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The Acute Toxicity of Selected Metal Mixtures to the Nematode *Caenorhabditis elegans*
(Under the direction of PHILLIP L. WILLIAMS)

Typically, a wide variety of chemicals are found concurrently in the environment, which makes identifying the contribution of individual toxicants difficult. For the purposes of this study, the toxicity of paired metals to the nematode *Caenorhabditis elegans* was investigated. Toxicity tests were designed to first determine LC_{50} values for single metal exposure and then the tests were rerun with a second metal present to observe the effect on the previous LC_{50} values. Four-day-old adult nematodes were exposed to paired combinations of Cd, Zn, Pb and Hg for 24 h without food. Exposures with paired metals showed a variety of interactions which ranged from antagonistic to synergistic effects.

INDEX WORDS: Caenorhabditis elegans, toxicity testing, metal mixtures

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by

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CHAPTER 1

INTRODUCTION

Research projects involving the toxic effects of a single compound upon a single organism are numerous and have generated an abundance of data over the last several decades. Many different types of organisms have been exposed to many different types and classes of toxicants for the purpose of determining or predicting chemical toxicity. The resulting data have been used to assess not only the toxicity to the test organism, but also the potential toxicity to humans. Safety factors are incorporated into assessments of risk and the toxic levels of these compounds are extrapolated to levels that are deemed safe for human exposure. Consequently, most "safe levels" or standards such as Water Quality Criteria (WQC), Maximum Contaminant Levels (MCL), chemical exposure standards, drinking water standards, etc., are based on data from single compound exposures. However, in a natural environment there is very seldom only one toxicant present. In fact, even a water or soil sample from supposedly "pristine" sites will typically yield a virtual soup of toxicants upon analysis, even if at barely detectable levels. However, there is evidence that the presence of an additional toxicant can greatly affect the toxicity of the first toxicant and vice versa. The resulting effects vary, but are generally described in terms such as additive, synergistic or antagonistic. If actual results can differ from expected results, it is possible then to wonder if the current safe standards are adequately protecting human health and the environment.

Research involving mixtures of toxicants is not a new trend. Though very limited when compared with single exposure research, there are data extending back decades. However, there has been an increase in the generation of mixture data over the last two decades. Much of the research has been in the field of aquatic toxicology. Some reasons for this are that water is a controlling factor in contaminant migration, many toxicants reside in an aqueous matrix and humans are vulnerable to exposure through contact or ingestion of contaminated water or contaminated aquatic organisms. Toxicants found in aquatic systems can be divided into two general types; organics and inorganics. Organics include all carbon-based toxicants such as hydrocarbons, polycyclic aromatics, biphenyls and many others. Inorganics include all non-carbon based toxicants such as heavy metals and other elements. A critical difference between metals and organic toxicants is that organics are molecules which can be transmuted or degraded over time and their toxicity may eventually be reduced or nullified. Metals are elements and cannot be transmuted except under extreme conditions (fission or fusion), which renders them practically indestructible. Consequently, metals tend to accumulate in the environment (Baird, 1995) as elemental metals, metallic salts and organometallic compounds (ie. methylated metals). Metals can enter an aquatic system through both natural and anthropogenic sources. Natural sources include weathering and leaching of metals from rocks and soils. Anthropogenic sources are man-made contributions of toxicants from industries such as smelting, mining, certain types of manufacturing, and the burning of fossil fuels (Baird, 1995). For the purposes of this study, mixtures of heavy metals were examined.

In this thesis, the nematode *Caenorhabditis elegans* was used to assess the toxic effects of the water soluble salts of the heavy metals lead (Pb), cadmium (Cd), mercury

(Hg) and zinc (Zn). The four metals were first tested individually to establish mortality curves and then retested in various pairs to observe the effects of metal interaction. Various parametric, nonparametric and nonstatistical methods were employed to assess any differences between single metal mortality and mixture mortality. Discussion of the data emphasizes interpretation of the findings in comparison to data from other studies.

This thesis consists of four chapters. This chapter describes the overall objectives of this project and how metals interaction will be assessed. Chapter 2 consists of a literature review of related research involving chemical mixtures. An emphasis was placed on metal mixtures and a review of different methods for assessment is derived also from various chemical mixture research. Chapter 3 describes the experimental design, materials and methods, results, and discussion describing the results of the metal mixture tests. Chapter 4 provides the overall conclusions drawn by this project and describes possible directions for further studies.

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CHAPTER 2

LITERATURE REVIEW

The nematode *Caenorhabditis elegans*

Caenorhabditis elegans is a free-living bacterivorous nematode that has been used extensively in the fields of biology and toxicology. Inhabiting the biofilm of water which surrounds soil particles, it functions in the decomposition process (Wood, 1988). Having no pelagic or free-swimming stage, these organisms cannot escape from pollution and thus are considered as potential indicator species (Hoss et al, 1999). The worm is about 1 mm long and is easy to grow and maintain on agar with a lawn of *Escherichia coli* bacteria as a food source (Brenner, 1973). Because of its small size, aquatic tests can be performed with minimal waste generation, using test volumes as small as 1 ml (per 10 worms). The use of *C. elegans* is economical, costing about 10% of the cost for mammalian tests (Williams and Dusenbery, 1988). Stocks can be kept with virtually no genetic drift because *C. elegans* generally is a self-reproducing hermaphrodite. Males exist at low frequencies and are slightly different in appearance from the hermaphrodites. Juveniles hatch from eggs and will develop to maturity through four life stages. After the fourth molt at about 3.5 to 4 days, the mature adult is fertile and has a normal lifespan of 15-20 days (Wood, 1988). Large numbers of the worms can be grown either on agar plates or axenically in liquid media (Wood, 1988). Observation and manipulation of the organism is easily performed using a dissecting microscope.

Many research organisms are obtained by collection from the environment or by other inconsistent means. However, *C. elegans* stocks are maintained for research and can be obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota. Quality of stock cultures can be maintained by procurement from this central repository and freezing in the laboratory. In addition, reference toxicity tests can be performed to ensure the quality of the stock cultures and to assess the reproducibility or precision of the assays (Cressman III and Williams, 1997, Freeman et al., 1998).

