CAENORHABDITIS ELEGANS AS A MODEL FOR ASSESSING BEHAVIORAL EFFECTS AND SOIL BIOAVAILABILITY OF ENVIRONMENTAL CONTAMINANTS

by

WINDY ANN BOYD

(Under the direction of Phillip L. Williams)

ABSTRACT

The effects of metal exposures on soil ecosystems are observed at all levels of biological organization from changes in enzyme activity at the cellular level to changes in nutrient cycling at the ecosystem level. A handful of standardized toxicity tests exist to assess the effects of toxicants on soil quality; however, knowledge of the effects on various species and functional groups of organisms would allow for more realistic regulations of soil use. Nematodes are the most abundant of soil mesofauna and play significant roles in nutrient cycling and availability via microbial grazing. The free-living nematode *Caenorhabditis elegans* is a popular toxicity test organism in aquatic medium, sediments, and soils. The studies in this dissertation were aimed at furthering the understanding of how the results of *C. elegans* toxicity tests may be affected by external factors such as soil physicochemical properties and food availability. Recently, a standardized guide on the use of *C. elegans* in soil toxicity tests has been published by the American Society for Testing and Materials. This procedure was used to quantify the lethality of 48-h exposures of five metals (Cd, Cu, Ni, Pb, and Zn) to *C. elegans* in soils

with varying texture, organic matter (OM) content, and cation exchange capacity (CEC). The toxicity of the metals decreased as OM and CEC decreased. A sequential soil extraction procedure was then used to recover the metals from different fractions of the soil in an attempt to estimate the bioavailability of the metals. The sensitivity and usability of two other nematode species, *Panagrellus redivivus* and *Pristionchus pacificus*, were compared to *C. elegans* using toxicity tests in soil and aquatic medium with various endpoints including lethality, reproduction, and movement. The sensitivity of the nematodes varied with the endpoint quantified. In a separate study, food availability alone was determined to affect the movement and feeding of *C. elegans* after 24-h exposures in aquatic medium. When 24-h metal (Cu, Pb, and Cd) exposures were performed at different food availabilities, the toxicity of all three metals appeared to increase as food availability decreased implicating starvation as a component of decreased movement in *C. elegans* behavioral assays. Decreasing the exposure to 4 h minimized the complicating effects of starvation.

INDEX WORDS: *Caenorhabditis elegans*, Nematodes, Metals, Soil, Toxicity, Bioavailability, Behavior, Movement, Feeding, Ingestion, Reproduction, *Panagrellus redivivus*, *Pristionchus pacificus*

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B.S., Kansas State University, 1995

M.S., Kansas State University, 1998

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2002

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DEDICATION

This work is dedicated to the memory of Dr. Paul H. Jennings, my mentor and inspiration. I will forever miss his advice and sense of humor.

ACKNOWLEDGEMENTS

I would like to thank Dr. Phil Williams for serving as my major advisor during the course of my PhD. I also extend sincere appreciation to my other committee members: Dr. Marsha Black, Dr. Malcolm Sumner, Dr. Chuck Jagoe, and Dr. Cham Dallas. I am grateful for Dr. Jeffrey Fisher's willingness to replace Dr. Dallas during my final defense. I appreciate all of the time and patience spent on me (and my EPA STAR funding) by Regina Davis as well as all of the assistance provided to me by Sandra McPeake over the years.

Over the years, I have had the pleasure of working and socializing with many EHS and Tox students. Because there are too many to mention, I wish to recognize a few of the students with whom I worked most closely: Deanna Conners, Chris Peredney, Ritu Dhawan, Wilson Everett, Ginger Stringer, and Russ Cole. I also express gratitude to Dr. Gary Anderson for all of his insight into my research and for serving as an unofficial member of my committee (willing or otherwise).

I am most grateful for my family and all of their support and love throughout my academic career. They have continually reminded me of what is most important in life and I can never thank them enough. I above all thank Tracey Crocker for her support, both academically and emotionally. We have spent countless hours together working in the lab, sitting in class, or discussing my dissertation. For all this and so much more, I thank you!

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CHAPTER 1

INTRODUCTION

With growing concerns of the preservation of the environment within the last half of the twentieth century, legislation protecting our air and water has been passed and well-defined regulations are currently in use. Likewise, sediment quality criteria are also being stringently examined. The regulation of soil quality, however, has only recently been considered. Toxicologists and soil scientists are now facing a most challenging and exciting future to determine the effects of polluted soils on soil organisms, food crops, groundwater contamination, and human health, as well as how to best regulate the use of soils.

One area of concern is how to assess the toxicity of soil pollutants. Limited options exist including extraction and chemical analyses of soil solutions, phytotoxicity tests, and earthworm toxicity tests. Major limitations exist when any one test is used alone. Chemical analysis may give a precise estimate of concentrations in soil solutions but offer no information as to the fraction that is bioavailable and thus toxic to organisms. Phytotoxicity, earthworm, and springtail testing give an estimate of the manifestation of toxic effects at specific concentrations for single species with specific ecological roles in the soil ecosystem.

The central hypothesis of this dissertation is that an equally relevant complement to the above methods is a nematode toxicity test. This hypothesis will be addressed throughout this dissertation with a variety of experiments designed to compare the

expression of the toxic effects of metals on the free-living nematode *Caenorhabditis elegans* using lethality, behavior, and reproduction as endpoints. In soil, existing toxicity testing procedures were refined to include estimates of the bioavailability of metals and the use of other nematode species in toxicity tests. In aquatic media, a variety of endpoints were used to assess the behavioral effects of metals on *C. elegans*. In Chapter 2, a summary of the literature reviewed throughout the dissertation is presented. After this general literature review, the dissertation is divided into two parts: 1) the use of *C. elegans* in soil toxicity testing; and 2) the feeding and locomotion of *C. elegans* in aquatic medium.

In order to evaluate the sensitivity of *C. elegans* relative to other soil organisms, 48 h LC50 values (the lethal concentration to 50% of the nematodes) of 5 metals were compared in 2 natural soils and one artificial testing medium to published *Eisenia fetida* LC50 values in artificial testing medium (Chapter 3). These data were also compared to regulatory values of soils in the US and the European Union (EU). Next the bioavailability of metals during soil exposures to *C. elegans* were estimated (Chapter 4). This study was a follow-up to the trends observed in Chapter 3. A sequential soil extraction procedure was used where increasing harshness of reagents led to the extraction of bound metal cations from negatively charged soil particles. The combined use of the *C. elegans* soil bioassay and the chemical extraction assay provided a more accurate means of estimating the toxic effects of metals in other soils based on the physicochemical characteristics of the soil of interest.

Besides estimating the relative sensitivity of *C. elegans* to metal exposures by comparison to published data, the responses of two other nematodes, *Panagrellus*

redivivus and *Pristionchus pacificus*, were compared to those of *C. elegans* after 24-h metal exposures (Chapter 5). Soil exposures used only lethality as an endpoint while aquatic exposures used lethality as well as the sublethal endpoints of reproduction, feeding, and movement. Nematode species were also evaluated by their ease of use and culturing as well as reproducibility in the toxicity tests.

The second major theme of this dissertation was the study of feeding and locomotion of *C. elegans* and how these behaviors were affected by food availability and metal exposures. First, the effects of food availability on feeding and, in turn, movement were investigated (Chapter 6). Also in this chapter control charts were constructed for movement of 3- and 4-day old nematodes in an attempt to quantify 'normal' *C. elegans* movement and provide a means of monitoring the health of *C. elegans* from test-to-test. Chapter 7 builds upon these findings and investigates the combined effects of food availability and metal exposures on *C. elegans* feeding and movement. Through the use of different sublethal endpoints as well as various exposure durations, effects of starvation on movement were separated from the toxic effects of metals. A description of a new method for measuring *C. elegans* ingestion is also provided in this chapter that allowed for the monitoring of the feeding behavior with no *E. coli* present.

The final chapter (Chapter 8) provides the overall conclusions from the entire dissertation and suggestions for future experiments.

CHAPTER 2

LITERATURE REVIEW

This chapter provides an overview of the literature reviewed on three main topics presented throughout this dissertation: the use of the nematode *Caenorhabditis elegans* in toxicity testing; the significance and effects of metals on soil invertebrates; and the behavior of invertebrates after environmental exposures to toxicants. Although some of the material presented is also described in following chapters, this chapter is intended to give a broader and more detailed perspective on these topics than is possible in scientific journal formats.

A. History of *Caenorhabditis elegans* as a toxicity testing organism

Because free-living nematodes feed on bacteria and other microbes within the soil matrix, they play significant roles in nutrient cycling and dynamics (Wood 1988). Soil nematodes living within the interstitial waters of soil particles are in direct contact with dissolved contaminants (Kammenga et al. 1994). After exposure to toxicants in the soil, the ability of soil organisms to perform necessary functions may be impaired, leading to deleterious effects at the ecosystem level (Donkin 1997). Besides their ecological roles, nematodes are also ideal fauna for laboratory toxicity testing. They reproduce in high numbers within short time periods allowing for mass culturing of individuals for toxicity tests. Because of their short life cycles and small size, toxicity tests can be performed rapidly and with small exposure volumes.

Caenorhabditis elegans, a free-living bacterivorous nematode, has been the subject of studies in genetics and developmental biology since the 1970s (Brenner 1974). The characterization of both the neural circuitry and cell lineage is more complete in *C. elegans* than in any other animal (Williams and Dusenbery 1988). The *C. elegans* Sequencing Consortium (1998) has recently sequenced the entire genome. *Caenorhabditis elegans* stocks are readily available from a national repository and can be cultured quickly and easily on agar plates (Williams and Dusenbery 1988) or in liquid media (Williams and Dusenbery 1990a) using *Escherichia coli*, another well-characterized organism, as a food source.

Caenorhabditis elegans has become a popular toxicity testing organism (Power and de Pomerai 1999; Peredney and Williams 2000a; Cioci et al. 2000; Anderson et al. 2001). Although a multitude of tests have been reported, in the interest of brevity, a select few are mentioned for illustrative purposes. Different endpoints of toxicity have been reported including mortality (Cressman and Williams 1997), transgenic expression (Candido and Jones 1996), reproduction (Middendorf and Dusenbery 1993), body length (Traunspurger et al. 1997), and behavior (Williams and Dusenbery 1990b). In addition to different endpoints, various exposure media have also been used including soil (Donkin and Dusenbery 1993, 1994; Freeman et al. 1999; Peredney and Williams 2000b), aquatic (Williams and Dusenbery 1990a; Freeman et al. 1998), agar plates (Williams and Dusenbery 1988), and sediment (Traunsperger et al. 1997).

Laboratory toxicity testing with *C. elegans* has included assessment of metal toxicity (Tatara et al. 1997), as well as recommendations for specific metals as reference toxicants in aquatic media (Cressman and Williams 1997). Besides metal studies, *C. elegans* has

also been used to estimate the lethality of such compounds as sodium pentachlorophenate (PCP) (Donkin and Williams 1995) and the breakdown products of glucosinolates (Donkin et al. 1995). Because the physiology and genetic makeup of *C. elegans* is well understood, the ability to quantify the lethality of toxicants can be supplemented by the possibility of determining the mechanisms associated with the toxic responses (Cressman and Williams 1997).

Recently, the American Society for Testing and Materials (ASTM) has published a standardized guide for soil toxicity testing with *C. elegans* that was prepared by individuals in the same laboratory where this dissertation research was performed (ASTM 2002). Before publication of this guide, the earthworm *Eisenia fetida* was one of the few internationally standardized soil testing organisms. However, many advantages of using *C. elegans* over *E. fetida* have been reported. The *C. elegans* test consists of a 24-48 h exposure time in 2.33 g dry weight soil while the *E. fetida* test has a 14-d exposure time in 200-400 g d.w. of soil (ASTM 1998). Previous studies have indicated that the sensitivities of *C. elegans* after 24-h exposures and *E. fetida* after 2-week exposures are comparable (Peredney and Williams 2000a). In situations where time and/or soil quantities are limited, the nematode test provides an attractive alternative.

B. Significance and Effects of Metals

Metals are important environmental pollutants that are unique from organic contaminants in that they are not created by humans and they often are pure elements that can not be degraded. However, anthropogenic activities significantly alter the amounts and chemical forms of metals in the environment, thereby increasing the risks of metal

exposures to soil and aquatic organisms as well as to humans. For example, the ratio of the amount of metal mined from the earth to the amount naturally-occurring in the environment is particularly high for several metals, including Pb, Cu, Cd, and Zn (Förstner 1995). Principal sources of metals to the environment include both industrial and agricultural practices as well as natural biogeochemical processes. Industrial sources of the greatest significance stem from power generation, battery use and disposal, mining and smelting of ores, municipal solid wastes, chemical manufacturing, and metal manufacturing and plating (Mukherjee 2001). Agricultural sources of concern include phosphate fertilizers, lime, application of sewage sludge and manures to soils, and irrigation with metal-contaminated water (Moore et al. 1998). Other anthropogenic activities may also increase metal concentration and bioavailability in the environment including automobile emissions, incineration of trash, and even cigarette smoke (Alloway 1995). Natural biogeochemical cycles can act to distribute metals globally via soil water erosion, wind-dispersal of soil particles, and atmospheric deposition of metal-contaminated particulate matter and gaseous metals. In fact, contaminated sediments have been designated as the single greatest pollutant of surface waters by volume on the planet (Menzer 1991).

Once present in the environment, metals must reach receptors in target organisms in order to exert toxicological effects. The highest risks of human exposures consist principally of occupational exposures; however, other exposures do exist from environmental exposures due to industrial and agricultural activities. Metals may become important contaminants of food products, as is the case with Cd accumulation in crops grown on fields enriched with contaminated fertilizers (McLaughlin et al. 1999). Before

national restrictions on the use of Pb as a gasoline additive, soils and waters adjacent to roadways were contaminated with high levels of Pb that may remain significant for years to come (Menzer 1991). Ecological consequences of soil and water contamination are the focus of countless studies on effects at all levels of biological organization from subcellular to ecosystem level.

The availability of metals in the soil environment is determined by chemical reactions with soil mineral and organic matter (SOM) surfaces that limit element solubility or solution phase reactions that determine element speciation (Peijnenburg et al. 1999). In soil, environmental availability is dependent upon interdependent factors including environmental conditions and chemical properties of the metals themselves (Sparks 1995). Clay and SOM content affect the number of sorption sites available and, thus, the cation exchange capacity (CEC) of a soil. CEC is consistently regarded as the most influential soil property in the prediction of metal availability (Janssen et al. 1997a; Lock et al. 2000). Increasing valence and decreasing hydrated radius of the metal of interest increase the affinity of sorption sites for the cationic species. Properties such as hardness/softness, electronegativity, and size determine the types and affinities of ligands to which metals can sorb.

Traditionally, the environmentally available metal fraction has been assumed to be equivalent to the unsorbed or solubilized fraction (i.e., the metal concentration in the soil pore water) (Donkin 1997). In aquatic media, the free ion activity model (FIAM) and biotic ligand model (BLM) have been proposed to account for exchangeable ions readily desorbed from solid phases and the competition for the sorption of these ions between biotic tissues and such ligands as dissolved organic matter (DOM) (Peijnenburg

et al. 1999; Di Toro et al. 2001). Because environmental availability cannot be measured directly by chemical analysis and because environmental availability is not always directly related to total metals in soils, measurements of exchangeable metals after extraction with salt solutions (e.g. $Ca(NO_3)_2$ or $Ca(Cl)_2$) have been indicated as better estimators of bioavailability (Conder and Lanno 2000; Janssen et al. 1997b). Several studies have made excellent attempts to identify the degree to which soil characteristics and uptake affect bioavailability or toxicity of metals to oligochaete species (Peijnenburg et al. 1999; Conder and Lanno 2000; Janssen et al. 1997a,b; Lock et al. 2000).

Once a metal is available in the environment, it still must be taken up or sorbed to an organism to be considered bioavailable (Newman and Jagoe 1994). Bioavailable metals may then elicit physiological or behavioral effects usually described in terms of a specific toxicological endpoint such as lethality, decreased reproduction, or increased enzyme activity (Mason and Jenkins 1995). The mechanism of action of metals in invertebrates is generally the result of the binding of the free metal ion with target receptors within the cells of the organism (Beeby 1991). Metals can target numerous receptors and these receptors appear to exhibit little specificity in the binding of metal ions, which can lead to compromised cellular functioning or membrane permeability (Mason and Jenkins 1995). A few of the cellular processes that may be affected by metal toxicity are decreased ATP synthesis, disruption of ionic balance, and impairment of neurological activity. The severity of the toxic effects of metals in the whole organism is largely dependent upon the dose and duration of exposure as well as on the ability of organisms to respond with detoxification mechanisms. In this dissertation, the effects of

dose and duration are observed to elicit different effects on *C. elegans* lethality and sublethal indicators such as movement and feeding.

C. Behavior of Invertebrates

An organism's ability to move is often impaired after exposure to contaminants at sublethal concentrations. Locomotion is a requirement for such activities as migration, food search, reproduction, and predator avoidance. Any changes in locomotion may affect the fitness of the organism and, in turn, lead to effects at the population, community, and ecosystem levels (Baatrup and Bayley 1993). Due to the size of invertebrates and the complexity of their behavior, initial studies on locomotion concentrated simply on whether an animal was moving or not, yielding only qualitative observations.

New computer-automated video tracking systems have allowed for detailed quantitative analyses of movement using such parameters as number of reversals, average rate of movement, and number of animals moving (Dusenbery 1992). Computer tracking of invertebrates may serve as a powerful biomonitoring tool, alleviating the need for more lengthy, tedious, and expensive toxicity endpoints such as mortality and reproduction. An overview of the principal types of behavioral endpoints used in ecotoxicological studies is presented in Table 2.1.

The aim of this work is to provide an overview of the types of behavioral studies that have been reported with terrestrial organisms in the literature and is the result of a book chapter recently published that also describes studies with aquatic organisms (Boyd et al. 2002). Each section focuses on a group of organisms by giving a brief description of their ecological role in the soil environment followed by discussions of the methods,

endpoints, and abbreviated results of specific experiments. The case studies are not an exhaustive list but an overview of the wealth of information in this area.

Nematodes

Nematodes make up a major proportion of the soil biota helping to maintain the soil ecosystem balance through decay of organic matter and nutrient cycling and are an important phylum for testing in terrestrial ecotoxicology studies. Discussed below are representative species of nematodes that have been used in behavioral toxicity testing.

Caenorhabditis elegans is a non-parasitic nematode that lives in the interstitial water of soils, feeding on bacteria and other microbes. The behavior of *C. elegans*, continuous feeding while moving in a wave-like motion (Dusenbery 1980), can now be monitored by a video camera interfaced to a computer tracking system. The rate of locomotion and change of direction of hundreds of subjects are recorded simultaneously (Dhawan et al. 2000; Anderson et al. 2001). After exposure to toxicants in the soil, aquatic or agar media, the worms are rinsed to eliminate debris and any juveniles are then transferred with a small volume of media to a thin layer of 1% agar that is poured directly onto a glass plate. Once they are placed on the agar surface, the worms are allowed to disperse evenly in a chamber over water in order to prevent desiccation. Then, the glass plate is placed upside down, directly above a stream of hydrated air and directly below the camera. A modified version of the NIH Image Tracker software (Dusenbery 1997) allows the user to manipulate parameters such as the threshold level (maximizing the contrast between the background and the worms), the color spectrum, and number of cycles tracked. The data are automatically transferred to an Excel spreadsheet.

Information is provided on the number of subjects moving, their individual rate of locomotion, and the frequency of change in direction.

Studies have been performed with nematodes exposed to metals, ethanol, and pesticides on agar plates and in liquid media (Williams and Dusenbery 1987; Williams and Dusenbery 1990; Dhawan et al. 1999, 2000; Anderson et al. 2001). Comparisons of the results of mortality tests to behavioral changes suggested that computer tracking provided a reliable quantification of toxicity effects. The behavioral mid-point (expressed as the toxicant concentration where the average movement of exposed worms is 50% of control movement) generally is from 25 to 50 times lower than the LC50, providing a very sensitive indicator of toxicity.

Entomopathogenic nematodes are used commercially to parasitize plant pest species. Some researchers have suggested the use of certain pesticides to increase the infectivity and activity of these nematodes. To examine this possibility, infective juveniles (IJs) of *Steinernema carpocapsae* and *S. feltiae* were exposed to two nematicides, oxamyl and fenamiphos (Patel and Wright 1996). A stereomicroscope was used to classify the movement of IJs as normal sinusoidal undulation, coiled and twisted, convulsive or violent twisting, uncoordinated movement, or inactive. *S. feltiae* movement was initially stimulated by oxamyl but this effect diminished with time. Oxamyl had no significant effect on the movement of *S. carpocapsae*. Both nematodes' non-sinusoidal movement was significantly and permanently increased by fenamiphos. Similar effects were observed on nictating behavior, the lifting of the anterior end from the substrate and waving from side to side. Infectivity of wax moths by IJs after 72-h exposures to the pesticides was determined by dissection of moth cadavers. Treatment

with both pesticides resulted in decreased infectivity in both species. For these reasons the authors cautioned against using these pesticides or similar pesticides to enhance nictation and infectivity of entomopathogenic nematodes.

Earthworms

Earthworms are among the largest and most important of the terrestrial invertebrates. Burrowing through the soil, they serve to mix organic and inorganic portions of the soil. Furthermore, their burrows help to increase soil drainage, which may decrease soil erosion and the occurrence of plant diseases. Three main types of earthworms are recognized including the litter dwellers, the vertical burrowing surface feeders, and the horizontal burrowing mineral soil species (Laskowski et al. 1998). *Eisenia fetida*, a litter-dwelling species, is a commonly used test animal in many ecotoxicological studies and an ASTM standard for lethality has been developed (ASTM 1998b).

In a short communication, Ramaswami and Subburam (1992) reported using earthworms to estimate the toxicity of two textile dyes, Navy blue M3R and Direct brown 2G, common waste products of the textile dye industry. In short, *Polypheretima elongata* failed to burrow into soils contaminated with even low concentrations of the dyes. In essence, these tests led the researchers to conclude that, in the field, the worms would avoid habitats contaminated with the textile dyes. This could prove to be detrimental as the dye effluent in India is sprayed on open lands or used as an irrigant in agricultural lands.

A similar design is the avoidance-response test used to assess the toxicity of contaminated soils. Because earthworms possess chemoreceptors, they are extremely sensitive to chemicals they contact in the soil environment. If they encounter highly contaminated soils, they will simply avoid them by moving to a more favorable environment. A sublethal test ranging form 7-72 h was designed to give a rapid, yet reliable, alternative to acute and chronic tests that range from 7-14 d and 28-55 d, respectively (Stephenson et al. 1998). The testing apparatus was a plexiglass container that was divided into six compartments, each of which held 300 g of different dilutions of contaminated or control soils. Holes in the base of the apparatus allowed for worm movement between different compartments. A central compartment without soil was loaded with *E. fetida* or *Lumbricus terrestris*. In both cases, the worms refused to enter only the most contaminated soils. Within 24 h of the start of the test, *E. fetida* had evacuated the two highest levels of contamination while *L. terrestris* took 72 h to do the same. In effect, this sublethal test was found to be more sensitive as well as much quicker than the acute toxicity test since worms avoided the soils that were contaminated at a level below that of the no observed adverse effect level (NOAEL) calculated from mortality data.

A third study focused on the tendency of earthworms to migrate throughout the soil in response to pesticide applications (Mather and Christensen 1998). This migration away from contaminated soils can seriously affect population density and thus the overall quality of the soil. Specially designed troughs were used in the field to trap worms that moved in from a single direction. Traps were lowered into the troughs so that the top of each trap was level with the soil surface. Benomyl, a carbamate fungicide, was used as

the soil contaminant. Of the seven species sampled, a 1.4 to 2.5 fold increase in trappings was observed at the lowest treatment levels of 0.5 and 1.0 kg a.i. ha^{-1}. However, at 2.0 kg a.i. ha^{-1}, there was a 1.3 fold decrease in captures. These differences were seen to be significant as early as 2 d for the two higher concentrations and 4 d for the lowest concentration. In addition, the species composition of trapped worms was changed by the two higher treatments.

Another test system investigated the electrical activity of neural units to give an estimation of the startle reflex, a mean of rapid escape from predators (Drewes and Vining 1984). *E. fetida* was used to test the effects of a neurotoxic insecticide, dieldrin, and two aromatic hydrocarbons, dimethyl phthalate (DMP) and fluorene. Giant nerve fiber conduction velocity was monitored in both the head and tail segments both before and after 48-h exposures. Because dieldrin is neurotoxic, it was shown to cause tonic spasms and hyper-responsiveness to touch. At low doses, dieldrin caused decreased firing of the medial giant nerve fiber (MGF) and, at higher doses, decreased the conduction velocity in both the medial and lateral nerve fibers. Interestingly, fluorene had an opposite effect with low doses decreasing conduction velocity in both nerve fibers and high doses only affecting the MGF. DMP showed only marginal effects even at the highest doses. Decreases in nerve fiber conduction could lead to animals being more susceptible to predation and avoidance of contaminants in the soil. The authors concluded that, although this tool is useful in predicting neurophysiological effects, it was not sensitive to other behavioral effects.

