	93	2	43	1
Cr	229	156	18	63	17	40	11	1
Ni	363	2916	200	185	30	3	6	1

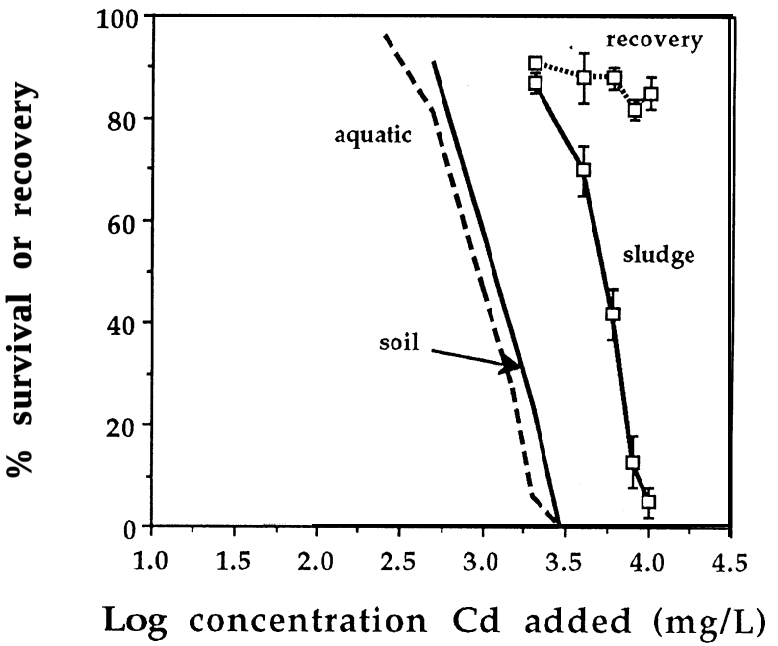
IPST = LC_{50}s (mg/L) obtained at the Institute for Paper Science and Technology.

GT = LC_{50}s (mg/L) obtained at the Georgia Institute of Technology (Williams and Dusenbery 1990).

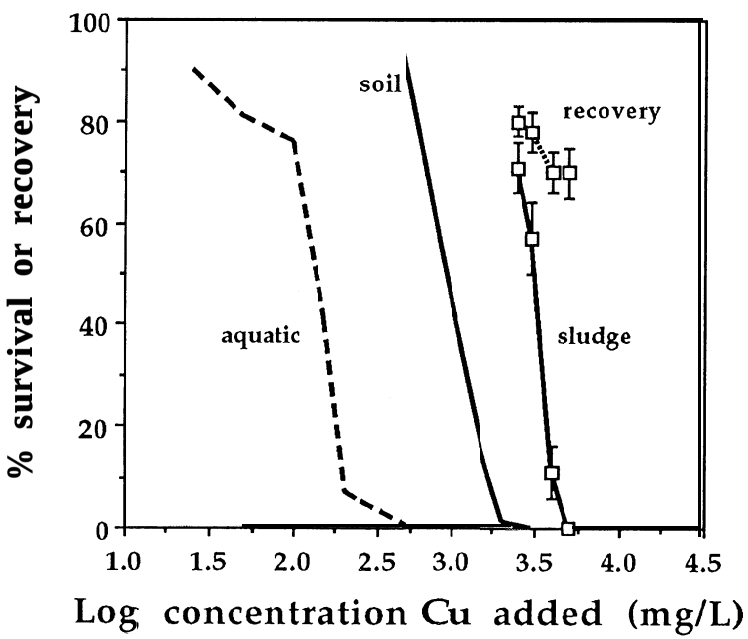
Zinc



Cadmium



Copper



Lead