C. elegans was first isolated and used for genetics research by Brenner (1973). Over the years, the use of *C. elegans* has increased dramatically in the fields of developmental biology, neurobiology and genetics. The genetic system of *C. elegans* is comprised of 8×10^7 base pairs of DNA. The genome size is small when compared with many other organisms used for genetic research. Its entire genome size is 20 times the size of the bacterium *E. coli*, but only half the size of the fruit fly *Drosophila* (Wood, 1988). *C. elegans* was the first multicellular organism to have its entire genome sequenced and has been included in the list of genomes that are important in deciphering human sequences for the human genome project. The entire somatic cell lineage as well as the connectivity of the nervous system has been mapped. The nervous system is simple and consists of only 302 neurons in the hermaphrodites and 383 neurons in the males (Wood, 1988).

In summary, *C. elegans* is ideal for use in toxicity testing for many reasons. Perhaps the most appealing traits are the anatomical simplicity of the organism and the thorough understanding of the nervous system and genome. The organism is cost effective and can be easily maintained and manipulated in the lab in sufficient numbers to

perform continuous testing with minimal effort. Unlike many test organisms, quality can be controlled by obtaining stocks from a central repository and by performing routine reference tests. These advantages combined with the size of the organism and the small volumes of toxicants required for testing make *C. elegans* ideal for performing toxicity research.

Toxicological Studies Using *Caenorhabditis elegans*

Though originally isolated for genetic research by Brenner (1973), *C. elegans* has been used extensively in research involving toxicological testing (Williams and Dusenbery, 1988, van Kessel et al., 1989, Williams and Dusenbery, 1990, Kammenga et al., 1994, Donkin and Williams, 1995, Hitchcock et al., 1997, Hoss et al., 1999, Kammenga, 1999, Anderson et al, 2001). The full applications of this versatile and well-understood organism are still being developed. *C. elegans* was used for toxicity testing by Williams and Dusenbery (1988) to predict mammalian acute lethality to metallic salts of Hg, Be, Al, Cu, Zn, Pb, Cd and Sr. Testing procedures were established initially, using agar plates with a bacterial lawn of the *Escherichia coli* variant, OP50, and later with plates devoid of a food source. The results indicated that the testing is cost effective and that data are comparable to mammalian data. Van Kessel et al. (1989) performed similar work by exposing juvenile stages of *C. elegans* to $CdCl_2$. The results indicated that the toxicity tests performed with *C. elegans* were highly reproducible and may even be performed in simpler media, such as buffer solutions.

An aquatic testing procedure was developed by Williams and Dusenbery (1990) to further illustrate the ease and benefits of using *C. elegans*. Worms were exposed in

tissue culture dishes and exposed to various soluble metal salts. A buffer (K-medium) was used to dissolve metallic salts of Ag, Hg. Cu, Be, Al, Pb, Cr, As, Tl, Zn, Cd, Ni, Sr and Sb for preparing various testing concentrations. The results were compared to various published invertebrate data.

Other researchers have used this information and incorporated *C. elegans* into various types of aquatic toxicity testing. Using previously published data, Kammenga et al. (1994) compared 24 h aquatic tests from 12 nematode species to Cd. The results indicated that six of the species (including *C. elegans*) showed a statistically similar response to Cd. Hitchcock et al. (1997) used *C. elegans* to assess the toxicity of municipal and industrial wastewater. Results of the 72 h mortality tests suggested that waste waters from certain industries may interact and increase the toxicity of both the influent and effluent of a wastewater treatment plant. Donkin et al., (1995) assessed the toxicity of glucosinolates and their enzymatic breakdown products using *C. elegans*. The results of the 24 h mortality tests confirmed that decomposition products of sinigrin rather than sinigrin itself, are toxic. The glucosinolate breakdown product isothiocyanate was found to be relatively toxic to nematodes and its possible use as a nematicide was suggested. Hoss et al. (1999) used *C. elegans* to compare various endpoints (body length, eggs per worm, and percentage of gravid worms) with respect to sediment particle size and organic content.

Methods for using *C. elegans* in toxicity tests using soils and sediments have also been developed over the years as a result of increased environmental awareness concerning polluted soils. The research of Donkin and Dusenbery (1993) is the first example of using *C. elegans* to assess toxicity in soils. Using lethality as the endpoint,

the worms were exposed in six soil types spiked with $CuCl_2$ for 24 hours. The results suggested that the tests with *C. elegans* may be comparable to 2-week earthworm tests in terms of sensitivity. An important aspect of this work was the development of an effective means for recovering the test worms from the soil. Both live and dead individuals were separated using centrifugation through a colloidal silica suspension without harming the live animals.

Peredney and Williams (2000) utilized similar techniques and further demonstrated the ease of using *C. elegans* for assessing heavy metal contamination in artificial soils and compared the results with earthworm data. The 24 h tests in ASTM artificial soils (ASTM 1997), in which *C. elegans* were exposed to the metals Ni, Cd, Cu, Zn and Pb, yielded comparable results to 14 day *Eisenia fetida* (earthworm) tests. *C. elegans* was found to be more sensitive to Pb toxicity than earthworms. The results demonstrated that *C. elegans* has great potential for assessment of contaminated soils and for use as a cost effective alternative to more lengthy test designs. This soil method has been standardized and is now available as an ASTM Standard Guide (ASTM E2172-01, 2001).

Caenorhabditis elegans **Endpoints**

C. elegans is a versatile test organism because various endpoints can be used for toxicity testing. Lethality is a commonly used endpoint, but sublethal endpoints such as reproductive impact, behavioral change effects, growth and feeding effects have been used as well. Lethality endpoints are typically assessed by counting the number of dead individuals versus live, after exposure. This common endpoint with *C. elegans* is

frequently used and well documented (Williams and Dusenbery, 1988, Williams and Dusenbery, 1990, Cressman III, 1997, Tatara et al., 1997, Donkin and Williams, 1995, Donkin et al, 1995, Hitchcock, 1997, Freeman et al, 1998, Dhawan, 1999).