Spiders

Spiders represent a diverse class of organisms. Their predatory behavior involves complex activities such as construction of various web designs to trap and immobilize insects. Because spiders are known to prey on pests, they must be considered beneficial organisms. Historically, research efforts determining the toxicity of pesticides and other toxicants to beneficials have concentrated on mortality as an endpoint. However, a toxicant does not have to be lethal to cause significant changes in behavior that may affect reproduction or predation and thus population dynamics.

Samu and Vollrath (1992) used the common cross spider *Araneus diadematus* to examine the effects of two fungicides (triadimenol and prochloraz) and two insecticides (cypermethrin and paraffinoil) on orb web building. The pesticides were administered orally and topically. Time to construction of the web was recorded and the web was photographed. Digitized images were used to measure web characteristics such as capture area, total length of radii, total length of spiral, and mean mesh size. Only topically applied cypermethrin, a pyrethroid insecticide, delayed the spiders building webs and affected web characteristics. In addition, spiders did not recycle cypermethrin treated webs due to taste aversion.

Using a similar test, Lengwiler and Benz (1994) investigated sublethal effects of primicarb, deltamethrin, diazinon, and dicofol on the gray cross spider *Larinioides sclopetarius*. Web structure was analyzed before and after topical application of the pesticides. Digitized images were used to characterize web parameters including web area, median angle, regularity of angles, width of web over length of web, and distances over spiral turns. No pesticide caused significant mortality at the tested concentrations. Deltamethrin and diazinon treatments resulted in significant delays in web building and

decreases in web area. No other significant effects on web building were observed. Spiders also recovered from pesticide application quickly.

In an attempt to quantify the effects of deltamethrin, a pyrethroid insecticide, on a non-target linyphiid spider, a study was carried out on plots of land sprayed with the insecticide (Jagers op Akkerhuis 1994). Environmental conditions such as air temperature, soil and air humidity, and quantity of rain were also monitored because these conditions are known to affect the behavior, in this case the walking activity, of animals. Linyphiid spiders live most of their time hidden from the direct spray of insecticides, either in cracks in the soil or under the plant canopy. However, as they search for mates or prey or a place to nest their eggs, walking puts them at greater risk to be exposed to spray or residues. Not only do the activities above depend upon environmental conditions, so does the speed at which the spiders walk. In the case that the spiders come in contact with residues of an insecticide, their walking activity decreases leading to possible death by predation or through water loss.

Round pitfall traps were placed at regular intervals from the outside of each treated or control plot. Spiders were emptied from the traps daily and, after the first 12 h after spraying, every 1.5 h. Insecticide residues were measured at ground level as well as the environmental conditions previously mentioned. Two parameters were developed to estimate the walking activity of the spiders: an instantaneous effect which measured the decline in trap catches and a recovery time which was a measurement relative to the observed instantaneous effect. After deltamethrin spraying, they found a 50-75% decrease in initial effects and recovery half-lives that ranged from 1-3 d.

Springtails

Collembolans, or springtails, are among the most abundant of the terrestrial soil invertebrates. Springtails feed on fungi and other microbes and, in highly acidic soils, serve as primary decomposers (Laskowski et al. 1998). In addition, springtails serve as prey for many larger soil invertebrates such as spiders, beetles, and centipedes. Because of their ecological significance, collembolans are used in a wide range of ecotoxicological tests. In fact, the springtail *Folsomia candida* is used as an international standard test organism for the testing of soil quality (Wiles and Krogh 1998). They also offer the benefits of a rapid life cycle, ease of laboratory cultivation, and a high parthenogenic reproduction rate.

Because springtails are prone to desiccation, their locomotory activity allows them to escape adverse environmental conditions. However, a means to measure their activity was difficult before the aid of computer tracking devices. In one study, a computer-automated video-tracking device was used to record and analyze the 2-dimensional movement of *F. candida* (Sørensen et al. 1995). Dimethoate, an organophosphate pesticide, was used as the contaminant. During the 36-h exposure period, springtails were observed to be increasingly hyperactive and displayed some difficulty in coordinated movement. For the first ten hours of the exposure, the only significant change was an increased turning rate. However, during the last ten hours of the exposure, significant decreases in average velocity and path length as well as increases in number of stops and turning rates were observed.

Other researchers also investigated the effects of dimethoate on another collembolan, *F. fimetaria* (Fábián and Petersen 1994). Unlike the computer tracking

studies, visual observations were made once every hour for 24 h. The activity state of the springtails was noted as at rest, walking, struggling, or motionless. The last two descriptors indicated abnormal activity associated with contaminant effects. Another experiment allowed springtails to choose between a contaminated or uncontaminated environment, similar to the earthworm study mentioned previously. Area-choice was recorded every hour for the first 10 hours and once a day for the remaining 7 days. A final experiment observed the dispersion and spatial distribution in a microcosm treated with five concentrations of dimethoate. Collembola location was noted after 1, 3, or 7 days. Abnormal activity (struggling and motionless behavior) was noted to increase with increasing doses. In the area-choice experiments, springtails either avoided or escaped from the contaminated side of the canisters. About half of the test organisms were located in the control soil within a few hours and, after a few days, nearly all were. However, no significant effect was seen on the spatial distribution or dispersion habits of the springtails in the microcosm test indicating that complete avoidance of contaminants is unlikely in a natural soil.

Aphids

Although not true soil inhabitants, aphids are known horticultural and agronomic crop pests and are routinely recovered in pitfall traps placed in agricultural fields. Harrewijn (1997) used EPG to study the effect of a novel aphidicide, pymetrozine, applied in several ways on different phases of stylet penetration and feeding activity on individual aphids. Pymetrozine was either topically applied, directly injected into the hemolymph, added to a chemical diet, sprayed onto plants, or plants were allowed to soak

for 24 h in a treated solution for root uptake. Topical applications of 150 ng pymetrozine/mg fresh weight inhibited stylet insertion into the plant. When injected, less than 30 ng/mg was sufficient to produce the same effect. When pymetrozine was systemically applied via plant spraying or root uptake, aphids fed normally. After feeding, they withdrew their stylets and appeared normal. However, at low doses aphids were able to resume feeding whereas aphids exposed to higher doses, appeared to have irreversibly disrupted feeding and were unable to re-insert the stylet for feeding. Aphid mobility was not affected up to an estimated hemolymph concentration of 1 mmol pymetrozine. Aphids that eventually stopped feeding on treated plants showed EPGs with distorted salivation and ingestion patterns, suggesting that pymetrozine selectively interferes with the nervous regulation of feeding behavior, which results in death due to starvation after a few days.

Beetles

In general, beetles feed on the larvae of other insects as well as on adult aphids and collembolans (Laskowski et al. 1998). In addition, they serve as prey to larger animals such as birds and mice. While there are a considerable number of beetles that live on or near the soil surface, the following description will focus on a group of cereal aphid predators including ground beetles, rove beetles, and a ladybird beetle.

A video camera was used to record the movement of the beetles across a sandy loam soil after treatment with the insecticide deltamethrin (Wiles and Jepson 1994). An index was generated that was intended to reveal the susceptibility of the beetles to pesticide residues. This index consisted of two parameters: an exposure function which

gave an estimate of how fast the animal walked, how wide its walking track was, and the proportion of the animal that came in contact with the soil surface; and a susceptibility function that was an LD50 after topical treatment with the insecticide. In this manner, a high index would indicate that an insect was vulnerable to contact with lethal doses of the toxicant while a low index would be indicative of a decline in contact and thus uptake and susceptibility. The large ground beetles, *Pterostichus melanarius* and *Nebria brevicollis*, medium-sized ground beetle, *Agonum dorsale*, small ground beetles, *Demetrias atricapillus* and *Bembidion lampros*, small rove beetle, *Tachyporus hypnorum*, and ladybird beetle *Coccinella septempunctata* were used as the test species in the study. First, the topical LD50 for each species was determined for the susceptibility function. Next, the mean walking speed for each species was determined by recording the movement of 5 individuals at a time with a video camera. After recording, the walking paths were traced onto acetate that covered the video monitor. The walking paths were then measured with an ipsometer. Finally, contact area and track width was measured by placing animals on kymograph paper covered with soot. As the animals moved across the paper, a clean trail was left behind on the sooted paper and image analysis was used to estimate the area swept clean. Although the index did not predict all of the beetles' susceptibility in the correct order, it was able to predict the extreme susceptibilities. The authors suggest other factors that must be taken into account before the index is able to predict mortality reliably.

The International Organization for Biological and Integrated Control of Noxious Animals and Plants (IOBC) has published an official guideline detailing the Carabid Beetle Laboratory Test using *Poecilus cupreus* to assess effects of pesticides before

registration (Heimbach 1992). This test was used to compare the effects of 126 compounds on beetle behavior. Test chemicals were misted onto a sand surface including beetles and their food (Römbke and Heimbach 1996). No significant mortality was observed with any substance tested. Abnormal behavior began as impaired movement of the mandible followed by uncoordinated walking and finally "lay(ing) on their backs with shivering legs". Feeding rate of the beetles was also recorded during testing. However, the majority of tests showed no significant difference in behavior or feeding.

Bees

Learning, an aspect of behavior, can also be affected by toxicants. An excellent review of learning in invertebrates is recommended to the reader desiring a more comprehensive coverage (Abramson 1994). As with spiders, toxic effects of insecticides on beneficial insects such as honey bees are of significance. Although most toxicity tests have been based solely on mortality data, effects on behavior in bees could lead to lower honey yields as well as decreased pollination.

The toxicity of dicofol, a chlorinated hydrocarbon acaricide thought to be relatively non-toxic, was estimated using the Africanized honey bee, *Apis mellifera* (Stone et al. 1997). Classical conditioning of proboscis extension was used to measure sublethal toxic effects on learning. Dicofol was mixed with a sucrose solution and offered as a food source. Three seconds of a cinnamon odor served as a conditioned stimulus scent (CS). After the CS, touching the antennae stimulated proboscis extension. Honey was then delivered as the unconditioned stimulus (US). Bees were considered to

learn if they extended their proboscis after delivery of the CS but before the US. Although dicofol-exposed bees did learn to some degree, a decreased response to CS as well as a shorter persistence of learning was observed when compared to the controls.

In a similar test, Abramson et al. (1999) tested the effects of endosulfan, decis, baytroid, and sevin, four pesticides commonly used on cotton crops in Brazil. The study showed that not only were *A. mellifera* not repelled by the pesticides, they consumed the pesticides when mixed with a sucrose solution. Proboscis conditioning was again used to test the ability of the bees to learn. At the recommended concentrations, all pesticides except decis were observed to disrupt learning of *A. mellifera*. Besides inhibition of learning, the insecticides also led to tremors and vomiting in the bees.

Woodlice

Woodlice act as primary decomposers of dead plant material and other organic material. Before fully digesting, they forward the organic matter back into the soil, making it available to other soil fauna (Laskowski et al. 1998). Woodlice and other isopods search for cool humid places for shelter during the heat of the day. If their ability to move to these shelters is impeded or slowed, the possibility of desiccation and thus lethality is increased.

Sørensen *et al.* (1997) performed a study on woodlice by use of a similar system to the one used for springtails. In this case, the effects of the resulting pollutants formed by a plastics fire were observed in the woodlouse *Oniscus asellus*. Suspected and known contaminants included polyvinyl chloride (PVC) and polyethylene (PE). The activity of 6 animals was recorded for 4 hours and digitized to obtain the path length, velocity, total

number of moves/ walked meter, turning rate, and turn bias. Woodlice collected directly from the plastic layer walked about half as far as those collected from a reference site. A decreased average velocity and time spent in activity were also observed. However, in woodlice collected from the edge of the plastic layer, there were no significant differences in behavioral parameters. Juveniles hatched from the collected woodlice did not exhibit any significant changes in behavior. Similar studies have been executed with dimethoate on the woodlouse *Porcellio scaber* (Bayley 1995; Bayley and Baatrup 1996).

Conclusion

Based on the literature reviewed in this chapter, there are numerous opportunities for research to evaluate the effects of toxicants to soil ecosystems. Therefore, the purpose of this dissertation work is to address some of these needs with a variety of toxicity tests and the nematode *C. elegans*. The relationship between soil physicochemical properties and the environmental availability of metals in soils is estimated using a sequential soil extraction procedure. The relative sensitivity of nematodes to metal exposures is compared with several endpoints of toxicity. Feeding and movement assays are used to investigate the sublethal effects of metals with varying food availability and exposure duration. The end result of these experiments will provide critical information on future directions of research with *C. elegans* as a model for assessing the toxicity of pollutants to soil organisms.

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Table 2.1. Principal behavioral endpoints employed in ecotoxicological studies.

Behavioral Endpoint	Test Media	Organisms	Toxicants Tested
Burrowing response	Sediment	Crustaceans	PCBs, metals, hydrocarbons
	Terrestrial	Annelids	Textile dyes
	Sediment	Annelids	PCBs, metals, and pesticides
Phototactic behavior	Aquatic	Amphipods and daphnids	Copper and lindane
Migratory activity	Terrestrial	Earthworms	Benomyl
Swimming activity	Aquatic	Daphnids, barnacle nauplii, protozoa and rotifers	Variety of organics, salts and metals
Locomotion	Terrestrial	Beetles, nematodes, springtails and woodlice	Variety of metals and organics
Walking activity	Terrestrial	Linyphiid spider	Deltamethrin
Feeding and ingestion rate	Aquatic	Daphnids and rotifers	Variety of metals and pesticides
Valve closure	Aquatic	Molluscs	Variety of metals and pesticides
Case building and net spinning	Aquatic	Caddisflies	Cadmium and copper
Web building	Terrestrial	Spiders	Pesticides
Mate guarding behavior	Aquatic	Amphipods	Variety of metals and pesticides
Ovipositing behavior	Aquatic	Midges	Cadmium
Predator-prey interactions	Aquatic	Rotifers	PCP
Infectivity and nictation	Terrestrial	Entomopathogenic nematodes	Pesticides
Learning	Terrestrial	Honey bees	Variety of pesticides

Table 2.2. Summary of Behavioral Tests using Terrestrial Organisms.

Organism	Species	Toxicants Tested	Endpoint	Reference
Beetle	*seven species*	Deltamethrin	Locomotion	Wiles and Jepson 1994
	Poecilus cupreus	Various pesticides	Locomotion	Römbke and Heimbach 1996
Caddisfly	*Hydropsyche angustipemnis*	Copper,heavy metal and toxic chemical effluents	Net-spinning activity	Pascoe et al. 1991; Petersen and Petersen 1983
	Agapetus fuscipes	Cadmium	Case-building activity	McCahon et al. 1989
Earthworm	*Polypheretima elongata*	Textile dyes	Burrowing preference and avoidance	Ramaswami and Subburam 1992
	Eisenia foetida	Benomyl,dieldrin, dimethyl phthalate, fluorene	Burrowing preference and avoidance, migratory activity, escape reflexes	Stephenson et al. 1998; Mather and Christensen 1998
Honey bee	*Apis mellifera*	Dicofol, endosulfan, decis, baytroid, sevin	Proboscis conditioning	Stone et al. 1997; Abramson et al. 1999
Linyphiid spider	*Oedothorax apicatus*	Deltamethrin	Walking activity	Jagers op Akkerhuis 1994
Nematode	*Caenorhabditis elegans*	Anesthesia, tumor promoters, copper, mercury, beryllium, mercury, lead, cadmium, nickel, malathion, vapona, various organic compounds, ethanol	Locomotion	Miwa et al. 1982; Morgan and Cascorbi 1985; Williams and Dusenbery 1987, 1990; Avery and Horvitz 1990; Terrill and Dusenbery 1996; Dhawan et al. 1999, 2000; Anderson et al. 2001
	Steinernema sp.	Oxamyl and fenamiphos	Locomotion, nictation, and infectivity	Patel and Wright 1996

Table 2.2 (cont.). Summary of Behavioral Tests using Terrestrial Organisms.

Organism	Species	Toxicants Tested	Endpoint	Reference
Spider	*Araneus diadematus, Larinioides sclopetarius*	Various pesticides	Web building activity	Samu and Vollrath 1992; Lengwiler and Benz 1994
Springtail	*Folsomia sp.*	Dimethoate	Locomotion	Fábián and Petersen 1994; Sørensen et al. 1995
Woodlouse	*Oniscus asellus*	PVC and polyethylene	Locomotion	Sørensen et al. 1997
	Porcellio scaber	Dimethoate	Locomotion	Bayley and Baatrup 1996

CHAPTER 3

METAL LC50s OF A SOIL NEMATODE COMPARED TO PUBLISHED EARTHWORM DATA[1]

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[1] Boyd, W.A., Stringer, V.A., and Williams, P.L. 2001. *Environmental Toxicology and Risk Assessment: Science, Policy, and Standardization – Implications for Environmental Decisions: Tenth Volume, ASTM STP 1403*, B. M. Greenberg, R. N. Hull, M. H. Roberts, Jr., and R. W. Gensemer, Eds., American Society for Testing and Materials, West Conshohocken, PA, pp. 223-235.

Abstract

A comparison of the acute LC50s for five metals between the standard test organism *Eisenia fetida* and the soil nematode *Caenorhabditis elegans* was made. Although the test with *C. elegans* is shorter (24 h vs. 2 wks) and uses less soil or testing medium (2.33 g vs. 200 g dry weight) than that for *E. fetida*, LC50s were comparable for the earthworm and nematode. The current study further investigated similarities by extending the exposure time to 48 h. Comparisons were made to 24-h *C. elegans* data, published *E. fetida* data, and U.S. Environmental Protection Agency (EPA) allowable concentrations. The nitrate salts of Pb, Ni, Cd, Zn, and Cu were used to generate LC50s. Two naturally occurring soils in Georgia were chosen to compare different soil properties on the metals' toxicity. Tifton soil was sampled from the southern region of Georgia and is characterized by relatively high sand and low clay and soil organic matter (SOM) contents. Cecil soil, in contrast, is found in the Piedmont region of Georgia and is characterized by relatively high amounts of clay and SOM. As anticipated, extending the exposure time to 48 h significantly increased the toxicity (i.e. decreased the LC50s) of the metals compared to published 24-h *C. elegans* data. Physical-chemical properties of soils are known to affect the binding of polyvalent metals and thus the bioavailability and toxicity of these metals. Increasing clay and SOM contents allow for an increased capacity to bind metals. For this reason, LC50s were higher in Cecil than in Tifton soil. Because tests using *C. elegans* are rapid, reliable, and generate data comparable to that of the earthworm, we suggest further studies that may lead to the standardization of the nematode for use as a soil toxicity-testing organism.

Introduction

In selecting an appropriate test species with which to assess toxicity of soils, both idealistic and realistic criteria must be considered (Lökke and van Gestel 1998). Ideally, a species should be ecologically and geographically relevant to the soil ecosystem, able to tolerate different soil conditions (pH, moisture content, etc.), and representative of multiple routes of exposure. Realistically, however, criteria for species selection often include consideration of the cost and duration of the test, ease of obtaining and culturing the test species, knowledge and understanding of the basic biology of the test species, and regulatory acceptance of the test. No single test species can meet all of the above criteria. However, nematodes appear to satisfy most of them.

Nematodes make up a major proportion of the soil biota. They help to maintain soil ecosystem stasis by contributing to the degradation of organic matter in soil and to the cycling of nutrients (Freeman et al. 1998). *C. elegans* is a non-parasitic nematode that lives in the interstitial water of soils, feeding on bacteria and other microbes. Since the 1970s, the advantages of using *C. elegans* for genetic studies have been known (Brenner 1974). The characterization of both the neural circuitry and cell lineage is more complete in *C. elegans* than in any other animal (Williams and Dusenbery 1988). The *C. elegans* Sequencing Consortium (1998) has now sequenced nearly the entire genome.

C. elegans is readily available from a national repository and can be cultured quickly and easily on agar plates (Williams and Dusenbery 1988) or in liquid medium (Williams and Dusenbery 1990) using *Escherichia coli*, another well-characterized organism, as a food source. Because the physiology and genetic makeup of *C. elegans* is well understood, the ability to quantify the lethality of toxicants can be supplemented by the

possibility of determining the mechanisms associated with the toxic responses (Cressman and Williams 1997). *C. elegans* is currently being used as a model test organism.

Laboratory testing with *C. elegans* has included assessment of metal toxicity (Tatara et al. 1997), as well as recommendations for specific metals as reference toxicants in aquatic media (Cressman and Williams 1997). Besides metal studies, the nematode has also been used to estimate the lethality of such compounds as sodium pentachlorophenate (PCP) (Donkin and Williams 1995; Cressman and Williams 1997) and the breakdown products of glucosinolates (Donkin et al. 1995).

C. elegans is ideally suited to assess toxicity associated with porewater exposures because it resides in water within the soil matrix. Soil sorption (i.e., the capacity of soil particles to bind chemical substances) may alter the bioavailability of contaminants in soils and soil porewaters and influence the results of soil toxicity tests. As a result, the use of the nematode toxicity test has increased over the last ten years (Donkin and Dusenbery 1993, 1994; Donkin 1997; Freeman et al. 1999; Peredney and Williams 2000). Given the many advantages of *C. elegans* and the need for acceptable soil bioassays, a standardized method for soil toxicity testing with the nematode would be very useful.

Currently, the earthworm *E. fetida* is one of the few internationally standardized soil test organisms. Our proposal is not to replace the earthworm but to strengthen the validity of soil toxicity testing with multi-species comparisons. Although the earthworm test is widely accepted, in some situations, a nematode test might prove more applicable. The proposed nematode test consists of a 24-48 hour exposure time in 2.33 g dry weight (d.w.) of soil. The standardized earthworm test, however, has a 14-day exposure time in

200-400 g d.w. of soil (ASTM Standard Guide for Conducting Laboratory Soil Toxicity or Bioaccumulation Tests With the Lumbricid Earthworm *Eisenia fetida* E1676). In situations where time and/or soil quantities are limited, the nematode test would provide an attractive alternative. Other considerations may include species sensitivity to contaminants and previous studies have indicated that the sensitivities of *C. elegans* and *E. fetida* are comparable.

The objectives of this study were to assess the effects of exposure time and soil type on the toxicity of five metals (Cd, Cu, Ni, Pb, and Zn) to *C. elegans*. These metals were chosen based on the availability of earthworm data and 24-h data using *C. elegans*. Further assessment of the applicability of the soil nematode test as a standardized testing protocol was also examined.

Materials and Methods

The wild type N2 strain of *C. elegans* was obtained from the *Caenorhabditis* Genetic Center (Minneapolis, MN) and used in all tests. Cultures were maintained as previously described to yield age-synchronized adult worms (Cressman and Williams 1997). Reference toxicant tests with Cu were performed to verify the health of the worms (Freeman et al. 1998). Adult worms (3 days old) were rinsed from culture plates and transferred to exposure dishes at three days old. After a 48-h exposure period, the supernatants of test solutions were measured for pH (Orion Model 720 pH meter and Ross combination electrode). The pH of each sample was between 4.5 and 5.0, well within the acceptable range of approximately 3-12 for *C. elegans* (Khanna et al. 1997).

Soil Toxicity Testing

A modification of the soil test procedure previously described was used (Donkin and Dusenbery 1993, Freeman et al. 1999, and Peredney and Williams 2000). Thirty-five mm petri dishes were loaded with 2.33 g d.w. Cecil or Tifton soil and spiked with 1.5 mL of testing medium containing the appropriate concentration of nitrate metal salt. Metal concentrations were determined from range finding tests. Nematodes were exposed to nitrate salts of Cd (0, 10, 30, 50, and 70 mg/L for Tifton and 0, 50, 100, 150, and 200 mg/L for Cecil), Cu (0, 10, 20, 30, and 40 mg/L for Tifton and 0, 150, 250, 350, and 450 mg/L for Cecil), Ni (0, 20, 40, 60, and 80 mg/L for Tifton and 0, 50, 100, 150, and 200 mg/L for Cecil), Pb (0, 35, 70, 105, and 140 mg/L for Tifton and 0, 250, 500, 750, and 1000 mg/L for Cecil), and Zn (0, 75, 112.5, 150, and 187.5 mg/L for Tifton and 0, 200, 300, 400, and 500 mg/L for Cecil). For each metal tested, five dishes were spiked, a control and four increasing metal concentrations diluted in K-medium (Williams and Dusenbery 1990). Each test was replicated three times. After spiking, the test soils were allowed to equilibrate at 20^{o}C for 7 days. Although 24-h exposures required no addition of bacteria as a source of food for the nematodes (Freeman et al. 1999 and Peredney and Williams 2000), it was necessary to add food for 48-h exposures to assure control survival. Fifty millilitres of an L-broth solution with *Escherichia coli* (OP50 strain) was centrifuged at 750 *g* for 10 minutes. The supernatant was removed and the pellet was re-suspended in 10 mL of K-medium for a 5:1 (L-broth to K-medium) concentration (Donkin and Williams 1995).