Reproduction is another common endpoint that can be utilized with *C. elegans*. Donkin and Williams (1995) used *C. elegans* development and evidence of reproduction as endpoints in an experiment designed to examine the effects of development, salts and food on various endpoints. For this study, development was assessed by monitoring the success of larval stage nematodes progressing to adulthood after 96 hours. Evidence of reproduction effects were assessed by simply scoring either the presence or absence of offspring to verify normal development to a reproducing adult. Other researchers have actually counted the broods and compared the numbers of eggs or viable offspring with control cultures (van Kessel et al., 1988, Middendorf and Dusenbery, 1993, Dhawan, 1999, Anderson et al., 2001). Another means of assessing reproduction has been performed by assessing the number of eggs per worm and the percentage of gravid worms (Hoss, 1999). Other research (Kammenga, 1999, Hoss et al., 1999) has used the time to reproduction or length of prereproductive period as an endpoint.

Growth has been assessed in various ways as an endpoint with *C. elegans*. Hoss et al. (1999) exposed adult worms to 26 different freshwater sediments and found that the endpoints of body length and percentage of gravid worms were effective measurements, making *C. elegans* a suitable test organism for sediment bioassay studies. Anderson et al. (2001) used computer tracking and image analysis to determine the length of the worms before and after 24 h exposure to Pb, Cd and Cu.

Feeding as an endpoint was also assessed by Anderson et al. (2001). This was accomplished by exposing *C. elegans* to Pb, Cd and Cu in a buffer solution (K-medium) with a bacterial food source (OP50) and monitoring the change in optical density (OD) of the suspension. Feeding was then calculated as a percentage of the controls. The method was found to be quick and effective, demonstrating inhibition of feeding after only 2 hours exposure to Cd. OD measurements required only five minutes reading time per culture plate. Since feeding is not a terminal endpoint, additional endpoints as well as reversibility of feeding can also be assessed.

Behavioral endpoints in *C. elegans* toxicity testing can refer to such traits as feeding, but typically refer to movement or rate of movement. Recent research has shown behavioral endpoints to be a promising means for assessing changes. Dhawan et al, (1999) used computer tracking to detect differences in worm movement after exposure to ethanol. Significant differences from the controls were clearly detected. An increase in movement was found in the concentration range of 0.8 g/l to 2.4 g/l whereas movement decreased at higher concentration ranges (8 g/l to 40 g/l). Anderson et al. (2001) performed similar research using computer tracking with movement as one toxicological endpoint. Changes in movement were detected when *C. elegans* was exposed to solutions containing Cd, Pb and Cu. After four hours of exposure, Pb was found to significantly reduce movement from control levels. Since feeding behavior data was also collected during this study, comparison between movement and feeding was also possible.

Sensitivity is perhaps one the most important aspects in acceptability of test organisms for toxicity testing. *C. elegans* has demonstrated multiple sensitive endpoints

which compare with the published data on other similar test organisms. Considering the cost effectiveness and multiple endpoints demonstrated, toxicity testing using *C. elegans* is a promising alternative to more costly and time consuming tests.

Chemical Interaction

The fact that chemical mixtures can have effects that differ from single chemical exposure is well known. This information is often engineered into the production of pesticides where certain chemical ingredients may be added to enhance the effects of other ingredients and thereby increase the potency of the formulation. One problem with using terms to describe effects is that the terms have been defined and redefined in the context of published papers and books to the point that wide variations may exist in the definition. Consequently, care must be taken when defining these terms. The following is a description of terms that are generally accepted. The term additive is commonly used to describe the effects of interaction when the sum of each agents effect alone is equal to the combined effects (ie. 1+1 =2). An example is found when two organophosphate insecticides are given together. In most cases, the result is an additive effect on cholinesterase inhibition (Eaton and Klaassen, 1996). Synergism is another term often used to describe combined effects that are much greater than the sum of the effects of each individual agent alone (ie. 1+1=10). An example of synergism occurs when the hepatotoxicants carbon tetrachloride and ethanol are administered together (Eaton and Klaassen, 1996). The resulting liver damage is much greater than would be expected based on the individual toxicities of the chemicals. The term potentiation refers to the effects of a non-toxic chemical administered with a toxic chemical. The non-toxic agent

has no effect alone but rather enhances the effects of a second agent (ie. 0+2=5). Potentiation occurs when the nonhepatotoxicant isopropanol is given with the hepatotoxicant carbon tetrachloride. The damage inflicted by the pair upon the liver is much greater than with exposure to carbon tetrachloride alone (Eaton and Klaassen, 1996, Rand et al., 1995).

The reduction of toxic effects is another common type of chemical interaction. When two or more agents are administered together and the effects are reduced (ie. 2+2=1), the common term used to describe the effects is antagonism (Rand et al., 1995). Antagonism can occur as a result of one agents interference with another or multiple agents interference with each other. Many antidotes are formulated to work by reducing the effects of a certain toxin through antagonism (Eaton and Klaassen, 1996). Antagonism can be further categorized into four types to describe different kinds of interference (Eaton and Klaassen, 1996 , Rand et al., 1995). When two chemicals have opposite effects on the same biological mechanism, the term functional antagonism is used. Chemical antagonism occurs when two chemicals react with each other and render a less toxic product. The agents are chemically inactivated. When antagonism occurs as a result of alteration of absorption, transport, excretion, distribution or biotransformation, the term dispositional antagonism is often used, because exposure to a particular organ is reduced. Receptor antagonism refers to lessened effects because both chemicals may compete for binding to the same receptor. The effect is less than the addition of their separate effects (ie. 2+2=3). The term blocker is often used to describe this type of antagonism (Eaton and Klaassen, 1996).

Assessment of Chemical Interaction

Researchers have developed many means of assessing interaction over the years. Various equations and models such as slope comparisons, TUs (toxic units), additive indices, product models and others have been developed. Bliss (1939) proposed three types of mixture models based on examination of response curves. Similar joint action occurs when the toxicants in a mixture produce similiar but independent effects. In this assessment, one component can be substituted at a constant proportion for the other. In this sense, variations in individual susceptibility to two components are correlated or parallel (ie. the response curves are parallel). Independent joint action occurs when the toxicants act independently and have different modes of action. Therefore, the susceptibility to one component may or may not be correlated with the susceptibility to the other. (Bliss, 1939). In this sense, each toxicant can contribute to the overall response only after a certain threshold is reached. The result is that the response curves for each toxicant may or may not be parallel (Pape-Lindstrom and Lydy, 1997). Synergistic action occurs when the effectiveness of the mixture cannot be assessed from that of the individual components but rather depends upon knowledge of their combined toxicity when used in different proportions. One component is assumed to synergize or antagonize the other (Bliss, 1939).

The toxic unit approach has been used extensively over the decades to describe mixture toxicity. The basic premise is that one toxic unit is assigned to a previously determined LC50 value. Typically experiments are designed ahead of time using one-half of a toxicants LC50 (0.5 TU). One half of a toxic unit of one chemical combined with one half of a toxic unit of a second chemical should sum to one. Deviations above or

below one would indicate interaction (Marking and Dawson, 1975, Newman, 1995, Pape-Lindstrom and Lydy, 1997). Since the probit scale is nonlinear, calculations were necessary to assign expected TU values for the mixtures using LC10s.

A variation of this approach was proposed by Marking and Dawson, (1975) in an effort to adapt methods and terminology and quantitatively describe additive toxicity. The additive index scale is more or less a toxic unit variation which allows for the summation of the ratios of a chemicals toxicity in mixtures to the chemicals individual toxicity. The method also adjusts for linearity and assigns a reference point of zero instead of one (Marking and Dawson, 1975). This method assesses data very well, provided the toxicities are known for both the mixtures and the individual chemicals.

Other models have been developed to fit different types of data sets. Christensen et al. (1979) proposed that a product model is more reasonable than the additive model. This is because the additive model may fall into the zero range. However, the experiment must be designed appropriately, such as in a two by two experimental design.

Metal Mixture Review

Research involving mixtures of heavy metals has produced various results in aquatic test organisms. The common assumption that if one metal exerts a certain effect, then an additional metal present would increase the toxicity, is not always true. Although some researchers may believe that an additive model of toxicity adequately describe the combined effects of metals, the type of action may deviate from additivity when low concentrations of one metal are combined with high concentrations of another (Harrahy, 1995). Studies have shown various results with different species, some of

which seem to contradict each other. Antagonism or less than additive effects have been observed in many organisms.

Kammenga (1999) found that the toxicity of a mixture of Cu and Cd to *C. elegans* increased with increasing concentrations of toxic units. In the case of Pb and Cd, low levels of Pb (0.036 mmol/L) mixed with Cd resulted in a decrease in time to reproduction whereas higher levels of Pb (0.36 mmol/L) mixed with Cd resulted in an increase in time to reproduction. Similar toxicity relationships between metals have been observed in fish. Parrot (1993) observed antagonism in fathead minnows (*Pimephales promelas*) between low concentrations of Cu (0-150 µg/L) and high concentrations of Zn (0-1500 µg/L). Assessing DNA, RNA and protein contents of newly hatched minnows after 96 hours exposure, less than additive effects were observed. In the same experiment, combinations of Cu (0-90 µg/L) and Zn (0-1200 µg/L) had an additive effect on bacterial (*Photobacterium phosphoreum*) luminescence. Keller and Zam (1990) found similar results for mixtures involving Cd and Zn. Both 48 h and 96 h tests using *Anodonta imbecilis* (juvenile mussels) resulted in antagonism. The combinations of Zn with Ni, Hg with Cr, and Cd with Cu produced similar antagonistic results. Hemelraad (1987) found that high levels of Zn inhibited the uptake of Cd in the freshwater clam *Anodonta cygnea*. Adult clams were exposed to Cd (25 µg/L) or to Cd (25 µg/L) plus Zn (2.5 mg/L) for 17 months. Zinc was found to exert a dual effect on Cd kinetics by offering antagonistic effects on cadmium uptake by the gills. It also proposed that Zn interaction with Cd occurs at the membrane transport level. In the gills, Zn competes with Cd for metal-binding sites within the cytosol. It is believed that zinc probably did not induce a large

synthesis of metal binding proteins. Consequently, Cd transport from the gills to internal organs was accelerated. In contrast, de March (1988) found that K or Mg paired with either Cu, Cd, or Zn had additive effects in *Gammarus lacustris* (freshwater amphipods). The combinations of Cu and Cd, Cu and Zn, and Cd and Zn had more than additive effects. However, the combination of Mg with K had less than additive effects. Antagonism or less than additive effects has also been demonstrated in terrestrial worms. Using toxic units, Khalil et al. (1996a) observed antagonistic effects from a tertiary mixture of Cd (300-800 μg/g), Cu (500-1000μg/g) and Zn (300-1600μg/g) on cocoon production of the earthworm *Aporrectodea caliginosa*. However, in a similar experiment using growth as the endpoint, Khalil et al., (1996b) observed a relative additive effect from a mixture of Cd (5-250 μg/g), Cu (25-300μg/g), and Zn (100-1600 μg/g). Other studies using worms have also produced additive effects. Conder and Lanno (2000) observed additivity in producing lethality to the earthworm (*Eisenia fetida*) in artificial soils containing mixtures of Cd, Pb, and Zn. Using the toxic unit approach, the mixture toxicity was assessed at 1.35 TU which suggested additive interaction. Weltje (1997) found mainly antagonistic toxic effects in earthworms (Oligochaeta) for total soil concentrations of Cd, Cu, Pb and Zn using tissue concentrations and sub lethal endpoints. However, when EC_{50}s are based on extractable metal soil concentrations, the mixture toxicity shifts towards concentration-addition. Zn and Cu had similar effects on the reproductive cycle of the terrestrial worm *Enchytraeus crypticus* depending on how the exposure of the worms was quantified. Additive effects were observed when the joint effect was judged by external concentrations. However, the effects were less than additive when judged by body concentrations (Posthuma et al., 1997).

Clearly the toxic effects of metals can be quite profound to various organisms. The effects of metal mixtures can be even more puzzling, producing antagonism in some cases and additive or greater than additive effects in others. Considering the complexity of interactions, more general information may not effectively clarify the problem. However carefully designed experiments to test the hypotheses relative to metal uptake are needed to determine the factors driving these interactions.