After the soil was allowed to equilibrate for 7 days, 1.5 mL of 5:1 L-broth was added to each test dish to re-wet the soils and provide food. Ten worms per dish were then

transferred with a platinum wire to the soil surface (Freeman et al. 1999). Test units were incubated at 20°C for 48 h (±1 h). After exposure, the soil and worms were rinsed from the petri dishes into 50-mL centrifuge tubes with Ludox® (Dupont, East Chicago, IN), a colloidal silica suspension. Each tube was mixed with a vortex at low speed for ~ 20 s to ensure thorough mixing of the soil suspension. After centrifuging for 2 min, tubes were set aside for ~ 15 min to allow time for the worms to float to the top of the solution.

The solution was then poured into 100-mm glass petri dishes and viewed under a light microscope. Worms were removed from the solution with a platinum wire, placed in a separate petri dish containing K-medium, and examined under a microscope. If the worm did not respond to gentle probing with the platinum wire, it was scored as dead. Live worms were either obviously swimming or moving after probing. In accordance with previous testing in our lab, unrecovered worms were scored as dead, control survival was greater than or equal to 90% and percent recovery of all treatment groups exceeded 80% (Freeman et al. 1999).

Initial metal spiking solutions were collected into 20-mL polyethylene vials, acidified with 250 µL of concentrated HNO_3, and stored at 4°C. Actual measured concentrations were verified to be within 10% of the nominal concentrations with atomic absorption spectrometry. LC50s from each replicate were generated using log-transformed data in Toxstat® 3.4 (WEST Inc. and Gulley 1994). The LC50s were reported as the mean value of three replicates. Standard deviations (SD) and coefficients of variation (CV) were also calculated from LC50s.

A three-way analysis of variance (ANOVA) was used to test for interactive effects of metal, soil type, and exposure time. All data passed the normal assumptions of ANOVA

(normality, homeoscedasticity, and independence). Because there was a significant interaction among all three factors, two-way ANOVA was then performed to test for interactive effects between soil type and exposure time within each metal. Paired comparisons were performed with a Student's t-Test. A p-value less than 0.5 indicated a significant difference in all statistical tests. All statistical operations were performed using SAS (SAS 1989).

Results

Average 48-h LC50s and CVs are presented in Table 3.1. All CVs for the 24-h test were <10% (Peredney and Williams 2000). In the current test, CVs were higher for two reasons: 1) two operators were performing the tests and 2) the average LC50s were lower.

Significant interactive effects were found between soil type, exposure time, and metal using three-way ANOVA for LC50s expressed in terms of mg/kg of soil or μmol/L (Figure 3.1). Two-way ANOVAs also found significant interactions between soil type and exposure time within each metal. The Student's t-Test detected significant differences in means between soil type and exposure time for each metal. Average LC50s and standard deviations are presented in Figures 3.2 and 3.3. Increased exposure time resulted in increased toxicity or decreased LC50s. At both exposure times, higher LC50s were observed in Cecil soil than in Tifton soil for all metals.

Table 3.2 compares LC50s in soil tests with those in K-medium without soil present (24-h soil data from Peredney and Williams 2000; aquatic data compiled from Williams and Dusenbery 1990a, Cressman and Williams 1997, and Tatara et al. 1997 and 1998).

In general, LC50s in Cecil soil were higher than in Tifton and Tifton LC50s were higher than aquatic values. The only exception for the 48-h data was Ni. The 48-h LC50 in K-medium only was higher than Cecil and Tifton values. This pattern was true for the 24-h data as well. In fact, all of the metals except Pb appeared to be more toxic in Tifton soil than in liquid medium at 24 h. In all exposure media, toxicity always increased with longer exposure time.

Discussion

Nematodes feed on bacteria and other microbes within the interstitial waters of the soil matrix, playing a substantial role in such processes as the nitrogen cycle, mineralization, decomposition, and nutrient mobilization (Wood et al. 1988). After exposure to toxicants in the soil, the ability of soil organisms to perform necessary functions may be impaired, leading to deleterious effects at the ecosystem level (Donkin 1997). Although the ecological significance of *C. elegans* has not been fully explored, the response of *C. elegans* to Cd is comparable to that of other nematode species (Kammenga et al. 1994). In addition, approximately 60% of *C. elegans* genome is shared by all nematodes (Blaxter 1998). A wealth of information exists on the biology, physiology, and genetics of *C. elegans* and stocks are easily cultured and maintained in the laboratory. These characteristics support the use of *C. elegans* in soil bioassays.

All metals appeared to be more toxic to *C. elegans* at an exposure time of 48 h in Tifton soil than to the earthworm *E. fetida* exposed for 14 days in ASTM soil (Table 3.3). When compared to the EPA's allowable limit for metals in land-applied sewage sludge, the ability of these limits to protect indigenous soil nematodes is questionable. While EC

(European Commission) limits for soils are typically more stringent than U.S. limits, they are still higher in some cases.

Acceptability criteria for 24-h soil testing have been established in our lab (Peredney and Williams 2000). The 24-h test required a percent recovery of live and dead worms >80% and control mortality <10%. Although the percent recovery of worms for the 48-h test was lower than that for the 24-h test (87% vs. >99%), each individual test had control survival ≥ 90%. A lower percent recovery with increased exposure time can be explained by the observation that most unrecovered worms were at the highest concentrations. If the worms died early in the exposure, decomposition processes would make the worms unidentifiable and, therefore, unrecoverable. For this reason, a lower percent recovery would be appropriate.

While the 48-h test produced lower LC50s than the 24-h test, the question remains: which exposure period produces results that reflect the actual toxicity of the soils? Our 24-h data were more comparable to those for the earthworm. Earthworm data are currently used to predict soil toxicity. If the earthworm test data accurately reflect metal toxicity then the 24-h test with nematodes should also be acceptable. Because nematodes perform such crucial roles in soil ecosystem processes, the 48-h test would more realistically reflect soil toxicity.

Besides lethality, additional endpoints of toxicity may also provide sensitive indications of sub-lethal toxic effects. Commonly-used sub-lethal endpoints used with invertebrates include reproduction, behavior, growth, and feeding and these have been previously explored with *C. elegans* in aquatic media (Anderson et al. 2001, Boyd et al.

2000, and Dhawan et al. 1999). Applications of these endpoints in soil toxicity tests require further study.

Toxic effects vary with soil conditions, species, and the specific contaminant and, thus, must be investigated on a site-specific basis. We feel that the nematode soil bioassay would prove useful as a supplement to existing standardized soil bioassays. The soil bioassay used in this study uses less soil (2.33 g) than the current standardized earthworm test (200g). In a situation where limited quantities of soil are available, the nematode soil bioassay would be an alternative to the earthworm test. Because a variety of species are involved with nutrient cycling and soil decomposition processes, single species tests may be an inadequate means of assessing toxicity of contaminants (Parmelee et al. 1997).

The bioavailability of metals in soil is dependent upon many factors. One of the most significant processes determining the fraction of chemical available in solution is sorption. Sorption is an electrostatic attraction between the positively charged metal cations in solution and negatively charged groups on SOM and clay particles. This phenomenon is dependent upon the physicochemical properties of the soil as well as environmental conditions. The pH of the soil is a most critical determining factor in soil sorption and soil speciation. Low pH values result in protonation of SOM functional groups and, therefore, in decreased sorption of cations.

The sorption of a particular metal is also dependent upon its chemical properties. Although the sorption selectivity is highly variable, in general, Cu, Pb, and Zn are sorbed more strongly than Cd and Ni (Sparks 1995). This could account for large differences between the 48-h LC50s for Cu and Pb (~ 12 and 11 times, respectively) between soil

types (Figures 3.2 and 3.3). Toxic responses are a result of dose (or exposure concentration) and exposure time. Increased exposure time was shown to increase toxicity of all metals within a soil type. However, 24-h LC50s for Cu, Pb, and Zn in Tifton soil were either less than or equal to 48-h LC50s in Cecil soil. If these metals are strongly sorbed by SOM and Cecil contains significantly higher SOM than Tifton, it is reasonable to assume that the nematodes must have been receiving a lower dose of these metals over the exposure period.

These observations stress the importance of soil type on the resultant toxicity of metals. In standardized soil bioassays, an artificial medium typically replaces a natural soil as the testing medium. Organic carbon content gives a relative measure of the SOM content. ASTM artificial medium contains approximately 5% organic carbon compared to 2.5 and 0.7% in Cecil and Tifton soils, respectively. If metals were less toxic in Cecil soil than in Tifton soil, these same metals would appear to be even less toxic in ASTM artificial medium. This effect would be greatest for the most strongly sorbed metals described above and may partially explain the differences seen between the earthworm and nematode LC50s (Table 3.3). These results indicate the need for site-specific determinations of toxicity and assessment of toxicity in natural soils. A synthetic-testing medium such as the ASTM 'soil' can not mimic the effects of soil formation factors on soil properties. This could lead to overestimation or underestimation of the actual risk (Parmelee et al. 1997).

Future studies with our soil bioassay will focus on the measurement of metals within the soil solution rather than the amount spiked into the soil. In this manner, we hope to investigate the relationships between soil sorption and lethality of metals. To further

validate our bioassay, we will also compare the toxic effects of different contaminants to *C. elegans* and other nematode species. Sub-lethal endpoints of toxicity are also being examined to provide a more sensitive indicator of soil toxicity.

Acknowledgments

We are grateful to the University of Georgia's Agricultural Experiment Station for financial support of this research. The nematode strain used in this work was provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

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Table 3.1 – Average 48-h LC50s and coefficients of variation (CV) of C. elegans *exposed to metals in two field-collected soils.*

Metal	CECIL Avg LC50 (mg/kg)	CECIL Avg LC50 (μM)	CV (%)	TIFTON Avg LC50 (mg/kg)	TIFTON Avg LC50 (μM)	CV (%)
Cd	80	1106	12	18	255	22
Cu	193	4716	8	16	383	17
Ni	71	1868	17	34	891	7
Pb	487	3650	3	43	322	8
Zn	219	5211	15	76	1810	10

Table 3.2 – Average 24- and 48-h LC50s in Tifton and Cecil soils and in K-medium (no soil present).

Metal	Tifton 24-h LC50 (mg/L)	Tifton 48-h LC50 (mg/L)	Cecil 24-h LC50 (mg/L)	Cecil 48-h LC50 (mg/L)	Aquatic 24-h LC50 (mg/L)	Aquatic 48-h LC50 (mg/L)
Cd	416	29	1454	124	1307	10
Cu	90	25	851	300	109	1
Ni	696	52	1870	110	4039	185
Pb	707	67	2102	754	54	0.81
Zn	244	118	838	313	425	2

Table 3.3 - LC50s and regulatory limits for metals in soils.

Metal	*C. elegans* 48 h LC50 mg/kg (Tifton soil)	*E. fetida* 14 d LC50 mg/kg (ASTM soil)	US EPA 40 CFR 503 mg/kg (Max. yearly loading)	EC Limit Values mg/kg
Cd	19	1843	20	1-3
Cu	16	641	750	50-140
Ni	33	757	210	30-75
Pb	43	5947	150	50-300
Zn	76	661	1400	150-300

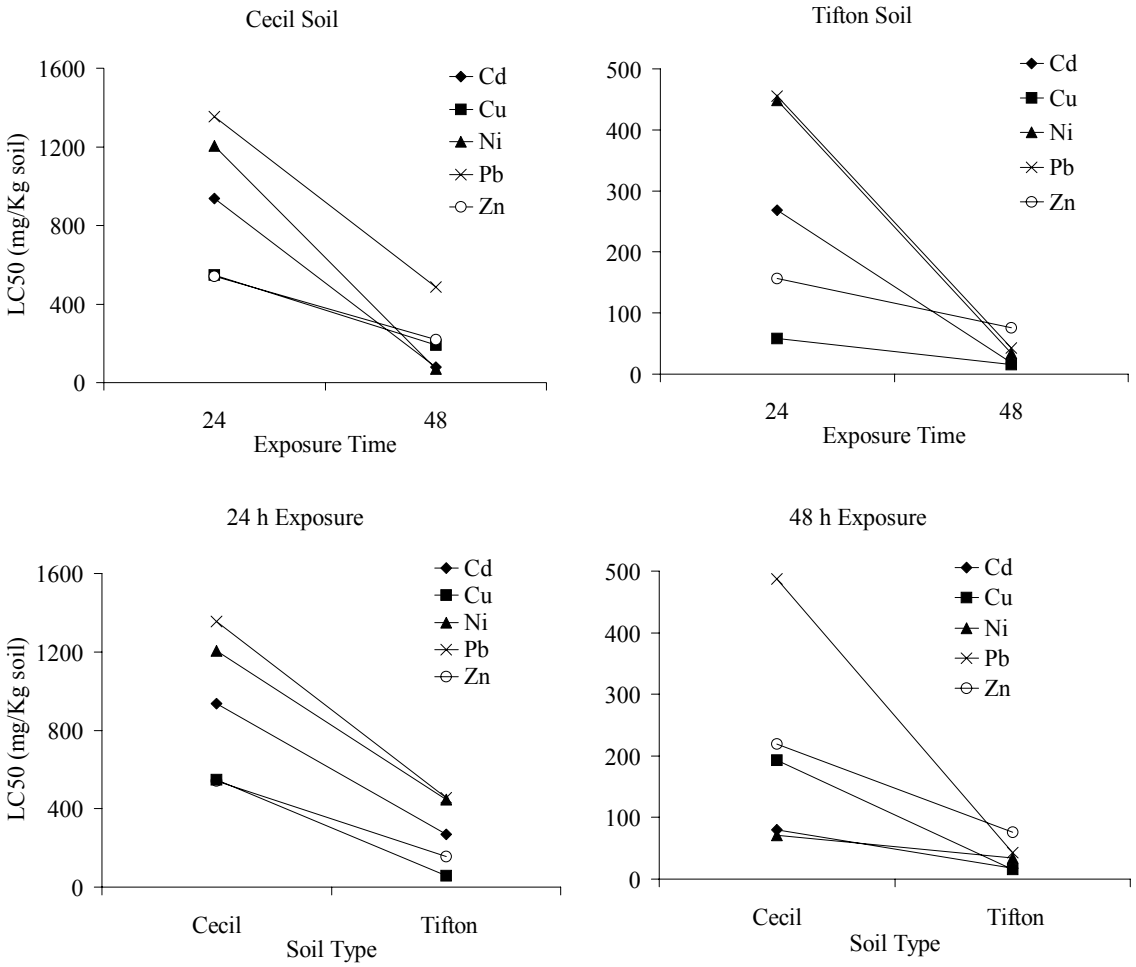

Figure 3.1 -- *Interactive effects of soil type and exposure time on the LC50s of five metals (Points joined for illustration only- not to imply a continuum).*

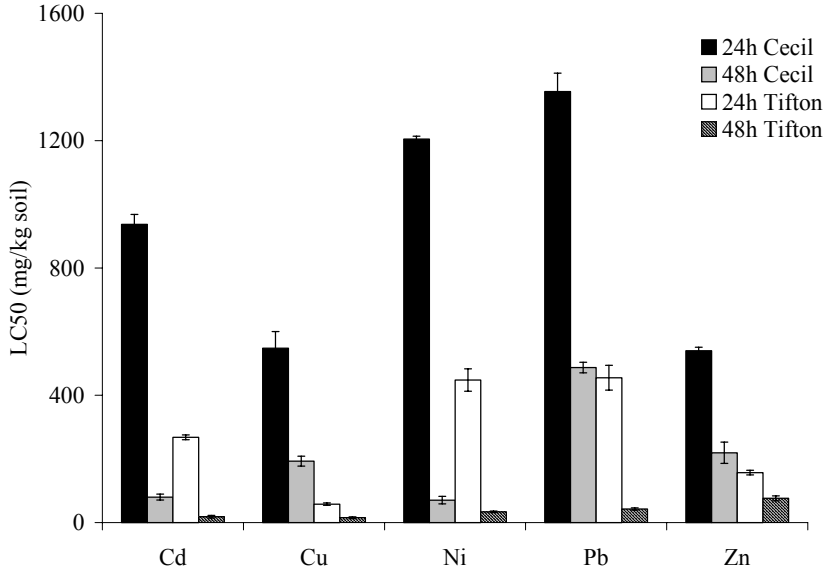

Figure 3.2-- *Average LC50s (mg metal/kg soil d.w.) for 24 and 48-h exposures of* C. elegans *to five metals in two field-collected soils.*

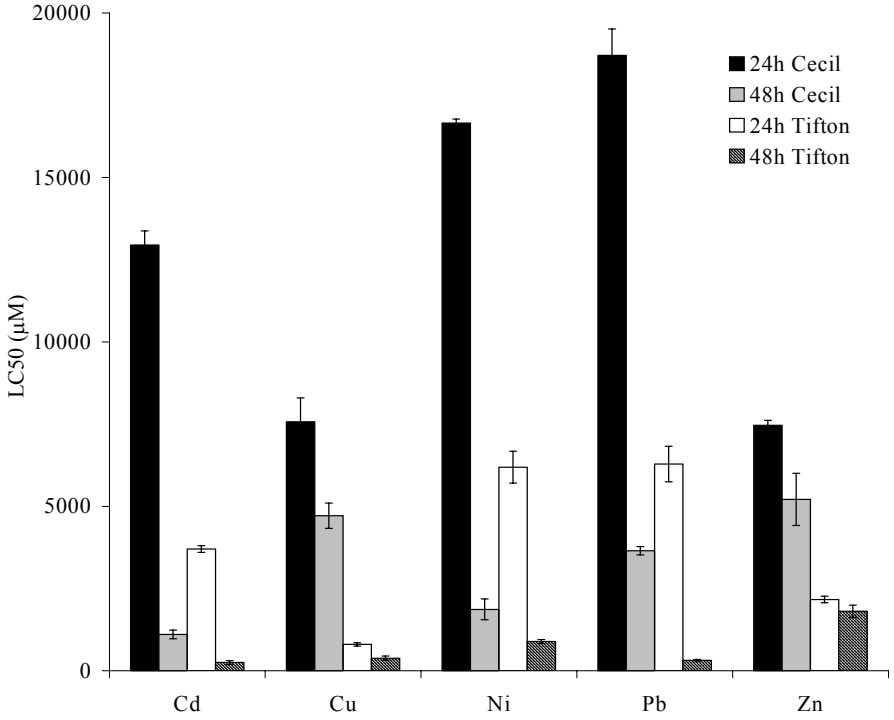

Figure 3.3-- *Average LC50s (µmole metal / L K-medium) for 24 and 48-h exposures of* C. elegans *to five metals in two field-collected soils.*

CHAPTER 4

THE AVAILABILITY OF METALS TO THE NEMATODE *CAENORHABDITIS ELEGANS*: TOXICITY BASED ON TOTAL CONCENTRATIONS IN SOIL AND EXTRACTED FRACTIONS[1]

[1] Boyd, W.A. and P.L. Williams. Accepted by *Environmental Toxicology and Chemistry*. Reprinted here with permission of publisher, 11/25/02.

ABSTRACT

Current regulation of metals in soils is based on total metal concentrations rather than actual exposure concentrations. Considering the extreme variation in soil physicochemical properties, total concentrations are not reflective of the availability and resultant toxicity of metals in different soils. In this study, the availability of Cd, Cu, Ni, Pb, and Zn to the free-living soil nematode *Caenorhabditis elegans* was assessed after 24-h exposures in three soils using a sequential soil extraction procedure. Albany soil, sampled from southern Georgia, is characterized by a high sand content while Cecil soil from the Piedmont region of Georgia contains higher fractions of clay and organic matter. The final soil was an American Society for Testing and Materials (ASTM) artificial medium composed of peat, kaolin clay, sand, and calcium carbonate. Based on their composition, metals would be most strongly sorbed in ASTM medium and least strongly in Albany soil. In fact, 24h lethal concentrations to 50% of nematodes (LC50s) of the five metals determined by the total metal concentration followed this trend. In addition, water-extractable metals were lowest in ASTM medium and highest in Albany soil when spiked at the same concentrations. Our data show the need to consider soil type when performing toxicological tests and establishing site-specific allowable metal concentrations in soil.

Keywords: *Caenorhabditis elegans*, environmental availability, metals, soil, toxicity

INTRODUCTION

Ecological risk assessments (ERAs) are often performed to provide maximum levels of exposure above which adverse effects are expected to occur to exposed organisms [1]. Although current ERAs in soils are based upon the total concentration of the contaminant(s) of concern, environmental scientists are now expressing the need for a more accurate reflection of the actual exposure concentration within the soil ecosystem [2]. A more appropriate approach should consider the entire soil matrix and how the physicochemical parameters of soils influence the bioavailability of contaminants to soil organisms.

Metals are important contaminants in a variety of soil ecosystems. Intensive soil management practices such as fertilization with animal wastes or land application of sewage sludge may lead to elevated levels of heavy metals in agricultural soils [3]. Transportation, industrial practices, and waste disposal may also cause increased levels of metals in the soil environment [4]. Metal ions have the potential to be taken up by crop plants causing adverse health effects to humans or may have deleterious effects on the entire soil ecosystem if accumulated at sufficiently high levels [5]. Although accurate measures of total metal content may be provided by chemical analysis, no information as to the resultant toxicity and biological relevance is offered. Analytical techniques used in conjunction with observations of toxicity to soil fauna may prove to be a more powerful means of predicting environmental and human health risks.

Bioavailability of metals is the degree to which a metal is free for uptake into or sorption onto an organism [6]. Two distinct phases of bioavailability, termed environmentally available and environmentally bioavailable, have been described [7].

Environmental availability is determined by chemical reactions with soil mineral surfaces that limit element solubility or solution phase reactions that determine element speciation. Environmental bioavailability is defined as the "physiologically driven uptake process requiring identification of a specific biotic species as an endpoint" [7]. Traditionally, the environmentally-available fraction has been assumed to be equivalent to the unsorbed or solubilized fraction (i.e., the metal concentration in the soil pore water) [8]. In aquatic media, the free ion activity model (FIAM) and biotic ligand model (BLM) have been proposed to account for exchangeable ions readily desorbed from solid phases and the competition for the sorption of these ions between biotic tissues and such ligands as dissolved organic matter (DOM) [7,9]. Because bioavailability cannot be measured directly by chemical analysis and because environmental availability is not always directly related to total metals in soils, measurements of exchangeable metals after extraction with salt solutions (e.g. $Ca(NO_3)_2$ or $Ca(Cl)_2$) have been indicated as better estimators of bioavailability [10,11].

Several studies have made excellent attempts to identify the degree to which soil characteristics and uptake affect bioavailability or toxicity of metals to oligochaete species [7,10-13]. However, there appear to be no reports of similar experiments with terrestrial nematodes, despite the fact that they are more numerous and species-rich than earthworms. Nematodes make up a major proportion of the soil biota helping to maintain soil ecosystem stasis by contributing to the degradation of organic matter in soil and to the cycling of nutrients [14]. Because nematodes occupy a key position in the soil food web, deleterious effects on their populations may lead to compromised soil quality. Within the phylum Nematoda, most terrestrial nematodes belong to the class Secernentea.

Within this class, the family Rhabditidae are small bacterivorous nematodes with a number of survival strategies that allow them to flourish even under less than optimal conditions. Formation of dauerlarva allows the rhabditid nematodes to survive periods of dessication and starvation. Because they live within the rhizosphere, the area directly surrounding the root where most soil microbial life can be found, rhabditids have also evolved to be rather tolerant to toxic plant and microbial exudates [15].

One representative rhabditid species that is both ubiquitous within the soil environment and easily utilized in laboratory toxicity tests is *Caenorhabditis elegans*. The many advantages of using *C. elegans* in laboratory experimentation include short life cycles, high reproductive rates, tolerance to a wide range of environmental conditions such as pH [16], and biological characterization exceeding any other multicellular organism [17]. Because of its small size and short life cycle, soil toxicity tests can be performed in relatively short time intervals (24 h) and with little soil volume (2-3 g) [18]. Previous experiments have determined the toxicity of a variety of contaminants in aquatic media and soils [14,19-26]. Recently, the American Society for Testing and Materials (ASTM) has accepted a guide describing the use of *C. elegans* in soil toxicity tests [27].

Because measurements of total metals are known to yield poor estimates of soil toxicity, identification and validation of alternative measures of toxicity and bioavailability must be considered [2]. Therefore in our study we compared the toxicity of five metals (Cd, Cu, Ni, Pb, and Zn) in two field-sampled soils and one artificial testing medium that were selected to include a range of physiochemical properties known to affect environmental availability. Because total metal concentrations may not be reflective of actual toxicity, our second objective was to examine the use of a sequential

extraction procedure to determine which extracted fraction(s) was most indicative of exposure concentrations across the soil types. Finally, linear regressions were used to further investigate the relationship between toxicity and soil characteristics (pH, cation exchange capacity (CEC), and soil organic matter (SOM)).

MATERIALS AND METHODS

Toxicity tests

The wild type N2 strain of *C. elegans* was obtained from the *Caenorhabditis* Genetic Center (Minneapolis, MN, USA) and used in all tests. Cultures were prepared as previously described to yield age-synchronized adult worms [28]. Toxicity testing was performed as previously described [27]. For each metal tested, there were five treatments, a control and four increasing metal concentrations. After spiking Cecil, ASTM or Albany soils (Table 4.1) with the appropriate concentrations of nitrate metal salts, the test soils were allowed to equilibrate at 20^{o}C for 7 days, after which time ten nematodes were added to each dish and exposed for 24 h. Nematodes were then recovered using centrifugation/flotation in a colloidal silica suspension and scored as live or dead. Lethal concentrations to 50% of nematodes (LC50s) were generated using Probit analysis. Each test was replicated three times.

Environmental availability studies

A comparison of the physicochemical properties of the three soils used in all experiments is given in Table 4.1. Cation exchange capacity (CEC) [29], percent organic matter (%OM) [30], pH in H_2O [31], and particle size distribution were determined by

the University of Georgia's Plant, Soil and Water Laboratory (Athens, GA, USA). Total recoverable metals analysis (microwave assisted acid digestion of soils) was also performed on each soil after field sampling but prior to the addition of metallic salts.

Soils were spiked at concentration ranges selected to include LC50s of each metal in all soils tested as described above ([Cd] = 0, 170, 600, and 1050 mg/L, [Cu] = 0, 150, 350, and 820 mg/L, [Ni] = 0, 290, 780, and 1600 mg/L, [Pb] = 0, 240, 870, and 1475 mg/L, and [Zn] = 0, 400, 350, and 1230 mg/L). A three-step sequential extraction method was modified from the methods of Tessier et al. [32] and Miller et al. [33]. Deionized water was added at ratios of 10:1 (solution:soil) into 50 mL centrifuge tubes. Tubes were shaken for 1 h and centrifuged for 10 min. The solution was then decanted, filtered, and analyzed for total metal content using flame atomic absorption spectrometry. The same procedure was repeated with 0.5 M $Ca(NO_3)_2$ to estimate exchangeable fraction and lastly with 0.44 M acetic acid + 0.2 M $Ca(NO_3)_2$ to estimate the dilute-acid extractable fraction. All treatments were replicated three times. Besides total metal concentrations within the water extract, the concentration of major cations (Ca, Mg, Na, and K) were determined by inductively coupled plasma mass spectrometry (ELAN 6000, Perkin-Elmer, Norwalk, CT, USA) after dilution up to 50 x. Quality control included initial and continuing blank and calibration standards and duplicate sample analysis.

Data analysis

LC50s from each replicate were generated using log-transformed data in Toxstat® 3.4 [34] and are reported as the mean value of three replicates. Standard deviations (SD) and coefficients of variation (CV) were also calculated for individual

LC50s. Correlations between soil physicochemical properties and LC50s were calculated from linear regression.

RESULTS

Toxicity tests

LC50s based upon the amount of metals spiked into the soil on a mg of metal per kg of soil and on a μmoles of metal per L bases are provided in Figure 4.1. LC50s were always highest in ASTM artificial medium and, generally, metals appeared to be more toxic in Albany than in Cecil soil when based on total metal concentrations (Fig 4.1). The two exceptions, Ni and Zn, had similar LC50s in either natural soil. Except for Pb, the order of metal toxicity was similar whether based on the amount of metals in the soil (Fig 4.1a) or on the concentration of the spiking solution (Fig 4.1b). In the case of Pb and to a lesser extent Cd, the high molecular weight of the element is taken into account when expressed as molarity but not mg/kg. In fact, Pb is one of the least toxic metals on a mg/kg basis and the most toxic on a molar basis (Fig 4.1).

Sequential extraction

The results of the sequential extraction experiments are summarized in Figure 4.2 where each of the soils were spiked at three concentrations meant to include the LC50 of all three soils in order to compare the amount of metals extracted at each soil's LC50. For example, the LC50 for Cu was 148 mg/L in Albany soil, 353 mg/L in Cecil soil, and 818 mg/L in ASTM soil. Therefore, each soil was spiked at 150, 350, and 820 mg/L Cu to include the range of the three LC50s (Fig 4.2b).

Regardless of metal, the extracted metal concentrations in the water-soluble fractions were always highest in Albany soil while the lowest water-soluble and highest carbonate fractions were found in ASTM artificial medium (Fig 4.2 a-e). Limited amounts of metals were extracted with water from ASTM soil or, in most cases, with $Ca(NO_3)_2$. The sorption of Cu and Pb was so strong that virtually no metal was extracted with water from either the Cecil or ASTM soil and very little metal was extracted with $Ca(NO_3)_2$ from the ASTM soil (Fig 4.2b&d). Summing all three steps, the highest recovery of spiked metals was always observed in the Albany soil except for Zn where equal amounts were extracted from Albany and Cecil soil (Fig 4.2a-e).

Figure 4.2f provides an example with Cu of the proportion of metals extracted at each extraction step expressed as a percentage of the total metal recovered (i.e., not on spiking concentration). At each extraction step, similar percentages of the extracted concentrations were observed within each soil type for all metals regardless of the initial spiking concentration. For example, whether Albany soil was spiked with 150, 350, or 820 mg/L Cu, 39-51% was extracted with water, 27-29% with salt, and 21-31% with acid (Fig 4.2f). This was true for all soil-metal combinations.

Corrected LC50s

LC50s were also calculated based on the concentration of metal extracted with water, salt, or acid rather than simply on total metal concentration in the soil. For the salt and acid extracts, the sum of all previous extraction steps was used for the metal concentration due to the increasing harshness of reagents with each extraction step. Therefore, the concentration for the salt extract was the sum of the water and salt extracts

and the acid extract concentration was the sum of all three steps. Furthermore, because only the environmental availability of the metal rather than the toxicity should change with soil type, the extraction step(s) with the lowest variability between soil types was assumed to be the best estimator of environmental bioavailability. The toxicity of metals from the water extraction alone was underestimated in Albany soil compared to Cecil soil and ASTM medium (Fig 4.3b). After weak acid extraction, the metal toxicity was higher in ASTM medium than in the two field-sampled soils (Fig 4.3d). The variation of corrected LC50s between the soils was lowest when based on the water-soluble and salt exchangeable fractions summed (Fig 4.3c).

Correlation of soil properties with LC50s

Because only three soils were tested, only limited information can be gathered from regression analyses. However, simple linear regressions were performed with CEC, pH, and SOM to determine any apparent trends. Future analyses with more field-sampled soils will include more intricate regression analyses. In general, the coefficients of determination were higher when CEC was regressed against LC50s than either pH or SOM (Table 4.2). CEC was a better predictor of LC50s based on total metal content than pH or SOM (Table 4.2). However, SOM better predicted LC50s based on the water-soluble and, to a lesser extent, exchangeable fractions. Within the exchangeable fraction, the best indicator of available metal, r^2 values varied between metals. SOM was most predictive for Cd, Ni, and Zn while CEC and pH were better predictors for Cu and Pb, respectively.

DISCUSSION

In soil, environmental availability is dependent upon interdependent factors including environmental conditions and chemical properties of the metals themselves [35]. Clay and SOM content affect the number of sorption sites available and, thus, the cation exchange capacity (CEC) of a soil. Although ASTM artificial testing medium and Cecil soil have similar sand, silt, clay, and SOM contents, their CECs are remarkably different (Table 4.1). Excessive fragmentation of mined clay particles as well as the use of peat as an SOM surrogate in ASTM medium may lead to significantly increased surface area and thus unnaturally high CECs. High CECs could account for the greater LC50s and low water-soluble fraction concentrations observed (Figs 4.1 and 4.2). Albany soil is composed of mostly sand and therefore most metals were easily extracted with only water (Fig 4.2). Of the three soil properties tested in our study, CEC was the most predictive of LC50s (Table 4.2). In fact, CEC is consistently regarded as the most influential soil property in the prediction of metal availability [12,13].

The chemical properties of the metal itself also affect environmental availability. Increasing valence and decreasing hydrated radius of a metal increase the affinity of sorption sites for the cationic species. Properties such as hardness/softness, electronegativity, and size determine the types and affinities of ligands to which metals can sorb. Although the affinity of soil sorption sites is inconsistent for divalent metals, in general, the order of selectivity of metal cations in soils (from most to least readily sorbed) is: Pb>Cu>Zn>Ni>Cd [35]. Although our data did not match perfectly, Pb and Cu were definitely sorbed more strongly than the other three metals in Cecil soil. In

Albany soil, sorption was only significant for Pb and Cu, while all metals were strongly sorbed in ASTM soil.

Surprisingly, toxicity of all metals did not decrease in the presence of soil when compared to previous aquatic tests with *C. elegans* [24]. In fact, Ni toxicity was lower in the aquatic medium than in any of the soils. This could be due to the fact that the concentrations of Ni tested approached the solubility limits where precipitates were visible. Only Cu toxicity was decreased in the presence of the Albany sandy soil. The order of metal toxicity was similar in all three soils where Ni was the least toxic and Pb was the most (Fig 4.1b). Two metals, Ni and Zn, appeared to exhibit similar toxicity in both natural soils even though CEC and SOM were higher in Cecil soil (Fig 4.1). However, in aquatic medium, Ni is much less toxic than Zn. Because soil sorption processes are so dynamic, Ni and Zn availability could have been affected by soil properties or processes not characterized in the current study. For example, the presence of dissolved organic matter (DOM) or hydroxides of Fe, Al and Mn may greatly influence the solubility of metals within the soil solution [36]. In sediment toxicity tests with *C. elegans*, increased DOM appeared to increase Cd toxicity possibly by increasing Cd solubilization [21].

When attempting to estimate the bioavailable fractions from the sequential extraction data, two assumptions were made. First, if the metal concentration in one extraction step was considered to be available, all preceding step(s) must also be available due to increasing harshness of extraction reagents. Secondly, the toxicity of an individual metal did not differ: only the chemical forms and bioavailability of that metal differed between soils. Therefore, the bioavailable fraction would best be represented by

the extraction step(s) that yielded the least variation in extracted concentrations between soil types spiked at their LC50s.

Water-soluble forms are assumed to be available to soil organisms that live within the interstitial water of the soil. However, LC50s based on the water-soluble fraction tended to underestimate availability in Cecil soil (Fig 4.3). Exchangeable fractions could be assumed to be potentially bioavailable to free-living soil nematodes. LC50s expressed as the sum of the exchangeable and water-soluble fractions generated the least variation between soils. The sum of all fractions appeared to overestimate the availability of metals in the ASTM artificial medium, as LC50s were significantly higher in ASTM than the other two soils. It is often difficult to make any generalizations of metal availability between artificial soils such as ASTM medium and natural soils because of structural differences between them [7]. However, when comparing only the two field-sampled soils in the current study as well as those in previous studies [10], the exchangeable fraction appears to be the most predictive estimator of available metals.

Although measurements of total metals are not indicative of actual exposure concentrations, CEC was highly predictive of toxicity based on total metals (Table 4.2). Previous findings also suggest that the availability of metals is a function of the total metal content and that bioavailability of metals is mediated by the same soil physicochemical properties that influence metal partitioning within the soil solution-solid phase [7,11]. Therefore, the CEC of a soil along with a measure of total soil metal may provide some predictive power across soil types. However, a number of additional studies must be performed before drawing this conclusion. Future studies in our lab will investigate the toxicity and bioavailability of metals in a wider range of soils while

measuring more soil physicochemical properties and soil pore water characteristics. We also plan to measure the body burden of metals and determine the relationships between toxicity, body burden, and soil physicochemical properties. Finally, validation of any findings from laboratory-spiked soils will be validated with field-contaminated, aged soils.

Acknowledgment - We are grateful to the University of Georgia's Agricultural Experiment Station for financial support of this research. Theresa Stiernagle at the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health (NIH) National Center for Research Resources (NCRR), provided the nematode strain used in this work. We also thank Dr. Brian Jackson for the inductively coupled plasma mass spectrometry analysis and editorial comments.

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Table 4.1. Soil physicochemical properties.

Soil	% Sand	% Silt	% Clay	% OM[a]	pH	CEC[b]
ASTM[c]	80	12	8	5.1	7.8	28.4
Cecil[d]	74	16	10	5.1	5.7	7.2
Albany[e]	98	0	2	1.4	6.1	2.4

[a] %OM = percent organic matter

[b] CEC = cation exchange capacity in mEq/100 g soil

[c] ASTM = American Society for Testing and Materials artificial testing medium

[d] Cecil soil collected in Athens, GA

[e] Albany soil collected in Waycross, GA

Table 4.2. Correlations of soil properties in three soils (Albany, Cecil and ASTM artificial medium) with LC50s (lethal concentrations of metals to 50% of nematodes) based on total metal, water-extractable, exchangeable, and carbonate fractions.

	Cd	**Cu**	**Ni**	**Pb**	**Zn**
CEC[a]					
r^2 Total Metal	0.86	0.98	0.97	0.87	0.99
r^2 Water	0.81	0.53	0.48	0.48	0.54
r^2 Exchangeable	0.78	0.99	0.11	0.72	0.41
r^2 Carbonate	0.94	0.59	0.62	0.92	0.90
pH					
r^2 Total Metal	0.55	0.78	0.96	0.57	0.94
r^2 Water	0.48	0.20	0.18	0.16	0.21
r^2 Exchangeable	0.44	0.84	0.01	0.96	0.11
r^2 Carbonate	0.67	0.25	0.98	0.64	0.99
SOM[b]					
r^2 Total Metal	0.78	0.54	0.26	0.76	0.30
r^2 Water	0.83	0.98	0.99	0.99	0.98
r^2 Exchangeable	0.86	0.47	0.88	0.02	0.99
r^2 Carbonate	0.66	0.97	0.01	0.69	0.13

[a] CEC = cation exchange capacity

[b] SOM = soil organic matter

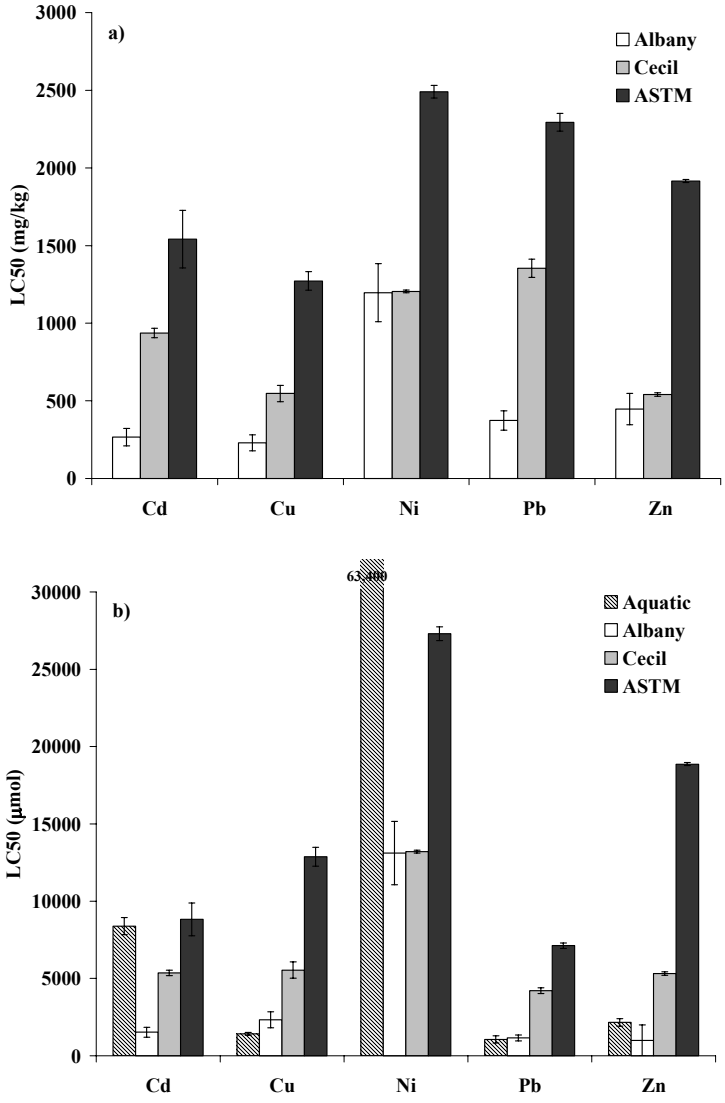

Figure 4.1. LC50s (lethal concentrations to 50% of nematodes) in three soils (Albany, Cecil and ASTM artificial medium) based on total metals in a) mg metal/ kg soil and b) µmoles/ L.

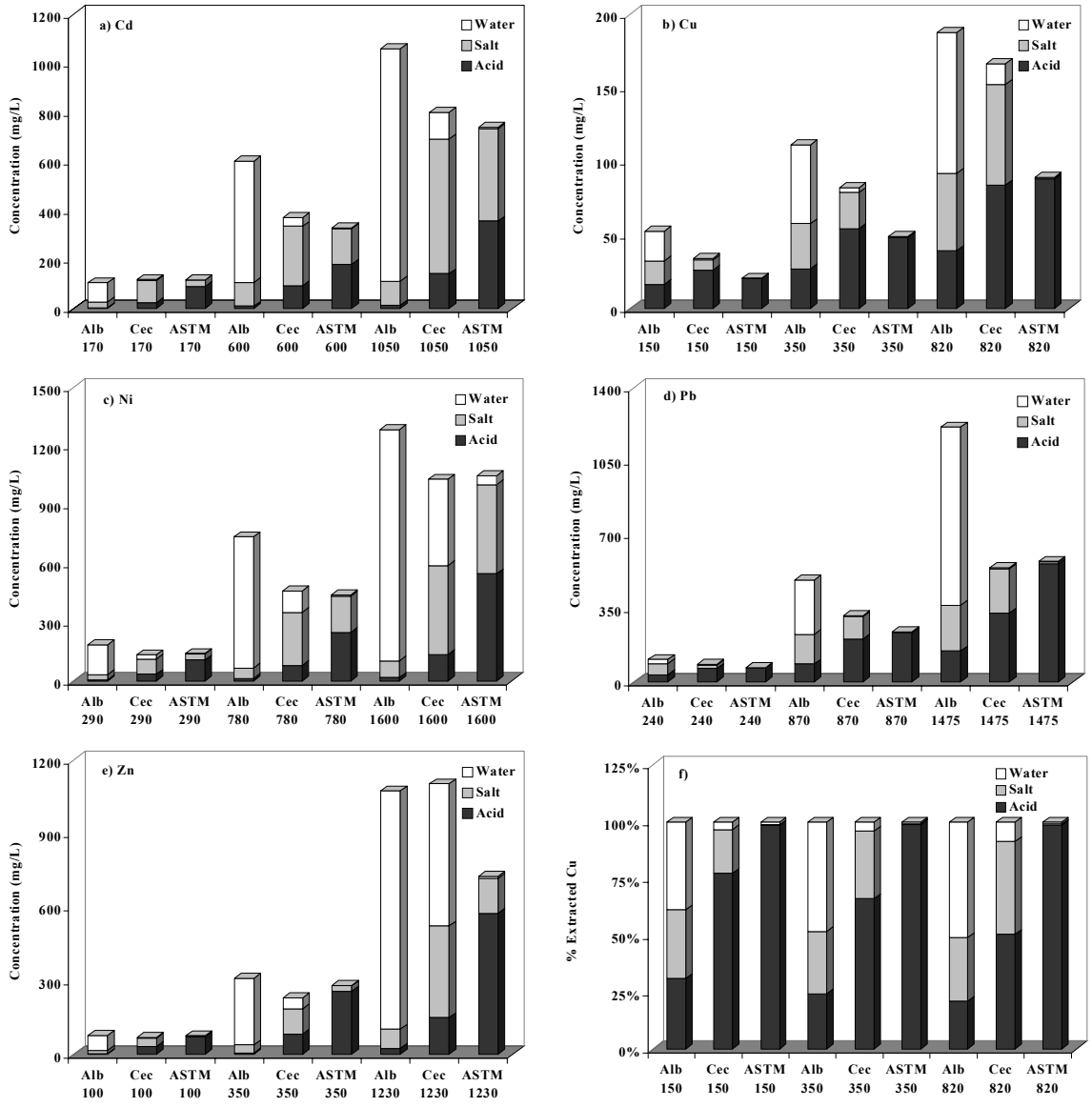

Figure 4.2. Concentrations of a) Cd, b) Cu, c) Ni, d) Pb, and e) Zn extracted from three spiked soils (Albany (Alb), Cecil (Cec) and ASTM artificial medium) in mg/ L and f) percentage of total extracted Cu measured in each extracted fraction. All soils spiked at the calculated LC50s (lethal concentrations to 50% of nematodes) of each soil (e.g. Alb 170 = Albany soil spiked at 170 ppm; Cec 600 = Cecil soil spiked at 600 ppm).

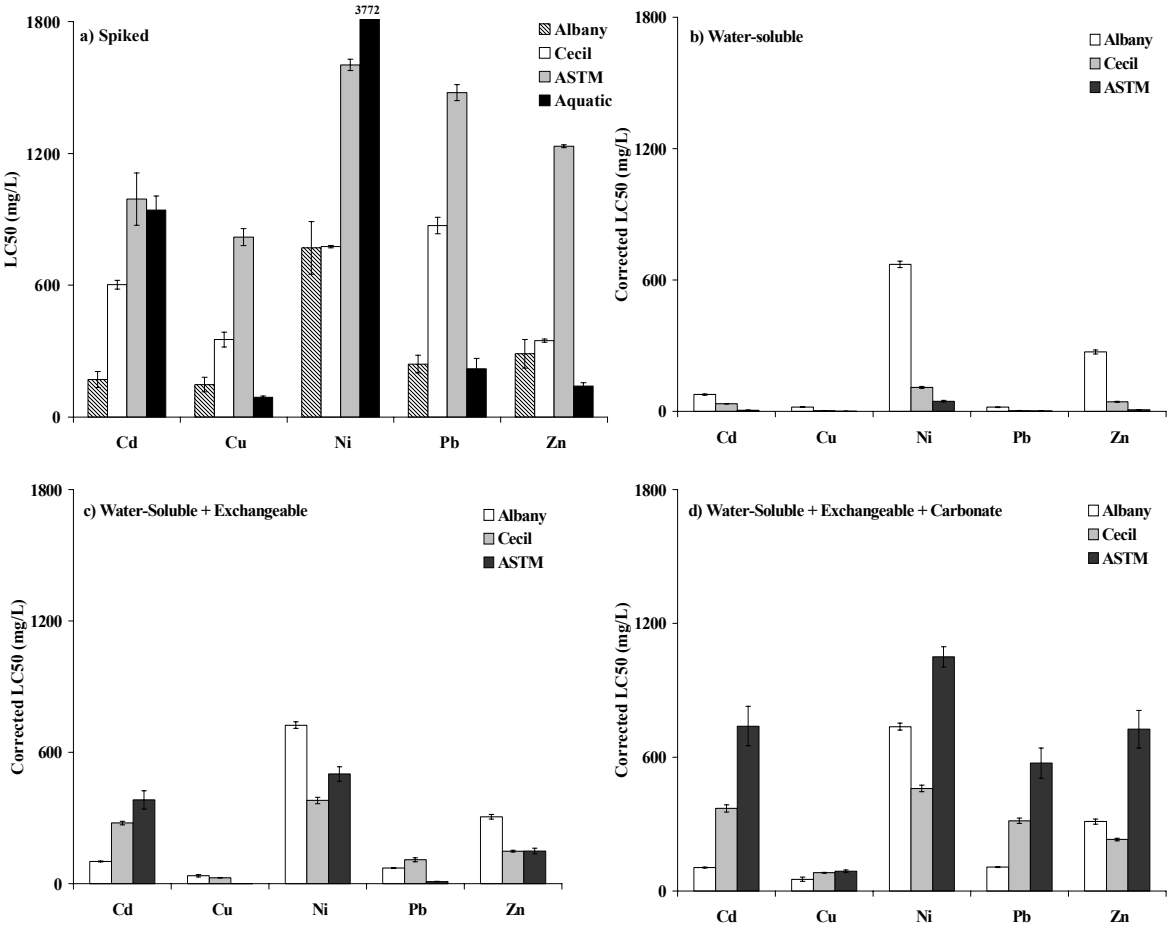

Figure 4.3. LC50s (lethal concentrations to 50% of nematodes) in three soils (Albany, Cecil and ASTM artificial medium) calculated from concentrations a) of spiking solutions, b) measured in water-soluble fraction, c) measured in water-soluble plus exchangeable fractions, and d) measured in all three fractions.

CHAPTER 5

COMPARISON OF THE SENSITIVITY AND USABILITY OF THREE NEMATODE SPECIES IN AQUATIC AND SOIL TOXICITY TESTS[1]

[1] Boyd, W.A. and P.L. Williams. To be submitted to *Environmental Toxicology and Chemistry*.

ABSTRACT

Nematodes are useful organisms for aquatic and soil toxicity testing because of their abundance and diversity as well as their ease of culturing and maintenance in the laboratory. The nematode *Caenorhabditis elegans* has been used extensively in toxicity testing but its sensitivity to metal exposures in relation to other nematodes remains unclear. In this study, we compare the sensitivity and ease of use of two other rhabditid nematodes, *Panagrellus redivivus* and *Pristionchus pacificus*, to *C. elegans*. Toxicity endpoints were chosen to investigate the effects of Cu on the survival of these nematodes after soil exposures and on the survival, reproduction, movement, and feeding behavior of nematodes after exposures in aquatic medium. In all lethality testing, *P. pacificus* was the most sensitive, *C. elegans* exhibited intermediate sensitivity, and *P. redivivus* was the least sensitive. Reproduction and movement of *C. elegans* and reproduction of *P. pacificus* were decreased 50% by similar concentrations of Cu (EC50s ~ 2 mg/L), but *P. pacificus* movement was less sensitive to Cu exposures (EC50 = 8 mg/L). Although all nematodes may be useful in lethality assays, using *P. redivivus* in toxicity tests is complicated by the presence of two sexes and difficulties in obtaining age-synchronized cultures. *Pristionchus pacificus* is an ideal acute toxicity testing organism because of its sensitivity and ease of culturing. However, *C. elegans* appears to be more sensitive and therefore most useful in sublethal behavioral assays. Knowledge of the usability and relative sensitivity of nematodes in toxicity testing could lead to more realistic approximations of soil ecosystem health.