Mechanisms of Metal Toxicity

It is generally accepted that metals exert toxicity through a variety of mechanisms. These include the blocking of essential biological functional groups of biomolecules, the displacement of essential metal ions in biomolecules and the modification of the active conformation of biomolecules (Calabrese, 1991, Mason 1996). A simple model for describing metal toxicity and detoxification suggests that upon entering a cell, most metals are sequestered rapidly by intracellular ligands (Mason, 1996, Goyer, 1996). Metallothionein is an example of a well-studied inducible metal binding ligand, which is commonly biosynthesized by invertebrates and other species in response to heavy metal exposure. The metals Cd, Zn, Cu, Hg, Co, Ni, Bi and Ag are all known to induce thionein synthesis (DiGiulio et al., 1995).

The partitioning of metals between these ligand pools is thermodynamically and physicochemically driven. Metal homeostasis can therefore be considered to be the biological control of binding of metals to these three ligand pools. Therefore, regulation of metals among these pools is central to maintaining metal homeostasis and optimal

cellular function (DiGiulio et al., 1995). Consequently, the detrimental effects of metal exposure can be viewed as a disruption in metal homeostasis.

Metals can exert toxicity through the displacement or substitution of essential metal ions in biomolecules. Metalloproteins are complexes that aid in metal detoxification. They have binding domains where the native metal can be occasionally substituted by an alternative metal. Thus, some metalloproteins are highly susceptible targets for metal perturbation. For example, *in vitro* studies have shown that the biological activity of the Zn-potentiated enzyme carboxypeptidase is lost when Zn is removed. The biological activity of the protein can be partially restored by the substitution of Mn, Fe, Ni, and Co for Zn, but not by Cu, Cd or Hg, although all the metals bind to the same site on the protein (Mason, 1996). Another case of metalloenzyme poisoning is with the Zn requiring enzyme δ-aminolevulinic acid dehydratase (ALAD). Pb has been shown to inhibit ALAD activity by displacing Zn (Mason, 1996).

The blocking of functional groups is another way that metal toxicity can be induced. Hg is capable of inactivating many enzymes by binding to sulfhydryl groups in the protein backbone. Glucose-6-phosphate is an example of an enzyme that can be inactivated by Hg (Mason, 1996).

The active conformation of biomolecules can be altered by some metals such as Hg. The functional conformation is allosterically altered, thereby inactivating the protein. Hg has been shown to accomplish this by reacting with disulfide bonds and through insertation between sulphurs (ie. -S-Hg-S-), which extends the bond length. This often

prevents normal biological activity by changing the geometry and structural integrity of proteins (Mason, 1996).

Information concerning the exact means of metal uptake and metabolism in *C. elegans* is limited. However, some studies do exist. Popham and Webster (1979) used electron microscopy (TEM) to determine that Cd modifies the mitochondrial structure in the esophagus and intestine cells. Alteration of the morphology of cytosomes in the intestinal cells and the formation of inclusion bodies in the nucleus of esophageal cells were also observed. Later research by Popham and Webster (1982), which used TEM analysis to observe the effects of Hg and silver (Ag), provided additional evidence that metal uptake in *C. elegans* is through the gut and esophagus. The results indicated that both Hg and Ag adversely affected intestinal cells in a manner similar to Cd. Ag also affected the morphology of hypodermal cell mitochondria and Hg was shown to create lesions in esophageal muscles cells.

Perhaps the macroscopic size of *C. elegans* has prevented many tissue distribution studies involving dissection. Tissue distribution data from dissection studies using larger marine nematodes suggests that metal uptake may be primarily surface adsorption through the cuticle. Howell (1983) examined heavy metal uptake and distribution in the marine nematodes *Enoplus brevis* and *Enoplus communis* using dissection and subsequent metal analysis on the different tissues. The external cortical layer of the cuticle contains disulphide and sulphydryl groups, which provide binding sites for metals. The effect of the presence of these groups is a net negative charge on the cuticular surface, which increases toward the tail. There were differences between species in metal accumulation through the gut versus the cuticle. However, differences

may be indicative of the form and bioavailability of the metals at different sites where the animals were collected. In general, the conclusions were that rapid uptake and loss of Cu and Zn was through the cuticle. However, these larger marine nematodes have pores or channels in the cuticle that are not present in *C. elegans* (Edgar et al., 1982, Bird and Bird, 1991). The cuticle of *C. elegans* is believed to be relatively impermeable (Wood, 1988) and Popham and Webster (1979, 1982) determined the point of metal uptake to be through the gut and esophagus. One structure, which is present in *C. elegans*, may be responsible for reducing metals uptake through the gut is the ventral or excretory gland (Wood, 1988, Bird and Bird, 1991). In larger nematodes, the ventral gland secretes a metal-binding acid mucopolysaccharide, which may have an important influence in metal uptake and loss in the gut. High molecular weight proteins have been observed in the secretion that may function in metal binding. (Howell, 1982, Howell 1983).

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CHAPTER 3

THE ACUTE TOXICITY OF *SELECTED* METAL MIXTURES TO THE NEMATODE *CAENORHABDITIS ELEGANS*[1]

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[1]Everett, R. Wilson, Marsha Black, Phillip L. Williams Submitted to *Archives of Environmental Contamination and Toxicology.*

Abstract. Typically, a wide variety of chemicals are found concurrently in the environment which makes identifying the contribution of individual toxicants difficult. Most chemical contaminant limits do not address chemical interactions and are based solely on single contaminant exposure data. For the purposes of this study, the toxicity of paired metals to the nematode *Caenorhabditis elegans* was investigated. Toxicity tests were designed to first determine LC50 values for single metal exposure and then the tests were rerun with a second metal present to observe the effect on the previous LC50 values. Four day old adult nematodes were exposed to paired combinations of Cd, Zn, Pb and Hg for 24 h without food. Probit analysis was used to predict LC50 values and a variety of parametric and nonparametric tests were used to assess binary interaction between the two metals. Exposures with paired metals showed a variety of interactions. When Hg was paired with either Cd or Zn, its toxicity was decreased. However, when Hg was paired with Pb, toxicity was increased. Zn had no effect on the toxicity of Pb, but both Cd and Hg interacted and increased the toxicity of Pb. Hg and Zn interacted with Cd and decreased the toxicity of Cd. Pb also interacted with Cd, but the effect was an increase in the toxicity of Cd. None of the metals tested changed the Zn LC50 value. The results indicated that combinations of metals can have a range of effects from increasing to decreasing toxicity of single metal exposure. This supports the need that potential chemicals combinations and interactions be further explored. This study further demonstrates the ease of using *C. elegans* in toxicological studies.