INTRODUCTION

Nematodes are considered excellent candidates for assessing the effects of pollutants on soil ecosystems for a number of reasons (Yeates and Bongers 1999). Nematoda is an abundant and diverse phylum, with individuals found in the millions per m^2 of soil surface, exhibiting a variety of feeding types and survival strategies (Korthals et al. 1996a). Soil nematodes live within the interstitial waters of soil particles and are therefore in direct contact with dissolved contaminants (Kammenga et al. 1994). By feeding on bacteria and fungi, soil nematodes act as regulators of decomposition processes and therefore play a key role in nutrient cycling and dynamics (Barker et al. 1994; Yeates and Bongers 1999).

Besides their ecological roles, nematodes are also ideal fauna for laboratory toxicity testing. A number of species are easily sampled from field soils, identified, and cultured either on agar plates or in liquid media (Bongers 1990). Opportunistic nematode species reproduce in high numbers within short time periods, allowing for mass culturing of individuals for toxicity tests. Because of their short life cycles and small size, nematodes are useful in toxicity tests that can be performed rapidly and with small soil volumes.

The nematode *Caenorhabditis elegans* has been used extensively as a model organism in studies of developmental biology and genetics (Riddle et al. 1997). Now *C. elegans* is also a popular toxicity testing organism in aquatic media and soils (Power and de Pomerai 1999; Cioci et al. 2000; Peredney and Williams 2000; Anderson et al. 2001; Boyd and Williams 2003). Terrestrial nematodes, such as *C. elegans*, are most often

classified as belonging to the class Secernentea (Blaxter et al. 1998). Within Secernentea, nematodes are classified into one of nine orders based on such characteristics as size, feeding type, and life cycle. The order Rhabditida is comprised of bacterivoruous soil nematodes including *C. elegans*. Rhabditid nematodes are generally characterized by high reproductive rates, small sizes, and formation of survival stages during times of stress termed dauerlarvae (Bongers and Ferris 1999).

Recently the American Society for Testing and Materials (ASTM) has published a standardized guide for soil toxicity testing with *C. elegans* (ASTM 2002). Although standardized toxicity testing with a single species is recognized as a useful first step in assessing the impacts of environmental contaminants, knowledge of the usability and sensitivity of other rhabditid nematodes in toxicity testing could lead to more realistic approximations of the effects of toxicants on soil ecosystem health. To this end, our lab has tested the lethal and sublethal effects of Cu on three nematode species: *C. elegans*, *Panagrellus redivivus*, and *Pristionchus pacificus*. Although all three species are from the order Rhabditida, each is from a different family: *C. elegans* belongs to the Rhabditidae family; *P. redivivus* belongs to the Panagrolaimidae family; and *P. pacificus* belongs to the Neodiplogastridae family. Therefore, although the worms are similar in many ways, they may differ in behavior, morphology, and sensitivity to toxicants.

Caenorhabditis elegans and *P. pacificus* are self-fertilizing hermaphroditic species with rare occurrence of males that are able to cross with hermaphrodites (Riddle et al. 1997; Sommer et al. 1996). Gravid hermaphrodites of both species can lay several hundred eggs over several days. *Panagrellus redivivus*, however, is an amphimictic species with ovoviviparous females giving birth to second stage larvae (L2) after being

fertilized by males (Samoiloff et al. 1980). All species tested have short life cycles that are dependent on food availability and temperature. At 20 ºC, both *C. elegans* and *P. pacificus* mature from egg to adult in about 3 days while *P. redivivus* matures after live birth to adult in about 4 days. While *C. elegans* and *P. redivivus* progress through four larval or juvenile stages to adult (L1-L4 or J1-J4), *P. pacificus* matures from a J1 after hatching from the egg to a J3 before maturing to an adult (Sommer et al. 1996). Both *C. elegans* (L3) and *P. pacificus* (J2) are able to form daeurlarvae if food or water are limiting. Adult *C. elegans* range from around 1-1.5 mm in length while *P. redivivus* adults are typically longer (0.9-1.8 mm) and *P. pacificus* adults are usually slightly shorter (0.75-1.2 mm) and wider in appearance.

Like *C. elegans*, *P. redivivus* has also been used as a model organism in biological studies (Sternberg and Horvitz 1981; Hood et al. 2000) as well as a toxicity testing organism (Samoiloff 1990). Methods exist for the determination of lethal and sublethal effects of toxicants in aquatic media including a rapid screen for mutagenicity in this nematode (Samoiloff et al. 1980).

The nematode *P. pacificus* was first described in 1996 (Sommer et al. 1996). Because of the ability to culture it identically to *C. elegans* as well as the ease of performing genetic studies and developing new mutants, *P. pacificus* has been proposed as a satellite organism for comparison to the *C. elegans* model system. In one study, the relative sensitivity of rhabditid nematodes was compared after exposure to *Burkholderia cepacia*, a nematicide designed to control plant-parasitic nematodes (Carta 2000). Although responses varied among different isolates of *B. cepacia*, *P. redivivus* was

generally the most sensitive and *P. pacificus* the least. *Caenorhabditis elegans* displayed intermediate sensitivity to *B. cepacia* nematicide.

The first objective of the current study was to compare the sensitivity of three nematode species (*C. elegans*, *P. redivivus*, and *P. pacificus*) to Cu exposures in aquatic medium and soil. Copper was chosen as the toxicant because of its prevalence in the soil environment and because we have used Cu as a reference toxicant in our laboratory for several years (Freeman et al. 1998; 1999). Also, Cu has been studied extensively with other soil invertebrates allowing for sensitivity comparisons to other species (Bogomolov et al. 1996). Our second objective was to construct control charts with Cu as a reference toxicant in aquatic medium so that nematode health and response could be monitored for future toxicity testing. Finally, we compared the ease of use of the three species as well as their potential to be included in future editions of the ASTM guide (ASTM 2002).

MATERIALS AND METHODS

Nematode culturing and maintenance

Wild type strains of *C. elegans, P. pacificus*, and *P. redivivus* were obtained from the *Caenorhabditis* Genetic Center (Minneapolis, MN) and used in all tests. Cultures of *C. elegans* and *P. pacificus* were maintained as previously described for *C. elegans* to yield age-synchronized adult worms (Cressman and Williams 1997). Briefly, eggs were harvested from agar plates after 3-4 days and rinsed with a 1% Clorox solution that killed all life stages except for eggs. Like *C. elegans* and *P. pacificus*, *P. redivivus* cultures were also cultured on agar plates seeded with OP50 strains of *Escherichia coli* as a food

source (Brenner 1974). However, because they are ovoviviparous rather than egg-laying, synchronized cultures were obtained by rinsing worm cultures through 35 μm filters every 96 h. For this reason, we refer to *P. redivivus* cultures as size-synchronized and *C. elegans* and *P. pacificus* cultures as age-synchronized.

Chemical exposures

Stock solutions of $CuCl_2$ were prepared with K-medium (0.032 M KCl, 0.051 M NaCl) as the diluent for each test replication (Williams and Dusenbery 1990). Aqueous test concentrations were then diluted from the stock solutions, collected, acidified and stored at 4 ^{o}C. Flame atomic absorption (AA) spectrometry was used to verify final metal concentrations.

Soil toxicity tests

Lethality toxicity tests. Soil toxicity testing with all nematode species was performed as previously described for *C. elegans* (ASTM 2002; Boyd and Williams 2003). Albany soil was sampled from the Coastal Plains region in Waycross, Georgia and was used in all soil tests (Boyd and Williams 2003). This soil is characterized by a high sand content (98%) and low clay (2%) and organic matter content (1.4%). After spiking Albany soil with the appropriate concentrations of the metal salt of Cu, the test soils were allowed to equilibrate at 20^{o}C for 7 days, after which time 10 nematodes were added to each dish and exposed for 24 h. Nematodes were then recovered with centrifugation/flotation in a colloidal silica suspension and scored as live if moving or

dead if not responding to gentle probing under a dissecting microscope (Donkin and Dusenbery 1993).

Aquatic toxicity tests

Lethality tests. All nematode species were exposed to concentrations (a control and 6 increasing Cu concentrations) in 12-well polystyrene tissue culture plates without food for 24 h (±1) (Dhawan et al. 1999). Each exposure well contained 10 (±1) worms in 1 mL of test solution and each treatment consisted of 6 exposure wells for a total of 60 worms per Cu concentration. Six reference toxicity tests from 2001-2002 were used to collect *C. elegans* aquatic lethality data. Copper concentrations in these tests were 0, 43, 53, 66, 80, 100, and 120 mg/L. Initial range-finding tests were performed on *P. pacificus* and *P. redivivus* at concentrations ranging from 1-1000 mg Cu/L dissolved in K-medium. Final Cu concentrations tested for *P. pacificus* were 0, 5, 10, 15, 20, 30, and 40 mg/L, and for *P. redivivus* 0, 50, 100, 150, 200, 300, and 400 mg/L. After the 24-h exposure period, worms were scored as live if moving or dead if unresponsive to gentle probing under a dissecting microscope. Aquatic lethality tests were replicated 6 times for the construction of control reference charts.

Reproduction tests. The effect of metal on the reproduction of *C. elegans* and *P. pacificus* was investigated as previously described for *C. elegans* (Anderson et al. 2001). Both nematode species were exposed to test concentrations (control and 5 increasing Cu concentrations) in 12-well tissue culture plates with OP50 *E. coli* for 72 (±1) h. For each Cu concentration, *E. coli* isolated from 20 mL of L-broth culture media via centrifugation at 750 x *g* was resuspended in 10 mL of testing solution (referred to as 2:1 L-broth).

Each exposure well contained one adult worm in 1 mL of the appropriate test solution and each treatment consisted of 6 exposure wells. Initial range-finding testes were performed at concentrations ranging from 0.5-25 mg Cu/L in K-medium. Final Cu concentrations tested for both species were 0, 0.5, 1, 2, 4, and 8 mg/L. After the 72-h exposure period, offspring were counted under a dissecting microscope.

Movement assays. Computer tracking of *C. elegans* and *P. pacificus* to assess movement was performed as previously described for *C. elegans* (Boyd et al. 2000; Anderson et al. 2001). Both species were exposed to testing concentrations (control and 4 increasing Cu concentrations) in 12-well tissue culture plates with OP50 *E. coli* for 24 (±1) h. For each Cu concentration, stock solutions of Cu were diluted with 2:1 L-broth (Donkin and Williams 1995). Each exposure well contained approximately 100 adult worms in 1 mL of the appropriate testing solution. Final Cu concentrations tested for *C. elegans* were 0, 0.5, 1, 5, 10, and 15 mg/L and for *P. pacificus* were 0, 4, 6, 8, 10, and 12 mg/L. After the 24-h exposure period, worms were rinsed from exposure wells with K-medium into 15-mL centrifuge tubes. After thorough rinsing with K-medium to remove any debris and disposal of excess supernatant, approximately 50 worms were transferred to a thin layer of cooled 1% agar on a glass slide. The agar slab was placed over a petri dish filled with H_2O to prevent dessication. Worms were allowed to distribute evenly on the agar slab. After approximately 30 minutes, the glass slide was placed within a hydrated air stream directly below a Hitachi CCD video camera interfaced with a PowerMac G3 computer equipped with a Scion LG 3 PCI frame-grabber board. The nematodes were then illuminated with a dark field illumination system where their movement and change in direction were recorded and arranged in an Excel spreadsheet

format by an NIH Image Tracker software program (Dusenbery 1997). Each tracking period consisted of 100 s and each sample was tracked for three periods.

Feeding assays. Feeding assays were performed on the same exposure plates used for computer tracking. Using the CCD camera and computer system described above, worms in each well were counted manually and recorded. The optical density (OD) of each solution was read at 570 nm and recorded immediately after worm transfer (0 h) and after the 24-h exposure period. Once the 24-h OD was recorded, worms were rinsed from the twelve well plates and transferred to 15-mL centrifuge tubes for computer tracking. Feeding was calculated in a similar fashion as described in Anderson et al. (2001), where the change in OD (ΔOD) in the wells with worms over the 24-h exposure period was corrected by the ΔOD in the test wells without worms.

Data analysis

All experiments were replicated at least three times. LC50s from each replicate were generated by Probit from log-transformed data in Toxstat® 3.4 after passing normality (X^2) and homogeneity of variance (Bartlett's) tests (WEST Inc. and Gulley 1994). The LC50s were reported as the mean value of three replicates. Standard deviations (SD) and coefficients of variation (CV) were also calculated for LC50s. Each CV was calculated by dividing the SD of each group by the grand mean and multiplying by 100%.

EC50 values, the concentrations at which the average responses of movement or reproduction were 50% of the control group from each replicate, were generated in a similar manner to LC50s. The only difference was that control responses were assigned

values of 100 and each treatment group was expressed as a fraction of control movement. After log transformation, EC50 values were computed by Probit analysis. Overall EC50 values were reported as the arithmetic mean value of the replicates.

RESULTS

Lethality tests. Control reference charts are presented in Figure 5.1. The order of toxicity (LC50s) observed after 24-h Cu exposures was *P. redivivus* (160 mg Cu/L) > *C. elegans* (85.4 mg Cu/L) > *P. pacificus* (18.9 mg Cu/L) (Table 5.1). Besides being the most sensitive, *P. pacificus* also exhibited the lowest variability between test dates (Fig 5.1) and therefore the lowest CVs (Table 5.1). Although slightly higher than *P. pacificus*, both the *C. elegans* and *P. redivivus* CVs were relatively low, indicating high reproducibility (Table 5.1). When Cu exposures were performed in soil rather than in aquatic medium, the same general pattern of LC50s was observed where *P. pacificus* (48.2 mg Cu/L) was most sensitive, followed by *C. elegans* (179 mg Cu/L), and finally by *P. redivivus* (251 mg Cu/L). However, only *P. pacificus* was statistically different from the other two.

Reproduction tests. Although *P. pacificus* appeared to be much more sensitive to Cu exposures in lethality tests, EC50s for both *C. elegans* (2.04 mg Cu/L) and *P. pacificus* (2.21 mg Cu/L) were almost identical (Table 5.1). The responses of *P. pacificus* reproduction were more variable than those of *C. elegans*.

Movement and feeding assays. Both *C. elegans* and *P. pacificus* experienced a concentration-dependent decrease in movement and feeding after 24-h Cu exposures at sublethal concentrations (Fig 5.2). The control groups of both species exhibited similar rates of movement (3.03 μm/s for *C. elegans* and 3.07μm/s for *P. pacificus*) and feeding (2.4 ΔOD units per worm over 24 h) on average. Both species showed a concentration-dependent decrease in feeding (Fig 5.2b). However, *P. pacificus* movement EC50s were significantly higher ($p < 0.0001$) than those for *C. elegans* (Table 5.1).

DISCUSSION

Nematodes are becoming popular bioindicators of pollution stress due to their ecological significance as well as the ease of culture and maintenance of large numbers of organisms in the laboratory. Because they share many of the same cellular processes and centralized nervous systems as mammals, their potential use as model systems in developmental biology studies has already been realized (Hood et al. 2000). Although nematodes are ideal candidates for toxicity testing, more comparative toxicology studies need to be performed to determine the relative sensitivity of nematode species in standardized toxicity tests.

Rhabditid nematodes are free-living bacterial feeders that are intimately involved in the regulation of microbial activity and therefore decomposition and nutrient cycling. Because they are in such close contact with microbes and their potentially toxic exudates, rhabditid nematodes are generally thought to be relatively insensitive to toxicants (Bongers and Ferris 1999). A recent study by Carta (2000) found differences in the

survival and feeding behavior of several rhabditid species, including the three used in our study, after exposure to the nematicide *B. cepacia.* Although the toxic effects varied between nematodes, in general *P. pacificus* was extremely tolerant, *P. redivivus* barely survived when grown on plates seeded with *B. cepacia*,. and *C. elegans* responses tended to be intermediate. Due to these findings, *P. pacificus* was then considered a toxicant-tolerant species. However, in our studies, *P. pacificus* Cu LC50s were considerably lower than those of *P. redivivus* in aquatic medium or soils (Table 5.1). Therefore sensitivity of species appears to vary with the specific toxicant tested.

In this study we used Cu to construct control reference charts for *P. pacificus* and *P. redivivus* similar to those already in use in our lab with *C. elegans* (Freeman et al. 1998;1999). Performing regular reference toxicity tests is an important step in the standardization of toxicity testing, most importantly to maintain high reproducibility over time and between different laboratories. As specified by the U.S. EPA (1993), the cumulative average is plotted with upper and lower boundaries at ± 2 SDs representing a 95% confidence interval (Fig 5.1). By ensuring that the test organisms' responses are similar, the precision (measured as a low CV) is maintained from test to test (McNulty et al. 1999; Yeardley et al. 1995). The responses of all three nematode species were highly reproducible with CVs ranging from 14.8 for *P. redivivus* to only 7.36 for *P. pacificus* (Table 5.1). Therefore it appears that Cu would be an excellent reference toxicant to use for these two species in the same manner as we have done with *C. elegans*.

In both aquatic and soil lethality tests, *P. redivivus* was the least sensitive to Cu exposures while *P. pacificus* was the most sensitive and *C. elegans* was intermediate in sensitivity (Fig 5.1 and Table 5.1). Similarly, response variation was highest for *P.*

redivivus and lowest for *P. pacificus*. Although all three species were used in lethality tests, *P. redivivus* was not used in the sublethal toxicity tests because of the difficulty in obtaining high numbers of age-synchronized nematodes. A few methods of synchronization were described in the literature (Bollinger and Willet 1978; Sternberg and Horvitz 1981) but they either resulted in limited numbers of organisms or in stressed organisms.

Comparisons between LC50s and sublethal EC50s for *C. elegans* have previously been performed with Cu using similar procedures as in the current study (Dhawan et al. 2000). For Cu, an LC50 of 91 mg/L and a movement EC50 of 3 mg/L were reported for *C. elegans*. These numbers are remarkably similar to the EC50s for *C. elegans* in the current study (85 and 2 mg/L Cu, respectively) suggesting high repeatability even between different users (Table 5.1). Another study found EC50s for Cu ranging from 2.8 to 4.1 mg/L for reproduction and movement (Anderson et al. 2001). In the current study, all of the *C. elegans* EC50s were almost identical at around 2 mg/L (Table 5.1). In all cases, the ratio of LC50:EC50 ranged from 30-40 for *C. elegans*. Although *P. pacificus* EC50s were lower than its LC50s, the ratio of LC50:EC50 was less than 10, much lower than that for *C. elegans* (Table 5.1). In addition, while all *C. elegans* EC50s were almost identical, *P. pacificus* movement EC50s were significantly higher ($p>0.05$) than its reproduction EC50s. Perhaps reductions in *C. elegans* movement at low exposure concentrations are offering some protection leading to reduced mortality apparent in lethality tests. Another possibility is that the energy reserves are greater in *P. pacificus* considering that feeding was affected similarly in both *C. elegans* and *P. pacificus*, even though movement was significantly greater in *P. pacificus* (Fig 5.2b). Previously we

have shown *C. elegans* movement to be dependent upon the nutritional status of nematodes (Boyd et al. 2000).

Few studies with *P. redivivus* or *P. pacificus* are suitable for direct comparison to the current one due to differences in testing procedures. Samoiloff (1990) described a developmental assay with *P. redivivus* that observed the growth from J2 to adults during toxicant exposure. Using this test, the response of *P. redivivus* varied with the metal tested (although Cu was not included) as well as the developmental stage (Samoiloff et al. 1980). No literature on the effects of Cu on *P. pacificus* was found.

The responses of nematodes to Cu exposures in the laboratory may or may not be related to the sensitivity of the same species in the field where life history traits may also come into play in their persistence. One convenient means of estimating the effects of pollutants on soil ecosystems is with a maturity index (MI) whereby the change in the nematode community structure after disturbance can be calculated (Bongers 1999). This index uses the colonizer-persister (cp) classification scheme that groups nematode families based on characteristics such as feeding types, reproductive potential, and formation of dauerlarva. Nematodes are classified along a continuum where cp-1 nematodes are termed colonizers or enrichment opportunists and at the far end of the scale the persisters are classified from cp-3 to cp-5. Nematodes classified as cp-1 are small, bacterial-feeders that are able to reproduce in high numbers, similar to *r*-strategists. All nematodes belonging to the Rhabditida order are classified as cp-1 (Bongers 1999). Bacterial-feeders that do not form dauerlarvae are classified as cp-2 nematodes or general opportunists. Because they are more tolerant to low food

availabilities, cp-2 nematodes are more likely to be found in soils that are polluted to the point that microbial activity is depleted (Bongers and Ferris 1999).

Persisters, nematodes with higher cp-rankings, can be likened to *K*-strategists. They tend to be larger nematodes that are parasitic or predatory and have lower reproductive capacities than cp-1 and cp-2 nematodes. During stressful conditions such as in polluted systems, the MI tends to decrease, indicating a decrease in the abundance of persisters and an increase in the number of opportunists (Bongers and Bongers 1998). Recently it was reported that the sensitivities of 70 nematode taxa to $CuSO_4$ in acute tests were positively correlated with cp classifications (Bongers et al. 2001).

Because cp-1 nematodes are the most abundant group and are often found at disturbed sites, their relative sensitivity to toxicant stress is of interest. They are thought to be the least sensitive nematode group to toxicants possibly because of resistance to microbial exudates along with their impermeable cuticle (Kammenga et al. 1994; Bongers 1999). Previous studies have found differences in sensitivity after metal exposures between cp-2 nematodes (Korthals et al. 1996b), however no evidence of similar studies with cp-1 species was found. The sensitivity of the cp-2 nematodes *Plectus acuminatus* and *Heterocephalobus pauciannulatus* to Cu exposures has been compared using reproduction as an endpoint (Kammenga and Riksen 1996). The EC20 values for reproduction were 3 and 9 mg/L Cu for *P. acuminatus* and *H. pauciannulatus*, respectively. Although these numbers are similar to the reproduction EC50s (2 mg/L) for *C. elegans* and *P. pacificus* (Table 1), differences in reproduction assays and calculations of EC20s rather than EC50s make direct comparisons impossible. We did attempt to use *P. acuminatus* and *H. pauciannulatus* in our assays. However, *P. acuminatus* has a

relatively long life cycle and lays few eggs over many days (Kammenga et al. 1996) making it difficult to obtain enough age-synchronized organisms for all tests. *Heterocephalobus pauciannulatus* cultures did not reproduce consistently on an *E. coli* food source and were therefore eliminated from all tests. Future tests with different bacteria as food sources (such as *Acinetobacter johnsonion*) should allow for inclusion of *H. pauciannulatus* in future testing.

In general, we recommend continuing the investigation of the sensitivity of *P. pacificus* to other toxicants. Because the culturing and testing procedures are so comparable to *C. elegans*, the addition of *P. pacificus* to the ASTM guide would require minimal description and would be a worthy supplement. *Panagrellus redivivus* are also useful organisms for toxicity testing, especially in soil tests, because the adults are somewhat larger than the other two species and are therefore easier to isolate. If an acceptable means of age-synchronization can be devised, *P. redivivus* will also offer a useful complement to the ASTM *C. elegans* standard guide.

Although it is unrealistic to think that any one species can serve as a universal bioindicator of soil health, we feel that *C. elegans* is a suitable organism for standardized, single-organism toxicity testing. Because the responses of *C. elegans* were intermediate in the current study, it appears to be representative of other rhabditid species. However, other rhabditids must be studied in order to further observe the relative sensitivity of *C. elegans*. In addition, we hope to compare the sensitivity of some cp-2 species to *C. elegans* and other cp-1 nematodes.

Acknowledgment - We are grateful to the US Environmental Protection Agency's Science to Achieve Results (STAR) Graduate Fellowship Program and the University of Georgia's Agricultural Experiment Station for financial support of this research. Theresa Stiernagle at the *Caenorhabditis* Genetics Center, which is funded by the National Institutes of Health (NIH) National Center for Research Resources (NCRR), provided the nematodes used in this work. We also wish to thank Dr. David Dusenbery for use of the NIH Image computer tracking software.

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Table 5.1. Comparison of the toxic effects of Cu on the survival, reproduction, and behavior of three nematode species (*Caenorhabditis elegans*, *Pristionchus pacificus*, and *Panagrellus redivivus*).

Species	Aquatic LC50[a]	Aquatic CV[b]	Soil LC50[a]	Soil CV[b]	Reproduction EC50[c]	Movement EC50[c]
C. elegans	85.4 ± 10.3	12.0 %	179 ± 47.4	26.5 %	2.04 ± 0.19	2.06 ± 0.30
P. pacificus	18.9 ± 1.39	7.4 %	48.2 ± 5.43	11.2 %	2.21 ± 0.93	8.07 ± 1.44
P. redivivus	160 ± 23.6	14.8 %	251 ± 52.5	20.9 %	n/a	n/a

[a] LC50 = lethal concentration to 50% of nematodes (± SD) in mg Cu/L

[b] CV = coefficient of variation

[c] EC50 = the concentration of Cu in mg/L required to reduce reproduction or movement by 50% relative to controls

[d] n/a = reproduction, movement, and feeding assays not performed on *P. redivivus* due to difficulties in obtaining age-synchronized cultures

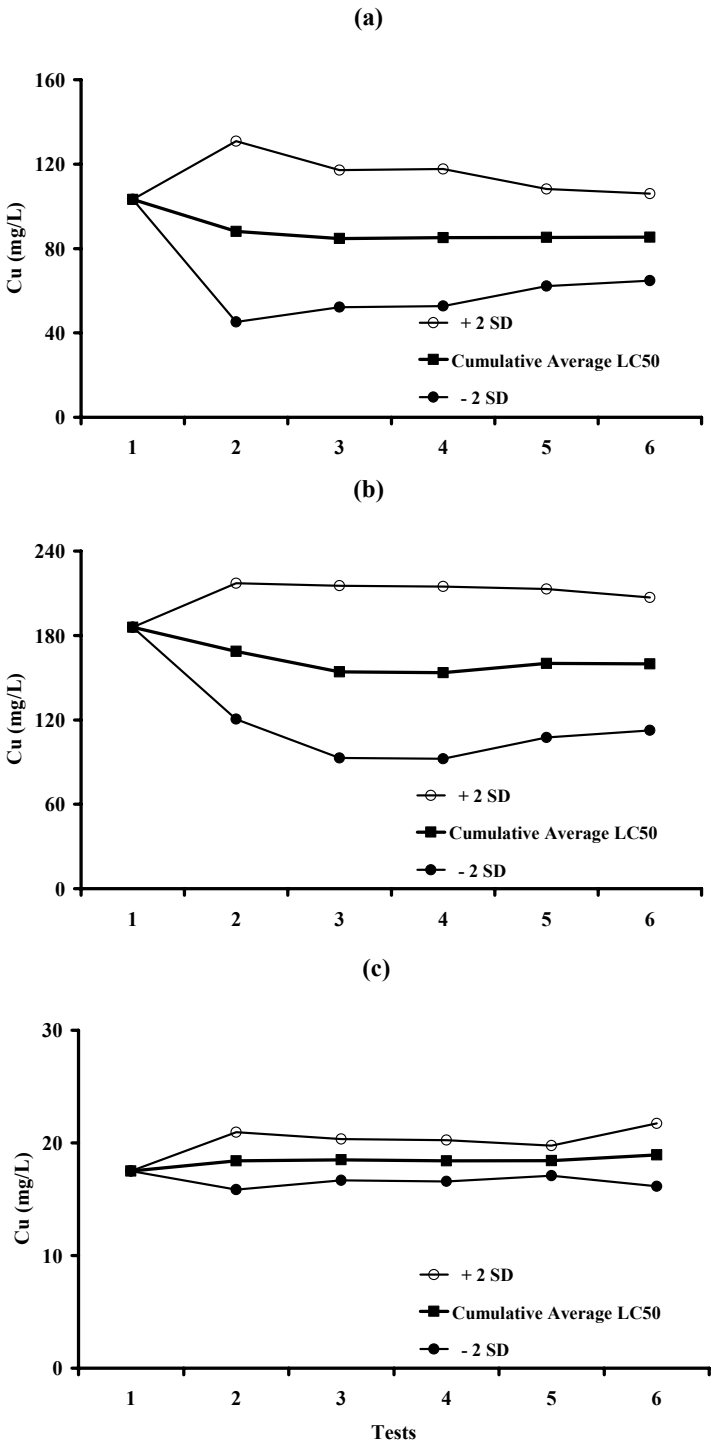

Figure 5.1. Control reference charts showing cumulative average LC50s (lethal concentrations to 50% of nematodes) ±2 SD of 24-h Cu exposures of (a) *Caenorhabditis elegans*, (b) *Panagrellus redivivus*, and (c) *Pristionchus pacificus*.

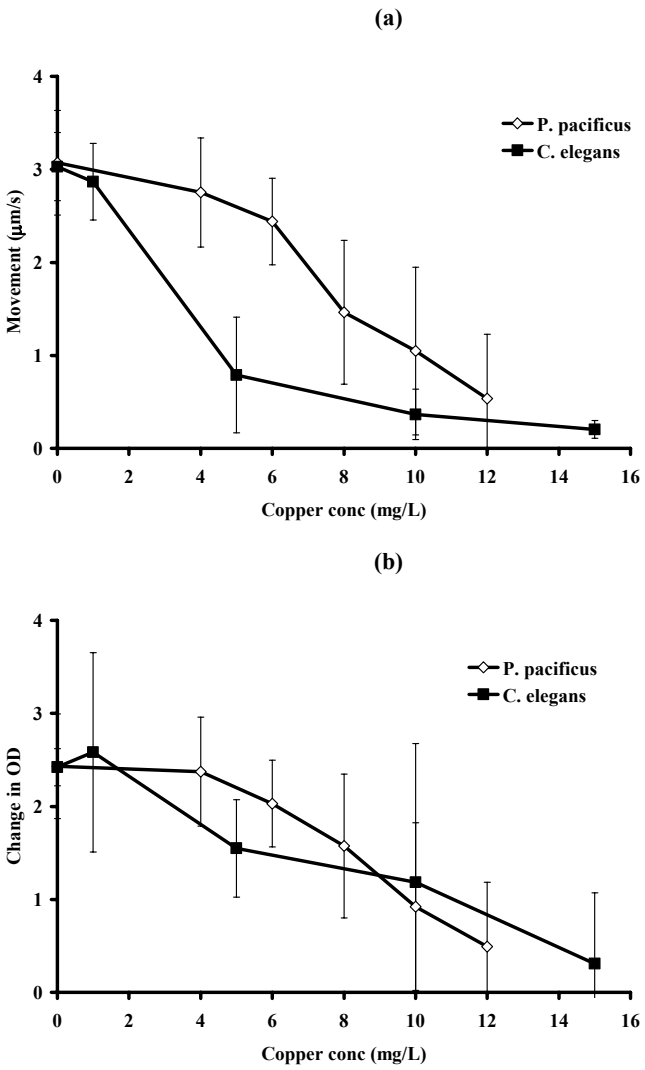

Figure 5.2. Effects of Cu exposures on *Caenorhabditis elegans* and *Pristionchus pacificus* (a) movement and (b) feeding after 24-h exposures.

CHAPTER 6

COMPUTER TRACKING METHOD FOR ASSESSING BEHAVIORAL CHANGES IN THE NEMATODE *CAENORHABDITIS ELEGANS*[1]

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[1] Boyd, W. A., Anderson, G. L., Dusenbery, D. B., and Williams, P. L. 2000. *Environmental Toxicology and Risk Assessment: Recent Achievements in Environmental Fate and Transport: Ninth Volume, ASTM STP 1381,* F. T. Price, K. V. Brix, and N. K. Lane, Eds., American Society for Testing and Materials, West Conshohocken, PA, pp. 225-238.

Abstract

Computer tracking of *Caenorhabditis elegans*, a free-living soil nematode, is a promising tool to assess behavioral changes upon exposure to contaminants. A short life cycle, a known genetic make-up, thoroughly studied behavior, and a completely mapped nervous system make *C. elegans* an attractive soil test organism with many advantages over the commonly used earthworm. Although many toxicity tests have been performed with *C. elegans*, the majority focused on mortality, a much less sensitive endpoint than behavior. A computer tracking system has been developed to monitor behavioral changes using *C. elegans*. Because conditions unrelated to specific toxicant exposures, such as changes in temperature, developmental stage, and presence of adequate food sources, can affect behavior, there is a need to standardize tracking procedures. To this end, we have developed reference charts for control movement comparing the movement of four and five day-old adult nematodes. The use of K-medium versus deionized (DI) H_2O for pre-tracking rinses was also investigated. A final reference chart compared the behavioral responses of nematodes at various food densities (i.e. bacterial concentrations).

Keywords: *Caenorhabditis elegans,* behavior, computer tracking

Introduction

For the past three decades, the use of *Caenorhabditis elegans* as a model organism in biological, physiological and molecular studies has expanded. Reasons for the popularity of the nematode as an experimental organism include a short life cycle (three days from egg to adult), a completely characterized and simple cell lineage, easy laboratory cultivation and maintenance, availability of known genetic mutants, and, most recently, an almost completely known genome sequence (Wood 1988, Riddle et al. 1997, Bargmann 1998). Nematodes also fill an important niche in the soil environment by feeding on bacteria and helping to maintain balance of nutrient cycling (Sohlenius 1980). Based on their abundance alone, nematodes make up a significant portion of soil macrobiota (Blaxter 1998).

Recently, researchers have begun to realize the potential for *C. elegans* as a model test organism in toxicity studies. To this point, *C. elegans* has been utilized in many lethality tests in media such as agar plates (Williams and Dusenbery 1988), aquatic media (Cressman and Williams 1997), and soil (Freeman et al. 1999). Although these tests provide relevant information on the survival of the worm, a means to assess sublethal effects of toxicants would also be useful. A computer tracking system which non-invasively monitors the movement and change of direction of tens to hundreds of worms has been developed (Dusenbery 1985). A video camera interfaced with a computer allows for a real-time assessment of changes in the nematode's simple behavior. Responses to metal exposures (Williams and Dusenbery 1987), organophosphate pesticide exposures (Williams and Dusenbery 1990a) and exposures to organic compounds such as isoamyl acetate (Terrill and Dusenbery 1996) have previously shown

the applicability of the computer tracking system. Because behavior is such a sensitive endpoint of toxicity, standardization of the tracking protocol is imperative. Changes in the availability of food, temperature, developmental stage of the worm, and/or other environmental factors must be taken into account.

The objectives of this study are to initiate the standardization of the computer tracking method by: 1) constructing control reference charts for four and five day-old worms; 2) comparing movement of 4 and 5 day-old unexposed worms; 3) comparing the use of K-medium with deionized (DI) H_2O as pre-tracking rinses; 4) assessing the effect of the availability of food on control movement; and 5) observing the temperature and relative humidity inside the tracking chamber.

Materials and Methods

The wild type N2 strain of *C. elegans* was obtained from the *Caenorhabditis* Genetic Center (Minneapolis, MN) and used in all tests. Cultures were cultured as previously described to yield age-synchronized adult worms (Cressman and Williams 1997). Monthly reference toxicant testing was performed with copper to verify the health of the worms (Freeman et al. 1998). Adult worms were rinsed from culture plates and transferred to exposure dishes at three or four days old. After a 24-h exposure period, the supernatants of test solutions were measured for pH with an Orion Model 720 pH meter and Ross combination electrode. Each sample's pH was found to be between 5.5 and 6.5, well within the acceptable range of approximately 3-12 for *C. elegans* (Khanna et al. 1997). Room temperature and relative humidity were recorded at the beginning and end of each tracking period.

Computer Tracking Procedures

For control reference charts, adult worms were rinsed with K-medium (Donkin and Williams 1995) or DI H_2O into 15-mL centrifuge tubes. After thorough rinsing to remove any debris and removal of excess supernatant, approximately 50 worms were transferred to a thin layer of cooled 1% agar on a glass slide. With the agar slab over a petri dish filled with H_2O, worms were allowed to distribute evenly. After approximately 30 minutes, the glass slide was placed within a hydrated air stream directly below a Hitachi CCD video camera interfaced with a Power Macintosh G3 computer equipped with a Scion LG 3 PCI frame-grabber board. The nematodes were then illuminated with a dark field illumination system where their movement and change in direction were recorded and arranged in a spreadsheet format by an NIH Image Tracker software program modified by Dr. David Dusenbery (Dusenbery 1996). Other parameters reported on the spreadsheet included number of subjects tracked and the number of subjects moving in each one second cycle. For the current study, each tracking period consisted of 100 seconds and each sample was tracked three periods. Experiments testing the effects of pre-tracking rinses and ages of worms consisted of two sub-samples per replication.

Food Density Studies

The food source for the nematodes was the OP50 strain of *Escherichia coli*, which was maintained in an L-broth solution (Donkin and Williams 1995). In order to pellet the bacteria, 20 mL of L-broth was centrifuged at 750 g for 7 minutes and the supernatant removed a total of three times to remove debris. The pellet was then re-suspended in 5

mL of K-medium for a 4:1 (L-broth to K-medium) concentration. From this solution, a series of volume to volume dilutions were made for final concentrations of 0 (no food), 0.25:1, 0.5:1, 1:1, 2:1, and 4:1. One milliliter of each solution was pipetted into each well of a twelve well culture plate. For each concentration, two wells were loaded, one with approximately 100 worms and one with no worms. Using the CCD camera and computer system described above, each well containing worms was counted manually and recorded. Then, the optical density (OD) of each solution was read at 570 nm and recorded immediately after worm transfer and after a 24-h incubation period. It should be noted that the actual concentrations between replications did vary somewhat due to loss of bacteria during rinsing procedures. In fact, almost half of the bacteria were lost during the three rinses so that a 2:1 volume to volume concentration actually contained approximately the same density of bacteria as the undiluted L-broth. However, for the sake of simplicity and because rinsing of bacterial pellets is a common practice without measuring the optical density, the treatments will be referred to as listed rather than their actual concentrations. After the 24-h incubation period and reading the optical density, worms were rinsed from the twelve well plates and transferred to 15-mL centrifuge tubes. From this point, the same computer tracking procedures were used as detailed previously.

Experimental Design and Statistical Analyses

All experiments were replicated a total of six times. Experiments investigating the effects of the age of worms and rinse mediums consisted of two tracking gels per treatment. Food density studies consisted of one tracking gel per treatment. Standard deviations (SD), average movements and changes in OD, and coefficients of variation

(CV) for each treatment group were calculated. The CV was calculated by dividing the SD of each group by the mean value and multiplying by 100%. The general linear model procedure in SAS was used to test for significant differences in mean values and multiple comparisons were performed with Scheffe's procedure (SAS Institute 1989).

Results

Age of Worms and Pre-tracking Rinse Media

The first objective of the study was to develop a control chart for five day-old worms, the most commonly used age of adult worm for toxicity testing (Figure 6.1), and for four day-old worms (Figure6.2). The figures present individual test's (replicate) average rate of movement, as well as the average rate of movement for all tests up to that point (cumulative). As evidenced in the figures, both groups exhibited moderate variation from the average movement. However, the movement of five day-old worms deviated less than the movement of four day-old worms. Table 6.1 provides a summary of the comparisons between four and five day-old worms. Although each group's coefficient of variation (CV) was relatively low, five day-old worms showed less variability in their movement. On average, five day-old-worms' movement was slightly lower than that of the four day-old worms. However, this difference was not found to be significant (Table 6.1). As pre-tracking rinse mediums, the use of K-medium or DI H_2O did not affect the variability or average movement of either age group of worms (Table 6.1, Figures 6.3 and 6.4). For all experiments, room temperature ranged from 20-25 ^oC and relative humidity ranged from 27-55%.

Food Density

A final experiment investigated the effects of available food, in the form of bacteria, on worm movement. Figure 6.5 compares the average movement per worm with the amount of bacteria eaten per worm. On only one of six replications, worms starved for the 24-h incubation period (0 concentration) were able to survive rinsing and transferring procedures and exhibited movement. Otherwise, these worms were alive but showed no movement when transferred to the tracking gel (Figure 6.5). When bacteria pellets were diluted to a concentration of 0.25:1 (L-broth:K-medium), the food source was diminished in the 24-h incubation period to the same level as the 0:1 group. Similarly, the movement of the 0.25:1 group was not significantly different from that of the starved group (Figure 6.5). Movement was not significantly affected by food density at concentrations higher than 0.5:1 (Figure 6.5, Table 6.2). However, feeding, as measured by a change in optical density (OD) at 570 nm, was significantly affected by food density (Figures 6.5 and 6.6, Table 6.2). In the control wells with no worms added, there was a decline in bacteria over the 24-h incubation period, but this decline was found to be less than that seen in the wells with worms at all concentrations, except the 0:1 and 0.25:1 groups (Figure 6.6). Because the bacteria were no longer in a suitable environment for growth and survival, some bacteria were dying and apparently lysing over the 24-h incubation period, thereby decreasing the OD. At the 0.5:1 level or higher, there were enough bacteria present for the worms to have an ample food source for the 24-h incubation period.

Discussion

The popularity of toxicity testing with *C. elegans* has risen in the past decade. Different endpoints of toxicity have been reported including mortality (Cressman and Williams 1997), transgenic expression (Candido and Jones 1996), reproduction (Middendorf and Dusenbery 1993), body length (Traunspurger et al. 1997), and behavior (Williams and Dusenbery 1990a). In addition to different endpoints, various exposure media have also been used including soil (Donkin and Dusenbery 1993, 1994 and Freeman et al. 1999), aquatic (Williams and Dusenbery 1990b and Freeman et al. 1998), agar plates (Williams and Dusenbery 1988), and sediment (Traunsperger et al. 1997). Because *C. elegans* exhibits such simple behavior, continuously searching for food while moving in a wave-like motion, the changes in direction and rate of movement provide effective and easy-to-quantify measurements of behavior (Dusenbery 1980, 1985). Although the measurements of all endpoints of toxicity provide useful information about the potential health threats of chemicals, neurobehavioral studies may prove to be a most effective means of testing new chemicals. As with all pre-market testing, neurobehavioral testing is generally an expensive and lengthy process. However, the computer tracking system described above is a relatively quick and inexpensive test system (Williams and Dusenbery 1990a). Before this system can become widely accepted, more investigation into a standardized testing procedure must be completed.

The first concern in standardizing the tracking protocol was the issue of how the age of adult worms might affect control movement. Because the 95% confidence intervals were so similar for both age groups, it appears that either four or five day-old worms could be used for 24-h toxicity testing. Although both groups showed an

acceptable degree of reproducibility (CV < 30%), the movement of the five day-old worms was less variable than that of the four day-old worms (Table 6.1). This is probably due to the fact that the smaller worms had a tendency to desiccate more quickly. After four day-old worms were transferred to the tracking gels, they had to be monitored very closely while dispersing on the gel to prevent them from drying out while five day-old worms could be left for several hours without decreases in movement (data not shown). For these reasons, if time permits, tracking of five day-old worms would be preferred.

Control reference charts were constructed for both age groups by plotting the test replications on the x axis and the average rate of movement per worm on the y axis. According to the Environmental Protection Agency (1993), control charts and their 95% confidence intervals (± 2 SD) can be used to determine the precision of tests as well as the overall fitness of test organisms. Currently, our lab has used monthly aquatic reference testing with copper to assess organism fitness (Freeman et al. 1998). Monthly computer tracking of control worms might prove to be an additional means of assessing worm health without the production of waste and potential health threat to lab workers.

Because the buildup of salts on tracking gels left after medium evaporation is known to attract *C. elegans*, we investigated the use of K-medium and DI H_2O as pre-tracking rinses (Donkin and Williams 1995). Worm movement and variability of movement were not significantly affected by the type of pre-tracking rinse medium used in either age group of worms (Table 6.1, Figures 6.3 and 6.4). These findings could be due to the fact that very low volumes of rinse medium were actually transferred to the tracking gels with the worms.

Because *C. elegans*' behavior is so closely linked to the abundance of food, our final investigation was into the effects of different bacterial concentrations on movement. *C. elegans* eats by pharyngeal pumping while moving through the surrounding media. Pharyngeal pumping is affected by various stimulations including touch, dauer larvae formation, and presence or absence of bacteria (Avery and Horvitz 1990). In their study, Avery and Horvitz (1990) observed a significant increase in the pharyngeal pumping rate when worms were starved for four or more hours or when bacteria were plentiful. Furthermore, they found that starved worms responded to lower concentrations of bacteria than those required to stimulate fed worms. Other experiments in our lab have shown that, in short-term lethality tests, it was not necessary to feed worms (Donkin and Williams 1995). In the current study, because starved worms could not survive rinsing and transferring to the tracking gel, we conclude that a food source must be present. In past computer tracking experiments, we have used a 2:1 (L-broth to K-medium) concentration of bacteria for a 24-h exposure period without measuring the actual concentration. The results of the current study show that even lower concentrations (greater than 0.5:1) are an ample food source and do not affect movement significantly (Figure 6.5, Table 6.2).

In conclusion, we recommend further investigations into the use of computer tracking procedures and similar procedures to quantify behavioral changes. Table 6.3 provides a summary of suggested procedures for computer tracking. Although K-medium or DI H_2O is equally suitable as a pre-tracking rinse, the use of DI H_2O is less costly and a larger volume of medium could be transferred without concern of salt build-up. In terms of feeding, no significant differences in movement are seen at

concentrations above 0.5:1. However, maintaining a 2:1 concentration would ensure that worms were well-fed. Future examinations into toxicant exposures at various bacterial concentrations may further clarify this picture. The effects of temperature and relative humidity, other factors known to affect behavior in many organisms, warrant further studies. Currently, we have no means of controlling either. We suspect that temperature plays a significant role in the worm's behavior and may account for some of the variability evident in the control charts.

Acknowledgments

We are grateful to the University of Georgia's Agricultural Experiment Station for financial support of this research. The nematode strain used in this work was provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

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Table 6.1 – *Comparisons of four and five day-old nematodes.*

Age (days)[a]	Rinse Medium	Coefficient of Variation[b]	Mean Rate of Movement (μm/s)	95 % C. I.
4	K-medium	24 %	3.39	1.73-5.06
	DI H_2O	24 %	3.20	1.65-4.75
5	K-medium	18 %	2.82	1.82-3.82
	DI H_2O	19 %	3.01	1.86-4.15

[a]Age of worms following 24 h exposure
[b]Coefficient of Variation (CV), [(SD/0) x 100]

Table 6.2 – *Multiple comparisons of feeding and average rate of movement of five day-old worms. Different letters indicate significant differences as determined by Scheffe's test.*

Bacterial Concentration	Mean Rate of Movement (μm/s)	Standard Deviation	Mean Change in OD/24 h (x 10000)	Standard Deviation (x 10000)
0	0.053 c	0.125	0.121 d	0.124
0.25	0.689 c	0.378	0.351 d	0.152
0.5	1.603 b	0.784	0.947 cd	0.402
1.0	2.854 a	0.723	1.426 c	0.319
2.0	3.098 a	0.896	2.446 b	0.522
4.0	3.326 a	0.745	3.640 a	0.807

Table 6.3 - *Summary of conditions and test acceptability criteria for* Caenorhabditis elegans *computer tracking system.*

Test Exposure	24 h Duration
Type	Static
Chamber	12-well tissue culture tray
Exposure Volume	1 mL/well
Organism Age	120 h (± 4h)[a]
Number of organism per well	~100
Food Source	2:1 ratio of L-broth[b]
Dilution medium	K-medium[c]
Allowable pH range	3.1 - 11.9[d]
Tracking medium	1% agar
Endpoint	Rate of movement
Software	NIH Image, Version 1.59[e]
Hardware	CCD camera, Power Mac
Test Acceptability	± 2 SD on control chart

[a] Age at time of tracking.
[b] Bacterial density should be confirmed spectrophotometrically to be ≥ 0.04 OD.
[c] Williams and Dusenbery 1990b.
[d] Khanna et al. 1997.
[e] Dusenbery 1996.