Anthropogenic pollution by metals has been increasing since the beginning of the industrial revolution. Sources include the burning of fossil fuels, mining, smelting and manufacturing activities, which have released tons of heavy metals to the environment

(Baird, 1995). Rarely are metals found individually in a system, and the multiple types of contamination that are found, add to the difficulty of identifying the individual contribution of metals to the toxicological profile. Overall, little is known about how multiple contaminant exposures differ from single contaminant exposures. However, most allowable exposure concentrations, including USEPA regulations, are based on data from single compound exposure. Interactions have been demonstrated in many organisms including the freshwater mussel *Anodonta imbecilis* (Keller and Zam, 1991) and other invertebrates including daphnids, gastropods and aquatic insects.

With concurrent exposure to two metals, it often is assumed that the combined effect, as compared to individual exposure, will be additive. However, research has shown metal combinations that decrease toxicity. Working with *Caenorhabditis elegans*, Kammenga (1999) found that low levels of Pb mixed with Cd resulted in a decrease in time to reproduction whereas high levels of Pb mixed with Cd resulted in an increase in time to reproduction. Antagonism has been observed between several metal combinations in freshwater mussels. Working with juvenile *Anodonta imbecilis* and using death as an endpoint, Keller and Zam (1991) found that mixture tests with Ni, Zn, Hg or Cu were generally more toxic than single metal tests. However with Cd, the toxicity decreased in 96-h mixture tests with Zn and also with Cu. Similar metal mixture toxicity testing by other investigators has produced various results. For example, the Zn-Cu effects on rainbow trout were additive at low concentrations and synergistic at higher levels (Loyd, 1980). Cd uptake in adult, *Anodonta cygnea*, was found to be inhibited by high levels of Zn (Hemelraad *et al*., 1987). Conder and Lanno (2000) found the toxicity

of a tertiary mixture of Cd, Pb and Zn to produce an additive effect in producing lethality in the earthworm *Eisenia fetida*.

For this study the omnivorous, bacterial feeding round worm *Caenorhabditis elegans* was chosen as the test organism based on several factors. Research using *C. elegans* has generated an abundance of LC50 values from single metal exposure (Williams and Dusenbery, 1988;Williams and Dusenbery, 1990; Tatara *et al*., 1997; Tatara *et al*., 1998). *C. elegans* is easy to maintain in the laboratory with virtually no genetic drift because *C. elegans* is generally a hermaphrodite. *C. elegans* can easily be supplied with bacterial food (*Escherichia coli*) grown on agar plates. The worm's life cycle is short (3-4 days at 20^{o} C) and the basic biology is well understood. However, there has been very little research performed to determine the effects of multiple metals exposure to *C. elegans*. As the use of *C. elegans* increases in genetic and toxicological research, it will become interesting to learn more about how this organism responds to more than one contaminant.

The heavy metals Pb, Cd, Hg, and Zn were selected to be tested in various paired combinations based on several reasons. Their abundance in the environment because of both natural and anthropogenic sources, has been well documented over the years. The elements Pb, Cd and Hg are considered nonessential to humans and are toxic to most living organisms. The element Zn, which is essential to most biological organisms in trace levels, is often associated with human and animal wastes and consequently is very abundant in aquatic systems. Zn has also been shown to cause inhibitory or toxic effects at elevated cellular concentrations (Mason, 1996).

The objectives of this research were: (1) To assess the effects of single metal exposures and measure LC50 values for each metal individually; (2) To retest each single metal with a low level (predicted LC10 value) of a secondary metal and observe any differences on the LC50 value of the first metal; and (3) To calculate chemical interaction values.

Methods and Materials

The organism used for testing was the wild type N2 strain of *C. elegans*. Stocks were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota and maintained in culture and used for toxicity testing per the methods described by Freeman *et al*., (1998). To insure worm quality, reference toxicant tests were conducted monthly, using $CuCl_2$, according to the methods described by Freeman *et al*., (1998) and Cressman III *et al*., (1997). All $CuCl_2$ LC50 values were within 2 SD of the published LC50 of 63 mg/l (Freeman *et al*., 1998). Each individual metallic salt of Cd, Zn, Pb, and Hg was first tested alone and the LC50 values were calculated with probit analysis software using a log transformation (Toxstat 3.4, West Inc., & Gulley, 1994). Each metal was then retested using the same concentration ranges from the single metal tests, but exposures were conducted with a low level of a different metal added to the test solutions. The projected LC10 from the single metal tests was chosen as the low testing level in an effort to prevent mortality from exceeding 100% in the binary mixture exposures. General range finding tests were required to establish test concentration ranges when preliminary mixture results differed dramatically from the single test results. The effect on the original LC50 value was then assessed and compared. Based on our

preliminary findings, some of the tests (Cd and Hg) were run a third time with a higher level (the projected LC50 from single metal tests) of a different metal present to determine if observed trends continued with increased concentration.

The dauerlarval stage of *C. elegans* was maintained under controlled conditions in a stock solution consisting of a M-9 buffer at 20^{o} C and renewed monthly (Cox *et al*, 1981). The dauers were used to obtain eggs, which were transferred to petri dishes containing K-agar (Williams and Dusenbury, 1988) and an established lawn of the bacterium *Escherichia coli* variant OP50 as a food source (Brenner, 1973). The dishes were incubated at 20E C for 4 days to produce age-synchronized adult worms. The 4 day old adult worms then were used to perform 24-hour toxicity tests.