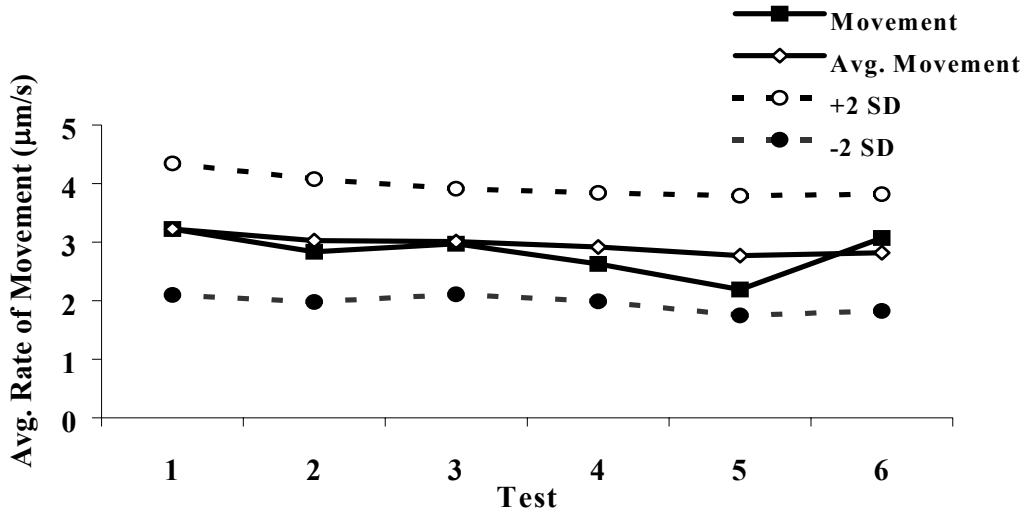

Figure 6.1-- *Control Reference Chart showing replicate and cumulative average rate of movement (µm/s) ±2 SD - five day-old worms with K-medium as pre-tracking rinse.*

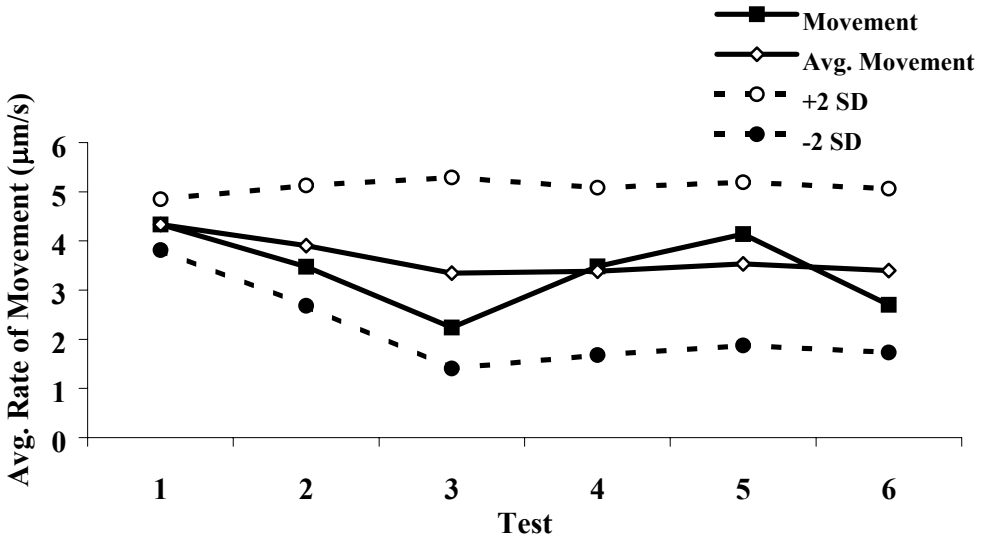

Figure 6.2-- *Control Reference Chart showing replicate and cumulative average rate of movement (µm/s) ±2 SD - four day-old worms with K-medium as pre-tracking rinse.*

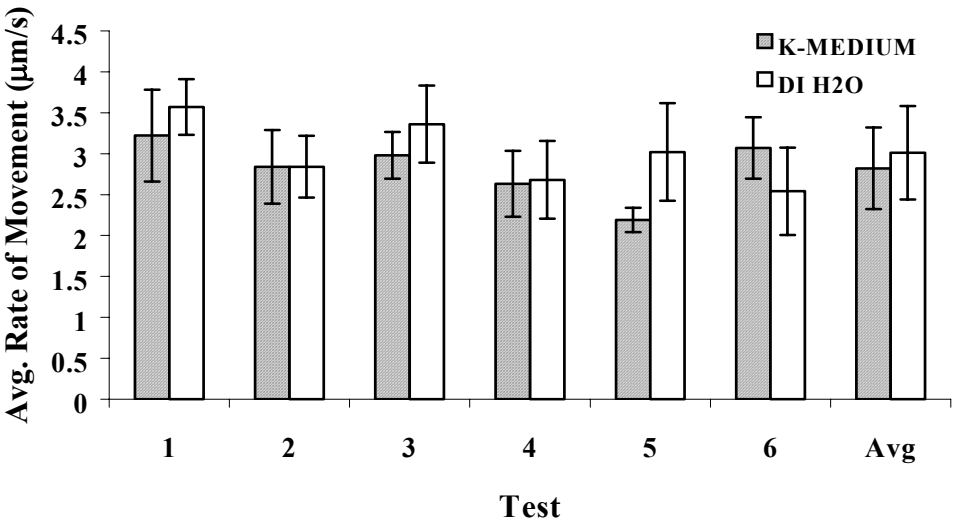

Figure 6.3--*Comparisons of K-medium and DI H_2O as pre-tracking rinses showing replicate average rate of movement (µm/s) ±SD of five day-old worms.*

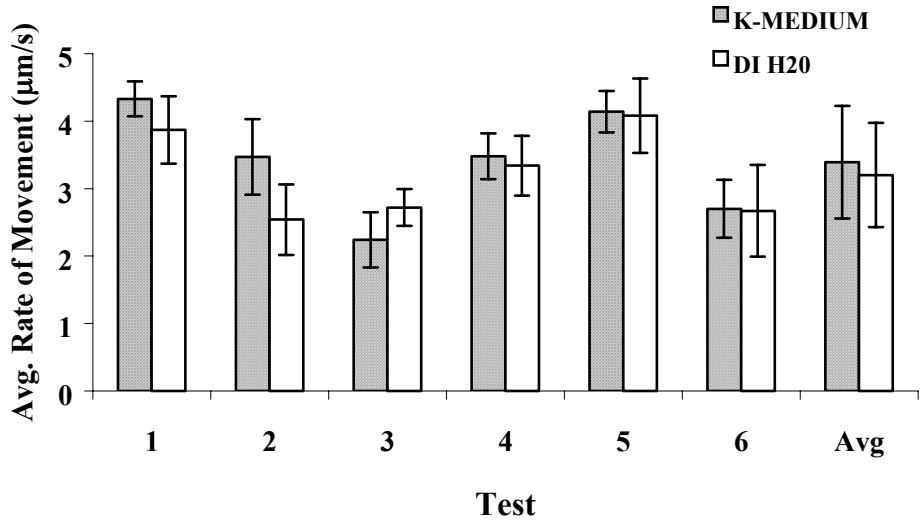

Figure 6.4--*Comparisons of K-medium and DI H_2O as pre-tracking rinses showing replicate average rate of movement (µm/s) ±SD of four day-old worms.*

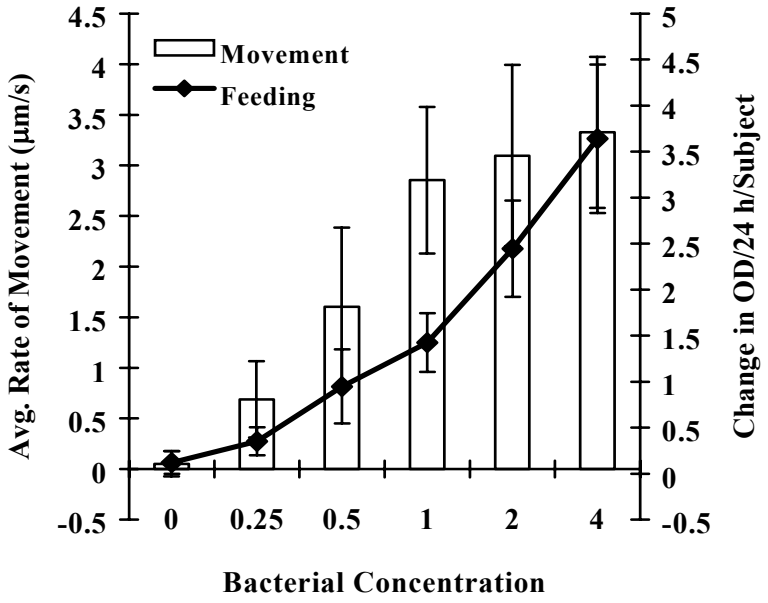

Figure 6.5--*Comparisons of feeding measured as the change in optical density (OD) at 570 nm (x 10000) and average rate of movement (µm/s) ±SD of five day-old worms.*

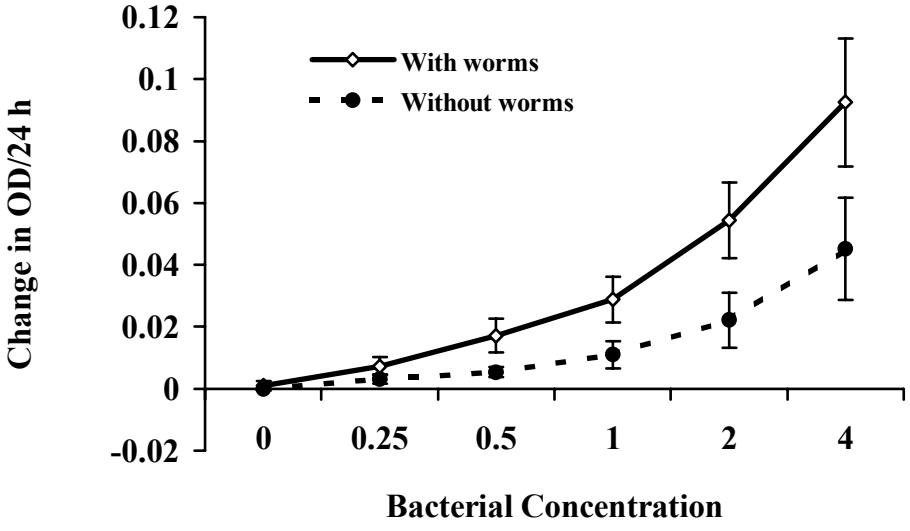

Figure 6.6--*Comparisons of the changes in optical density (OD) at 570 nm ±SD with five day-old worms and without worms for 24 h. Range for # worms/well was 90 to 187 with an average of 124.*

CHAPTER 7

THE EFFECTS OF METALS AND FOOD AVAILABILITY ON THE BEHAVIOR OF *CAENORHABDITIS ELEGANS*[1]

[1] Boyd, W.A. Cole, R.D., Anderson, G.L., and P.L. Williams. To be submitted to *Environmental Toxicology and Chemistry*.

ABSTRACT

Caenorhabditis elegans, a non-parasitic soil nematode, was used to assess the combined effects of metal exposures and food availability on behavior. Movement was monitored using a computer tracking system after exposures to Cu, Pb, or Cd while feeding was measured as a change in optical density (ΔOD) of bacteria suspensions over the exposure period. After 24-h exposures at high and low bacteria concentrations, movement was decreased in a concentration-dependent fashion but feeding reductions were not directly proportional to Pb or Cd concentrations. Concentration-dependent declines in feeding and movement of Cu-exposed worms, however, were observed at both bacteria concentrations. The impact of 24-h metal exposures was apparently reduced by increasing food availability. Therefore, exposures were shortened to 4 h in an attempt to minimize starvation effects on movement. Although nematodes exposed for 24 h required food for movement, when the exposure duration was shortened to 4 h worms not fed during exposure tended to ingest more beads and move more after exposure, but these tendencies were statistically insignificant. A bead ingestion assay after 4-h exposures was also used as an additional means of assessing the effects of metals on feeding behavior. Ingestion was significantly reduced by all concentrations of metals tested indicating its sensitivity as a sublethal assay. Feeding (ΔOD) during exposures exhibited similar trends as ingestion but was slightly less sensitive while movement was the least sensitive assay of 4-h metal exposures to *C. elegans*. Assessment of multiple sublethal endpoints allowed for the determination of the separate and interactive effects of metals and food availability on *C. elegans* behavior.

INTRODUCTION

Toxicant-induced behavioral changes of invertebrates such as decreased movement or feeding rate are sensitive endpoints in toxicity testing (Boyd et al. 2002). As computer-automated video tracking systems become more affordable, behavioral data can be generated quickly and more efficiently (Juchelka and Snell 1995). The challenge is to demonstrate that the changes in behavior seen in the individual are connected to the survival of the population as a whole (Little et al. 1985). The acquisition of food is important not only to the survival of the organism but also to their ability to grow and reproduce, factors that if reduced will affect population size (Charoy and Clement 1993).

The potential behavioral responses of nematodes to environmental disturbances are important to study because of nematode abundance in soil ecosystems and their key roles in decomposition and nutrient cycling processes (Yeates and Bongers 1999). The free-living nematode *Caenorhabditis elegans* feeds via pharyngeal pumping while moving through aquatic media or water surrounding soil particles (Avery and Horvitz 1990). The rate of pumping and locomotion within the medium can affect the acquisition of food by *C. elegans*. If the availability of food and/or the presence of a toxicant were to decrease mobility, feeding could also be reduced. Therefore, feeding rate and locomotion are complimentary and may be sensitive endpoints for determining sublethal toxicity of toxicants to *C. elegans* that are relevant to adverse effects at the population level.

The behavioral effects of toxicants on a number of invertebrate species have been reported. Several studies have used the freshwater rotifer, *Brachionus clayciflorus*, to examine the sublethal effects of toxicants on behavioral endpoints (Janssen et al. 1993; Janssen et al. 1994; Juchelka and Snell 1994; Charoy and Clement 1993; Charoy et al.

1995; Charoy and Janssen 1999). Early methods of observing swimming activity under a microscope consisted of counting the number of squares an individual rotifer entered within a grid (Janssen et al. 1993, 1994). With the aid of computer tracking, more sophisticated studies investigated the individual components of rotifer swimming behavior such as the swimming speed, sinuosity, and period of swimming (Charoy et al. 1995; Charoy and Janssen 1999). Similar computer tracking studies have been performed with the nematode *C. elegans* (Dhawan et al. 1999, 2000, Anderson et al. 2001) except that, instead of tracking an individual's movements, tens to hundreds of nematodes are simultaneously tracked with the aid of a modified version of the NIH Image Tracker software (Dusenbery 1997). Computer tracking systems have also been used to monitor the locomotion of the collembolan *Folsomia candida* (Sørensen et al. 1995) and the woodlouse *Oniscus asellus* (Sørensen et al. 1997).

Early estimates of rotifer feeding were calculated as the difference in the concentration of algae, the rotifers' food source, measured before and after a 5-h exposure period with a hemacytometer (Janssen et al. 1993). More recent methods quantified the ingestion of fluorescently-labeled microspheres during a 5-min exposure period (Juchelka and Snell 1994). A similar method was also used to assess the sublethal effects of several toxicants to *Ceriodaphnia dubia*, *Paramecium aurelia*, and *Brachionus plicatilis* (Juchelka and Snell 1995). Feeding in the nematode *C. elegans* has been determined spectrophotometrically as a change in optical density (ΔOD) of bacterial suspensions during exposure (Boyd et al. 2000; Anderson et al. 2001).

By employing movement and feeding assays simultaneously, the independent effects of bacterial density and metal exposures on the behavior of *C. elegans* have been

investigated. Movement and feeding of *C. elegans* were found to increase as the concentration of *Escherichia coli*, the nematodes' food source, increased (Boyd et al. 2000). Nutritionally-deprived worms exhibited virtually no movement after 24-h exposures without food. At higher bacteria densities, feeding continued to increase while movement rates appeared to plateau. In another study, movement and feeding were depressed to similar extents after 24-h exposures to Cu, Cd, or Pb (Anderson et al. 2001). In 4-h tests, where potential effects of starvation are minimized, Pb, but not Cu, decreased *C. elegans* movement. However, feeding was significantly lower with both metal exposures.

As starvation is an important factor that may modify metal toxicity in the laboratory or during environmental exposures, the purpose of the current study was to further clarify the relationship between sublethal metal toxicity and starvation in the nematode *C. elegans*. First we explored the combined effects of food availability and metal exposures on the behavior of *C. elegans* after 24-h exposures. Sublethal concentrations of Cd, Cu, and Pb were used with varying bacteria concentrations in an attempt to determine whether one or both of the treatments affected nematode feeding and locomotion. These metals were chosen due to their prevalence as environmental contaminants and their use in the aforementioned behavioral studies. Secondly, after shortening the exposure duration to 4 h, we observed the relationship between two different methods of measuring feeding and determined whether movement or feeding was a more sensitive endpoint. Although it was necessary to feed worms that were exposed for 24 h in order to perform computer tracking, we were able to quantify the effects of 4-h Cd, Cu, and Pb exposures with both fed and unfed worms. Finally, we

compared the relative toxicity of the three metals after 4- and 24-h exposure periods with nematodes fed varying amounts of food.

MATERIALS AND METHODS

Nematode culture

Wild type N2 strain of *C. elegans* was obtained from the *Caenorhabditis* Genetic Center (Minneapolis, MN) and used in all tests. Cultures were prepared as previously described to yield age-synchronized adult worms (Cressman et al. 1997). Quarterly reference toxicant testing was performed with Cu to verify the health of the worms (Freeman et al. 1998). Adult worms were rinsed from culture plates and transferred to exposure dishes at three days old.

Chemical exposures

Stock solutions of $CuCl_2$, $Pb(NO)_3$, and $CdCl_2$ were prepared with K-medium (0.032 M KCl, 0.051 M NaCl) as the solvent for each test replication (Williams and Dusenbery 1990). Although chloride salts have typically been used to assess toxicity of metals to *C. elegans*, the nitrate salt of Pb was chosen for use in this study due to its higher solubility in K-medium. Testing concentrations were then diluted from the stock solutions, collected, acidified, and stored at 4 ^{o}C. Flame atomic absorption (AA) spectrometry was used to verify final metal concentrations. Copper concentrations tested ranged from 10 to 240 μM for 24-h exposures and from 79 to 632 μM for 4-h exposures. Cadmium concentrations varied with bacteria concentration for 24-h exposures (20 to 160 μM at 1:1 bacterial dilutions and 63 to 625 μM at 4:1 bacterial dilutions), and were

higher for 4-h exposures (222.5 to 1780 μM). Lead concentrations ranged from 15 to 75 μM for 24-h exposures and from 60 to 960 μM for 4-h exposures.

Feeding studies

Escherichia coli (OP50 strain) (Brenner 1974) maintained in L-broth culture medium was used as a food source for the nematodes (Donkin and Williams 1995). To pellet bacteria, 20 mL L-broth was centrifuged at 750 x *g* for 7 min and the supernatant removed to eliminate debris. The pellet was then re-suspended in 5 mL K-medium for a 4:1 OP50 (L-broth to K-medium) concentration. From this solution, a series of volume to volume (v:v) dilutions were made with the appropriate metal solutions for final dilutions of 0 (no food), 1:1 OP50, and 4:1 OP50. One mL of each solution was pipeted into each well of a 12-well tissue culture plate. Two wells were loaded for each concentration, one with approximately 100 worms and one with no worms. Using a CCD camera interfaced to a computer, the exact number of worms was counted in each well for feeding calculations. The optical density (OD) of each solution at 570 nm was recorded immediately after worm transfer (0 h) and after the 4- or 24-h exposure period. Once the post-exposure OD was recorded, worms were rinsed from 12-well tissue culture plates and transferred to 15-mL centrifuge tubes for computer tracking of movement. Feeding was calculated in a similar fashion as described in Anderson et al. (2001) where the ΔOD in the wells with worms over the exposure period was corrected by the ΔOD in the test wells without worms to account for any bacterial death or growth not associated with feeding.

Movement studies

Computer tracking was performed as previously described (Boyd et al. 2000). Briefly, adult worms were rinsed with K-medium into 15-mL centrifuge tubes. After thorough rinsing with K-medium to remove any debris and disposal of excess supernatant, approximately 50 worms were transferred to a thin layer of cooled 1% agar on a glass slide. The agar slab was placed over a petri dish filled with H_2O and worms were allowed to distribute evenly. After approximately 30 min, the glass slide was placed within a hydrated air stream, directly below a Hitachi CCD video camera interfaced with a Power Macintosh G3 computer equipped with a Scion LG 3 PCI frame-grabber board. The nematodes were then illuminated with a dark field illumination system where their movement and change in direction were recorded by an NIH Image Tracker software program (Dusenbery 1997). For the current study, each tracking period consisted of 100 s and each sample was tracked for three periods.

Ingestion studies

Nematode ingestion rates were measured only after 4-h metal exposures. Polychromatic latex microspheres (1.755 μm diameter) were obtained from Polysciences, Inc. (www.polysciences.com). One milliliter of intermediate stock solution with a bead concentration of $3x10^8$ beads/mL was made with de-ionized water, and refrigerated until use.

Ingestion studies were carried out in 12-well tissue culture plates. Ingestion rates immediately before and after 4-h metal exposures were measured as snapshots of worm feeding in the time course of toxicity. All pre-exposure measurements were made in the

absence of any toxicant while the suspensions used in post-exposure measurements contained the same metal concentration used in the exposure. In each test, 500 μL 4:1 OP50 were added to one well followed by 10 μL K-medium containing approximately 20-40 worms. In a second well, 10 μL of the $3x10^8$ beads/mL intermediate were suspended in 480 μL 4:1 OP50. After a 10 min worm acclimation period, the bead suspension was mixed thoroughly into the first well containing the worms yielding a final test suspension of $3x10^6$ beads in 1 mL. Each well was allowed to sit for 5 min at which time 1 mL of 5% formalin solution was added to the test well. This appeared to immediately stop pharyngeal pumping, and killed the worms within 30 min without causing visible cellular damage.

The worms were transferred to small centrifuge tubes and rinsed with K-medium to remove bacteria and un-ingested beads. Once clean, the worms were transferred by pipette onto microscope slides for counting. The slides were placed on a compound microscope equipped with a lamp emitting light in the range of 510-550 nm. In this light, the fluorescent microspheres appeared as bright red dots against the dull red, almost transparent worm carcass. The number of beads ingested by each of 15 worms per microscope slide were counted.

Data analyses

All experiments were replicated at least three times. Standard deviations (SDs), average movements, ΔOD, and coefficients of variation (CVs) for each treatment group were calculated. Each CV was calculated by dividing the SD of each group by the grand mean and multiplying by 100%. EC50 values, the concentrations at which the average

rate of movement was 50% of the control group, from each replicate were generated with log-transformed data in Toxstat® 3.4 (WEST Inc. and Gulley 1994). All data passed normality (X^2) and homogeneity of variance (Bartlett's) tests. Overall EC50 values were reported as the arithmetic mean value of the replicates. The general linear model procedure in SAS was used to test for significant differences in mean values due to interactive or additive effects and multiple comparisons were performed with Tukey's multiple comparison test (SAS Institute 1989).

RESULTS

24-h exposures

Movement and feeding were decreased by Cu exposures in a concentration-dependent fashion at both bacteria concentrations (Fig 7.1). Additionally, EC50s for movement were significantly affected by changing bacteria concentrations (Table 7.1). Increasing food apparently decreased the toxicity of Cu as measured by EC50s of 20 and 50 μM at respective 1:1and 4:1 OP50. With Cu and bacteria concentrations considered as independent factors, significant interaction was observed for both movement ($p<0.0001$) and feeding ($p=0.039$) indicating that the effect of Cu on behavior depended on the amount of food available to *C. elegans*.

In Pb-exposed nematodes movement but not feeding decreased in a concentration-dependent manner (Fig 7.2). Although movement EC50s increased from 20 to 46 μM as bacteria concentrations increased from 1:1 to 4:1, this difference was not significant by Tukey's multiple comparison tests. Interactive effects between Pb and bacteria concentration occurred for movement ($p=0.0073$) but not feeding ($p=0.9221$), although

Pb and food did exert significant main effects on feeding individually ($p<0.0001$). So decreases in feeding of Pb-exposed worms were similar regardless of food availability.

Cadmium-exposed nematodes responded similarly to Pb-exposed nematodes in that movement but not feeding decreased with increasing metal concentrations (Fig 7.3). Because the highest test concentration for 1:1 exposures did not reduce nematode movement by 50% or greater, Cd concentrations were increased four times at 4:1 bacteria concentrations. For this reason, statistical tests for the interactive effects of metal exposure and food availability could not be performed in the same manner as for Cu and Pb. However, movement EC50s increased dramatically with higher food availability from 78 to 187 μM at respective 1:1 and 4:1 OP50 (Table 7.1). Although feeding decreased dramatically with increased Cd concentrations at low food availability, feeding at the 4:1 OP50 was not significantly affected by Cd.

4-h exposures

Initially, the movement and ingestion rates of fed (4:1 bacterial dilution) and unfed (only K-medium) controls were compared after 4 h. All control values were similar over all test dates (data not shown). For this reason, we felt that starvation was minimal in the absence of food at 4-h exposures. By contrast, nematodes held without food survive for 24 h but exhibit virtually no movement when tracked (Boyd et al. 2000).

Preliminary studies on ingestion investigated the length of ingestion periods, the size of microbeads utilized, and the concentration of microbeads suspended in the liquid medium. A range of microbead sizes were chosen to be inclusive of the stoma size of rhabditid nematodes of 1-4 μm in diameter (Yeates 1998). Microbeads with a diameter

of 5 μm or more were not ingested by *C. elegans*. However, they did ingest beads of 3.4 μm diameter or less. Microspheres with an average diameter of 1.755um were used because preliminary tests showed that they were readily ingested by *C. elegans* and could also be easily distinguished using a compound microscope. After testing ingestion levels in several dilutions of 1.755 uM beads over a 5 min period, a bead concentration of $3x10^6$ beads/mL was chosen to provide an average number of ingested beads that were manageable to count while still providing the possibility of distinguishing reduced ingestion from total cessation. Nematode ingestion before metal exposures (immediately after transfer from culture plates) was found to be significantly lower than the ingestion of control nematodes (either fed or unfed) after 4-h liquid exposures (i.e., 4-7 beads/5 min vs. 9-13 beads/5 min).

Metal concentrations tested were selected in an attempt to observe effects on all three parameters (movement, feeding, and ingestion) simultaneously. Feeding and ingestion proved to be much more sensitive to metal exposures than movement. To prevent complete suppression of feeding and ingestion, metal concentrations tested were not high enough to result in greater than 50% reductions in movement. For these reasons, it was not practical to obtain movement EC50s for 4-h exposures.