Preparation of Test Solutions

Metal chloride salts of Pb, Hg, Cd (98-99.999% purity, Aldrich Chemical, Milwaukee, WI, USA) and Zn (98.7% purity, Fisher Chemical, Fair Lawn, NJ, USA) dissolved in K-medium (Williams and Dusenbery, 1990) were used to prepare six test concentrations and a control. Test solutions for the single metals were made by weighing out the appropriate weight of chloride salt, transferring to a 1000 ml flask to make a stock solution. Appropriate aliquots were then calculated, measured volumetrically, transferred to 100 ml flasks and diluted to volume with K-Medium. Mixture test solutions were prepared in a similar fashion except the K-medium used for the stock solution and final dilution was first amended with the secondary metal. In this fashion, the levels of secondary metal present was assured throughout the different test levels. All control solutions consisted of K-Medium only.

Toxicity Testing

Prior to performing a test, four-day old worms were gently washed from the petri dishes using K-medium and then transferred, with a fire-polished pipet to a watch glass. A 15 µl pipet was used to load the worms into the test wells of 12-well dishes (Costar-3512, Corning Incorporated, Corning, NY, USA) containing 1 ml of test (or control) solution in each well with no food source. There were six test wells for each concentration. Ten worms were loaded into each well for a total of sixty worms for each test concentration plus controls (total of 420 worms per test). The test dishes were incubated at 20^{O} C for 24 hours. Mortality was assessed by counting dead versus live worms under a compound microscope. Each test was replicated three times.

Data Analysis

Only tests with control mortalities of less than 10% were kept for further analysis (Freeman *et al*, 1998). The raw data were initially log transformed, tested for normality and homogeneity using X-square and Bartlett's tests. Only data that passed these tests were used for further statistical analysis. Probit analysis was used to generate LC50 and LC10 values. For the single metal tests, samples of each test concentration for Zn, Cd and Pb were collected, preserved with HNO_3 for atomic absorption analysis (AA-flame). Because there were no significant differences between the AA analyzed data and the nominal data for the single metal tests, the mixture samples were not analyzed by AA and the values are presented as nominal rather than measured. Because of potential hazards posed by analysis and lack of appropriate analysis equipment, all data for Hg are nominal values.

Both parametric and nonparametric tests were used to assess metal interactions. Analysis of variance was performed by ANOVA. The Wilcoxon Rank Sum test was used to assess interaction from a nonparametric approach. Because of the small number of replicates (n=3 per test), a nonstatistical approach was also employed. Though there are many models that have been developed and used to assess various types of interaction, a straight forward additive index is probably the best known. The approach developed by Marking and Dawson (1975), which requires data for components individually and in mixtures, was used to assess interaction between metals from a non statistical approach. A value S is calculated from the summation of the ratios of mixture LC50 values to single LC50 values. The method is summarized as follows:

$S = A_m/A_i + B_m/B_i$

Where A_i and B_i are the toxicities (LC50 values) of the individual metals, A_m and B_m are the toxicities (LC50 values) of the metals in mixtures and S is the sum of the biological activities. To achieve linearity and a reference point of zero, the reciprocal of the values of S are used and adjusted for all values of S that are greater than one by 1/S - 1. For all values of S that are less than one, linearity is established by the adjustment S(-1) + 1. Therefore, if the sum of toxicity is additive, then S = 0. Values of S that are greater than zero indicate less than additive toxicity and values of S that are less than zero are interpreted as greater than additive.

Toxic units for mixture test assessments were calculated based on the predicted LC50 and LC10 values obtained from the single metal tests. The assigning of toxic units were based on an individual metals LC50 or LC10 value, which was considered as one toxic unit. Probit analysis was used to determine the LC50 values. However, since the

probit scale is nonlinear, calculations were necessary to assign expected TU values for the mixtures (Table 1).

Results

Figures 1 through 4 summarize the results of the single and metal-mixture toxicity tests. The numbers are the mean of three replicates and are presented in mg/l. For comparison purposes, the mg/l units were converted to μmol/l prior to analysis, but were found to be proportionally the same as the mg/l units and yielded the same results . The single metal tests indicated a order of toxicity to *C. elegans* as follows; Hg>Pb>Zn>Cd, with Hg as the most toxic and Cd as the least toxic. This order is roughly similar to other test organisms and comparable to other published data using *C. elegans* (Williams and Dusenbery, 1990; Tatara *et al*., 1997).

Lead

Single metal toxicity tests using Pb yielded an LC10 and LC50 of 56 mg/l and 115 mg/l, respectively. The presence of low levels of Cd (Cd LC10 of 113 mg/l) resulted in a significant decrease ($p<0.05$) of the Pb LC50 from 115 mg/l to 24 mg/l. When combined with low levels of Hg (Hg LC10 of 3.7 mg/l), the Pb LC50 significantly ($P<0.05$) decreased from 115 mg/l to 20 mg/l. The presence of Zn (Zn LC10 of 99 mg/l and Zn LC50 of 179 mg/l) had no significant effect on the LC50 of Pb (Figure 1).

Zinc

Single metal tests using Zn yielded a LC10 and LC50 of 99 mg/l and 179 mg/l, respectively. Neither the presence of low levels nor of higher levels of Cd (Cd LC10 of 113 mg/l or Cd LC50 of 393 mg/l) had any significant effect on the Zn LC50. Similarly, addition of either low or higher levels of Hg (Hg LC10 of 3.7 mg/l or Hg LC50 of 16.2 mg/l) or the addition of the Pb LC50 (56 mg/l) had no effect on the Zn LC50 (Figure 2).

Cadmium

Cadmium single metal tests yielded a mean LC10 and LC50 of 113 mg/l and 393 mg/l, respectively. When low levels of Hg (Hg LC10 of 3.7 mg/l) were present, a marginally significant difference ($0.05>p<0.10$) was detected and the Cd LC50 was raised from 393 mg/l to 618 mg/l. Higher levels of Hg (Hg LC50 of 16.2 mg/l) resulted in no statistical change in the Cd LC50. The presence of low levels of Pb (Pb LC10 of 56 mg/l) had the opposite effect of Hg and greatly lowered the LC50 of Cd. The change in the Cd LC50 from 393 mg/l to 0.74 mg/l was significant at $p<0.05$. The presence of Zn (LC10 of 99 mg/l and LC50 of 179 mg/l) had no statistical difference on the Cd LC50 (Figure 3).