The movement of nematodes was only significantly affected by the highest Cu concentration (632 μM) while feeding was significantly different from controls at 316 μM Cu. (Fig 7.4a). Ingestion was more than 7 x more with significantly fewer beads (5 beads/5 min) ingested in the lowest Cu concentration (79 μM) than in the controls (12 beads/5 min). Exposures of both fed and unfed worms to Cu resulted in identical effects on movement and ingestion ($p>0.05$).

After 4-h Pb exposures, nematode movement was significantly decreased by Pb concentrations of 480 μM and higher while ΔOD was lower at 240 μM and higher (Fig 7.4b). Ingestion was almost completely inhibited at all Pb concentrations tested (<1 bead/5 min).

Cadmium exposures up to 1780 μM did not result in significant decreases in movement after 4 h (Fig 7.4c). Similar to 24-h Cd exposures, feeding was reduced significantly from the controls at all exposure concentrations but not in a concentration-dependent fashion. Ingestion was also significantly less than controls (9 beads/5min) at all Cd concentrations (≤ 1 bead/5 min). Exposures of both fed and unfed worms to Cd resulted in similar effects on movement and ingestion.

DISCUSSION

Sublethal endpoints are not only extremely sensitive and meaningful means of estimating the effects of toxicants on exposed populations but may also provide insight into the mechanisms underlying these effects. The results from sublethal assays, however, may be confounded by effects of environmental factors, nutritional status of organisms, and duration of exposures (Mason and Jenkins 1995). The apparent toxicity of Cd, Cu, and Pb after 24-h exposures was greater when less food was available to *C. elegans* (Figs 7.1-7.3). Higher food concentration (4:1 OP50) increased the movement EC50s for all three metals by a similar factor (Table 7.1) and, although feeding was decreased by all metals at either food concentration, nematodes within any given exposure group fed more in 4:1 OP50 than those in 1:1 OP50 (Figs 7.1-7.3).

In a previous study, the effects of food availability alone (without toxicant exposure) on *C. elegans* behaviors were investigated to further the standardization of a computer tracking movement assay (Boyd et al. 2000). Initially, higher food availability increased both movement and feeding. However, at bacterial concentrations above 1:1 OP50, movement plateaued as feeding continued to increase up to the highest concentration tested (4:1). This indicates the presence of a possible critical feeding level with corresponding ΔODs of 0.95 (± 0.40) for 0.5:1 OP50 and 1.43 (± 0.32) for 1:1 OP50. Put simply, feeding below these levels could lead to decreased movement possibly due to energy depletion. Considering these data, the question was posed in the current study: is reduced locomotion after 24-h exposures to metals the result of starvation, toxicity, or both?

If the assumption is made that worms exposed to metals in 1:1 OP50 were starved if the ΔOD was < 0.95 and those exposed in 4:1 were starved if the ΔOD was < 1.43, then any movement effects observed in nematodes when feeding above these levels should be due to metal exposures. Nematodes exposed to metals in 4:1 OP50 did not appear to be starved because the concentrations of metals that led to feeding below critical levels were greater than their movement EC50s (Table 7.1). Therefore, the observed decreases in movement were due to toxic effects of the metal exposure at 4:1 OP50. However, the responses of the nematodes' movement at 1:1 L-broth are not as easily deciphered. Both Pb- and Cd-exposed nematodes fed below critical feeding levels at or below their movement EC50s indicating that starvation could not be completely eliminated. However, the movement EC50 for Cu-exposed worms was higher than their critical feeding level, possibly indicating that starvation was not a factor with Cu

exposures. Feeding was actually stimulated at the lowest level of Cu exposure (Fig 7.1b). Cu exposures also resulted in the clearest concentration-dependent decreases in feeding while Pb and Cd diminished feeding significantly but not in concentration-dependent fashions (Figs 7.2b and 7.3b).

Nematode behaviors can be separated into exogenous behaviors, those stimulated by environmental factors outside of the organism, and endogenous behaviors, those that are regulated internally (Croll and Sukhdeo 1981). The integration of both types of behavior is required for complex behavioral sequences such as feeding. For example, feeding is driven by a combination of many basic behaviors including pharyngeal pumping and forward movement, but it can also be stimulated by the exogenous presence of bacteria. Because of the complex nature of feeding in *C. elegans* and the intimate link between feeding and movement, the separation of the effects of food availability and metal toxicity on these behavioral parameters is complicated. In fact, when the effects of 24-h metal exposures on movement and feeding within a given food availability were compared as a percent of control behavior, the responses were virtually identical in magnitude and shape. The only exception was Pb exposure in 1:1 OP50 where movement was more sensitive to Pb than feeding.

When comparing the sensitivity of several endpoints, Anderson et al. (2001) found that Cu and Pb had similar effects on *C. elegans* movement after 24-h exposures, but Pb inhibited movement at much lower concentrations than Cu after 4-h exposures. Because food availability has such a critical effect on apparent 24-h metal toxicity in this study, the exposure period was shortened from 24 h to 4 h in an effort to eliminate starvation effects. In this way, the toxic effects of the metals could then be separated

from the effects of energy depletion on locomotion, a process known to require high amounts of energy. The resultant hypothesis for 4-h exposures was that no differences in metal toxicity would be observed at different food availabilities if starvation was not a factor.

Although unfed worms could not be tracked after 24-h exposures, the movement of worms not fed during 4-h exposures could be compared to those fed 4:1 OP50. In addition, the bead ingestion assay was used to quantify feeding in unfed worms when it was not possible to measure ΔOD. Although movement and ingestion tended to be greater in unfed nematodes, the differences were statistically insignificant. Therefore, starvation was indeed minimized at 4-h exposures as evidenced by similar responses in fed and unfed control worms. In fed worms, the sensitivity of the endpoints of feeding during the exposure (ΔOD) and ingestion after the exposure were contrasted. Ingestion exhibited similar patterns of response but was slightly more sensitive than ΔOD, while both methods of determining feeding were more sensitive than movement assays in all 4-h metal exposures (Fig 7.4).

Although the presence of bacteria is well known to stimulate pharyngeal pumping in *C. elegans*, this response has proven to be quite complex and dependent upon the nutritional status of the worms as well as the bacterial concentrations (Croll 1975; Croll and Smith 1978; Avery and Horvitz 1990). In fact, in depth observations of the feeding behavior of *C. elegans* in dense and dilute bacterial suspensions found two distinct types of ingestion depending on food availability (Seymour et al. 1983). Because reliable ingestion of microbeads required the presence of bacteria, unfed worms may have been stimulated to ingest beads simply by the sudden increase in bacterial density.

Interestingly, control worms did ingest significantly more beads after 4 h in liquid bacterial suspensions than did worms that were transferred directly from *E. coli* seeded agar plates.

Except at the lowest metal concentrations, metal-exposed worms ingested fewer beads than pre-exposure worms that were transferred directly from OP50 plates. Because pharyngeal pumping is controlled by a complex, integrated neural system made up of 20 neurons, the pharynx is a likely site of action for neurotoxins such as Pb and Cd. However, low concentrations of $HgCl_2$ did not affect pumping rates suggesting that worms were not able to avoid metal exposures by reducing pumping (Stringham and Candido 1994).

The fungicide captan was used to characterize the stress response of transgenic strains of *C. elegans* (Jones et al. 1996). Worms decreased feeding in a concentration-dependent fashion after exposure to captan. Upon closer examination, worms appeared starved and had no gut contents even in the presence of bacteria. Similar to Cd, captan led to retarded development. Because the pharynx is the first organ to come in contact with toxicants, the authors speculated that it acts as the "first line of defense against ingested toxicants". Therefore, despite the fact that $HgCl_2$ did not reduce pumping (Stringham and Candido 1994), the possibility remains that the worms were able to sense the presence of one or more of the metals used in this study and decrease their pumping rates. The higher the concentration of bacteria, the more food was ingested per pump and therefore worms were healthier and able to move faster after termination of the exposure.

Although it is clear that the behavioral effects of metals on *C. elegans* can be affected by the availability of food, the mechanisms of these occurrences are largely

unknown. Exposure of *C. elegans* to Cd led to degenerated mitochondria in esophageal muscle cells and intestinal cells (Popham and Webster 1979). In addition, microvilli in intestinal cells were significantly shortened by Cd exposure. Because Cd led to effects on the digestive system and Cd-exposed worms appeared starved, they concluded that Cd likely interfered with the uptake and metabolism of nutrients. Juvenile *C. elegans* exposed to low concentrations of $Pb(NO_3)_2$, $CuCl_2$, or $CdCl_2$ accumulated metals in the pharyngeal region as indicated by staining patterns (Stringham and Candido 1994). Cd was also a strong inducer of the heat shock protein (*hsp 70*) as measured by β-galactosidase activity (Guven et al. 1994). Again, Cd-exposed juveniles were delayed developmentally when compared to control worms. In our study, worms exposed to Cd or Pb for 24 h were consistently smaller than control worms. Additionally, within the same metal exposure concentration, worms exposed to metals in higher bacterial concentrations were larger. Although these observations were completely qualitative, starvation again is indicated as a factor in the expression of 24-h metal toxicity.

CONCLUSIONS

Without the use of multiple sublethal endpoints, the interactive effects of starvation and metal exposures on the behavior of *C. elegans* would never have been apparent. If only movement assays had been performed after 24-h metal exposures, the resultant decreases in movement could have been assumed to be solely due to metal exposures and not to depletions of energy reserves. Decreasing the exposure time from 24 to 4h minimized the effects of starvation on the movement of *C. elegans*. Upon

comparison of the sensitivity of sublethal endpoints, ingestion appeared to be the most sensitive indicator of toxicity. Therefore, we recommend decreasing the exposure time from 24 to 4 h in future experiments on the movement of *C. elegans* and including ingestion as a complementary measurement of toxicity.

Metal toxicity may be affected by a multitude of environmental factors such as temperature, pH, and moisture as well as the quality and quantity of available food. Because these factors are easily manipulated in the lab, toxicity tests are usually designed to minimize the influence of external factors by holding them constant at every testing date. In reality, organisms experience a wide range of environmental conditions when subjected to toxicant stress under field conditions. Knowledge of the responses of organisms in the lab to toxicant exposure with varying environmental conditions allows for more accurate predictions of organisms' responses to toxicants in the environment. We are now investigating the site of action of Cd, Cu, and Pb in *C. elegans* and hope to relate these back to patterns observed in the current study and in others in our laboratory.

Acknowledgement - We would like to thank Dr. David Dusenbery for use of the computer tracking software program. We are grateful to the US Environmental Protection Agency's Science to Achieve Results (STAR) Graduate Fellowship Program and the University of Georgia's Agricultural Experiment Station for financial support of this research. The nematode strain used in this work was provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

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Table 7.1. Effects of food availability on Cu, Pb, and Cd toxicity after 24-h exposures. Food concentration is expressed in terms of OP50 L-broth:metal salt solution (v:v).

	1:1 OP50			4:1 OP50		
	Cu	**Pb**	**Cd**	**Cu**	**Pb**	**Cd**
Movement EC50[1]	20	20	78	50	46	187
95% CI	16-24	13-	58-	40-60	28-	95-279
Critical feeding	56-	0-37	16-	135-	78-	442-

[1]EC50 values (and respective 95% confidence intervals) indicate the metal concentrations (μM) at which movement was decreased by 50% relative to controls.

[2]Critical feeding levels are the range of metal concentrations (μM) tested above which movement was affected by decreased feeding.

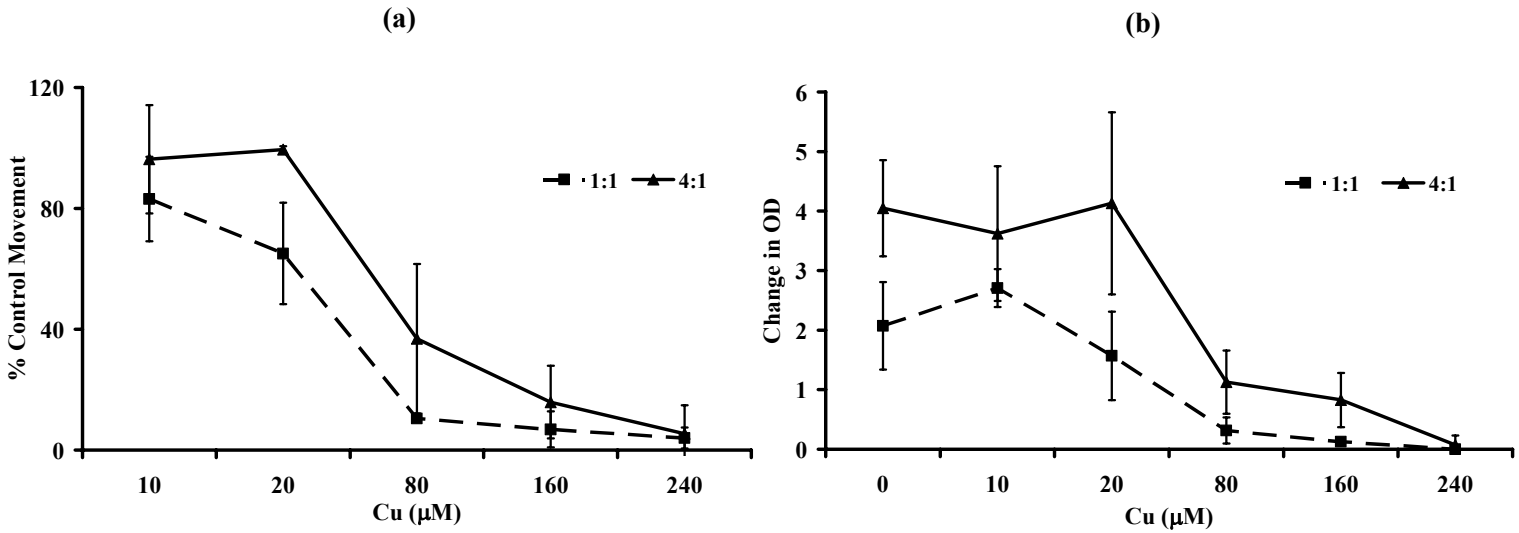

Figure 7.1. Effects of food availability and Cu exposure on nematode (a) movement and (b) feeding after 24-h exposures. Food concentration expressed in terms of L-broth:Cu solutions (v:v).

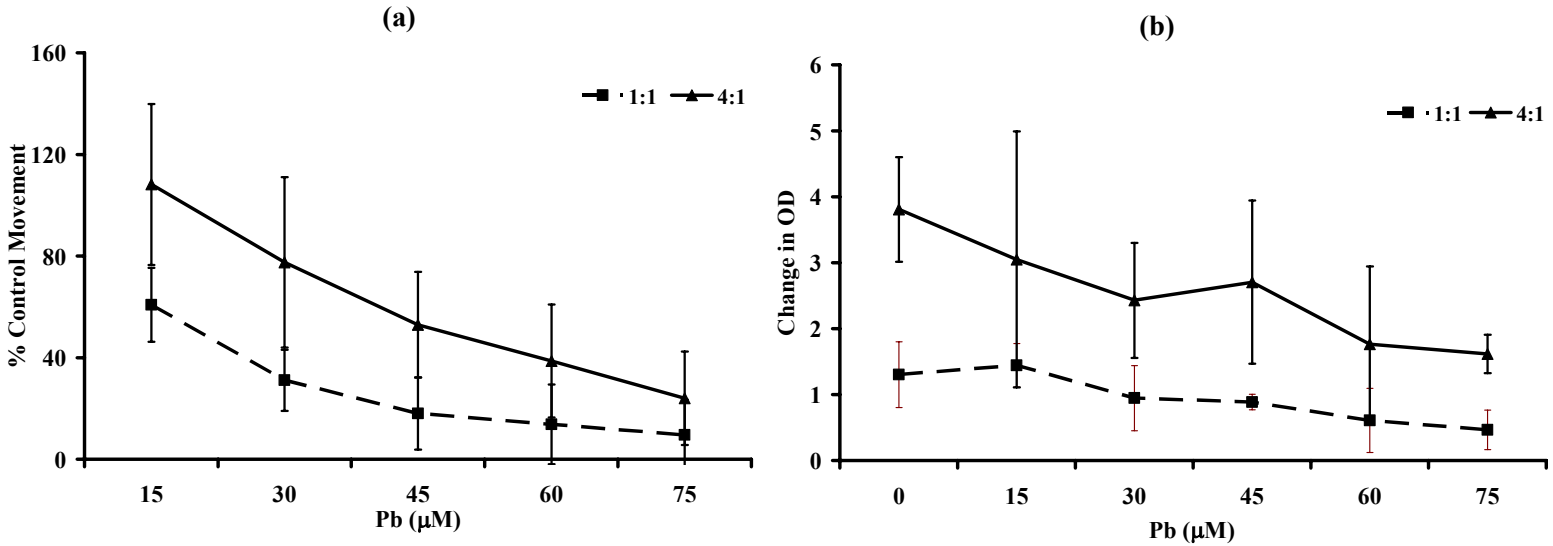

Figure 7.2. Effects of food availability and Pb exposure on nematode (a) movement and (b) feeding after 24-h exposures. Food concentration expressed in terms of L-broth:Pb solutions (v:v).

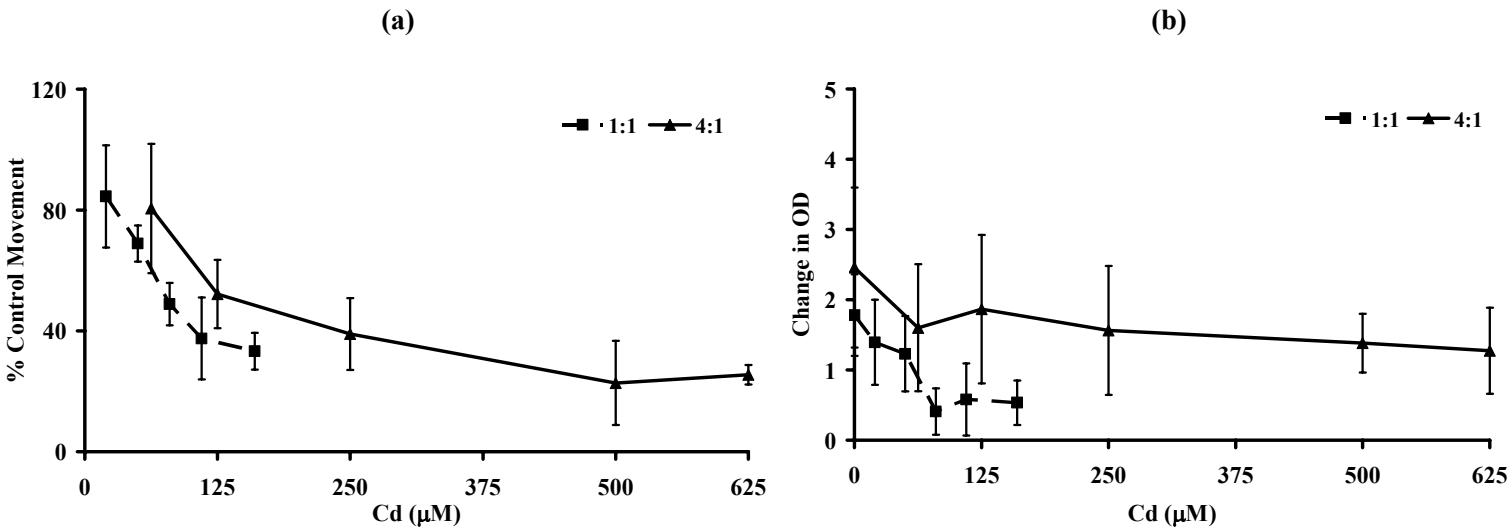

Figure 7.3. Effects of food availability and Cd exposure on nematode (a) movement and (b) feeding after 24-h exposures. Food concentration expressed in terms of L-broth:Cd solutions (v:v).

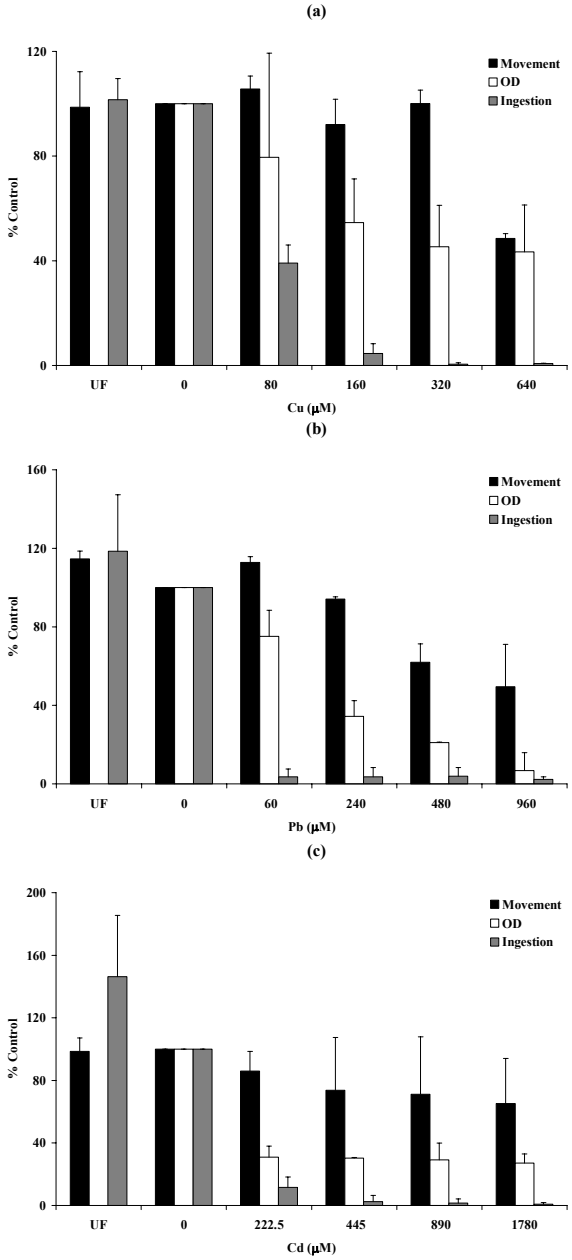

Figure 7.4. Effects of (a) Cu, (b) Pb, and (c) Cd on movement, feeding, and ingestion in nematodes after 4-h exposures. The responses of worms not fed during exposure (UF) are compared to control worms (0) fed during exposure.

CHAPTER 8

CONCLUSIONS

This dissertation compared the toxic effects of metals on the free-living nematode *Caenorhabditis elegans* using lethality, behavior, and reproduction as endpoints. Soil toxicity tests were refined to include estimates of the bioavailability of metals and the use of other nematode species in toxicity tests. In aquatic medium, a variety of endpoints were used to provide assessments of the behavioral effects of metals on *C. elegans*. The following list summarizes the major results and conclusions of the dissertation and provides suggestions for future research directions.

1. Conclusion: *Caenorhabditis elegans* is a useful toxicity testing organism in either aquatic medium or soil and with a variety of endpoints including lethality, movement, feeding, and reproduction. During the course of the experiments described in this dissertation, a standardized guide for the assessment of soil toxicity using *C. elegans* has been accepted and published by the American Society for Testing and Materials.

   Future research: The assessments of sublethal endpoints after soil exposures such as those described for aquatic toxicity tests would provide more sensitive estimates of effects.

2. Conclusion: Although it is possible to expose *C. elegans* to toxicants in soil for 48 h, it is necessary to add food, which can complicate the results of toxicity tests. Because 24-h exposures of *C. elegans* result in similar toxicity estimates to 2 week earthworm tests and avoid the necessity of the addition of food, 24-h exposures for soil toxicity tests are recommended.

   Future research: Testing the effects of other toxicants besides metals is necessary to further compare the sensitivity of *C. elegans* to other soil invertebrates.

3. Conclusion: The bioavailability and resultant toxicity of metals (Cd, Cu, Ni, Pb, and Zn) to *C. elegans* exposed in soils is dependent upon the physicochemical parameters of the soil, namely the cation exchange capacity.

   Future research: To this point, the relationship between toxicity and sequentially-extracted metal fractions has only been performed for two field-sampled soils and an artificial testing medium. Expanding the number of soils tested could lead to predictive models of soil toxicity after determination of the critical variables across soil types.

4. Conclusion: Useful complements to the *C. elegans* standardized guide for soil toxicity testing would be additional nematode species so that researchers could eventually use indigenous species making estimates of soil toxicity more site-specific and thus more realistic. *Pristionchus pacificus* and *Panagrellus redivivus* proved useful in 24-h lethality tests.

   Future research: The sensitivity of more nematode species should be evaluated for inclusion in the *C. elegans* standardized guide.

5. Conclusion: Sublethal metal toxicity to *C. elegans* is affected by food availability after 24-h but not after 4-h exposures. Therefore, exposures should be shortened to 4 h for behavioral testing to minimize these interactions.

   Future research More research is needed to investigate the time course of metal toxicity over 24-h exposures to metals, and in particular to Cd as this metal appears to lead to starvation and eventually developmental delays. The testing of organic chemicals, and in particular more neurotoxic agents, using 4-h behavioral assays is suggested to further characterize the interactions between feeding and movement in *C. elegans*.