Mercury

Single metal tests for Hg yielded LC10 and LC50 values of 3.7 mg/l and 16.2 mg/l, respectively. When Hg was rerun with low levels of Cd (Cd LC10 of 113 mg/l) present, the Hg LC50 increased significantly from 16.2 mg/l to 32 mg/l ($p<0.05$). Higher levels of Cd resulted in a similar increase, but were only marginally significant

(0.05>p<0.10). The presence of low levels of Pb significantly (p<0.05) decreased the Hg LC50 from 16.2 mg/l to 0.52 mg/l. Low levels of Zn (Zn LC10 of 99 mg/l) had no effect on the LC50 of Hg. However, higher levels of Zn (Zn LC50 of 179 mg/l) did have a statistical effect and raised the Hg LC50 from 16.2 mg/l to 26.2 at p<0.05 (Figure 4).

Additive Index and Toxic Units

In general, the results of the additive indices analysis and toxic unit calculations agreed with the statistical analysis (Table 1). Where significant differences were noted, both systems indicated either antagonistic, additive or greater than additive effects on the metal pairings. Using the Marking and Dawson equation, S-values were calculated and adjusted to zero (Marking and Dawson, 1975). The pairing of Zn and Pb resulted in less than additive (Pb w/ Zn LC10) or greater than additive (Zn w/ Pb LC10) relationships using the Tus approach. However, the assessment using the additive index was less than additive. Zn and Hg also interacted in a less than additive fashion with the exception of Hg w/ Zn LC10, which was assessed as additive by the TU scale. Similarly, the pairings of Zn with Cd and then Cd with Hg showed less than additive interaction, except for Zn w/ Cd LC50, which indicated greater than additive interaction on the TU scale. The pairings of Pb with Cd and Pb with Hg indicated greater than additive interaction by both scales.

Discussion

The results of the mixture tests indicated relationships, which ranged from greater than additive to antagonistic. An interesting relationship regarding Pb and Cd was

observed. With the exception of Zn, any other metal combination with Pb resulted in greatly increased toxicity. A possible explanation for this may be elucidated by examining the nematodes mechanisms of uptake and toxicity. Limited research has been performed using transmission electron microscopy to examine metal uptake in *C. elegans*. The metals Cd, Hg and Ag have been shown to produce similar adverse effects to various cells located in the gut and esophagus (Popham and Webster, 1979; Popham and Webster, 1982). Though information is limited on specific mechanisms in *C. elegans*, data does exist for larger (dissectible) marine nematodes, which may offer limited comparisons.

Howell (1983) examined heavy metal uptake and distribution in the marine nematodes *Enoplus brevis* and *Enoplus communis* using dissection and subsequent metal analysis on the different tissues. The cuticle, which is very metabolically active and rich in binding sites for metals (disulphide and sulphydryl groups), was found to be responsible for the rapid uptake and loss of metals (Cu and Zn). The pharynegal pumping of water through the nematodes has been shown to permit exposure through the gut as well. However, these large marine nematodes have pores or channels in the cuticle, which are not present in *C. elegans* (Edgar *et al*., 1982; Bird and Bird, 1991). The cuticle of *C. elegans* is believed to be relatively impermeable (Wood, 1988) and metal uptake is believed to be primarily through the gut (Popham and Webster, 1979; Popham and Webster, 1982). A primary mechanism used by *C. elegans* to handle gut exposure may be through the use of a secretory system or excretory gland. In larger nematodes, the execretory gland is known to secrete a acid mucopolysaccharide, which

may have an important influence in metal uptake and loss through the gut (Howell 1982; Howell 1983).

The metals Zn, Cd and Hg are all known to induce thionein synthesis. However, Pb is known to induce glutathione synthesis, suggesting possibly that Pb may be causing toxicity by more than one means or route. There is evidence that Cd may be have an additional detoxification pathway. A novel Cd-inducible gene, designated CDR-1, has been detected in *C. elegans* which responds specifically to Cd, but not Zn, Cu, Hg or Pb (Liao and Freedman, 1999). In another recent report, Vatamaniuk *et al*. (2001) found that the ce-pcs-1 gene in *C. elegans* encodes a functional phytochelatin synthase that is required for Cd tolerance. The tolerance conferred by the ce-pcs-1 gene was not restricted to Cd, but also extended to other soft metals and metalloids, including Hg and As.

Our findings from *C. elegans* concerning the antagonistic relationship between Zn and Cd are roughly consistent with the results from other researchers. Hemelraad (1987) found an antagonistic relationship with Zn and Cd in *A. Cygnea*, suggesting that Zn slows the uptake of Cd by the gills. The study suggested that Zn interaction with Cd occurs at membrane transport and also on the intracellular level. Research has shown that the presence of another divalent cation can trigger competition for binding sites. Khalil *et al*, (1996a), also observed antagonistic effects from mixtures containing Zn and Cd. A tertiary mixture of Cd, Cu and Zn antagonized coccoon production in the earthworm *Aporrectodea caliginosa*. However, when growth was used as the endpoint, an additive effect was observed from exposure to the same tertiary mixture (Khalil *et al*, 1996b).

The relationship between Cd and Hg is similar to that of Zn and Cd above. Low levels of Hg (Hg LC10 of 3.7 mg/l) decreased the toxicity of Cd. However, higher levels (LC50 of 16.2 mg/l) had no effect on Cd.

The results of this study regarding the interaction between Cd and Hg and between Cd and Pb are comparable to other findings including mammalian data. A study using rats found that Cd treatment increased the acute toxicity of Pb and decreased Hg toxicity (Schubert *et al*, 1978). Studies using mice have also shown that prior treatment with Hg markedly diminished the toxic effects of Cd (Calabrese,1991).

The relationship between Zn and Hg is more subtle, lower levels of Zn (Zn LC10 of 99 mg/l) had no effect on the toxicity of Hg, but the higher levels of Zn (Zn LC50 of 179mg/l) decreased the toxicity.

A better understanding of the various mechanisms of detoxification is crucial to determining how individual metals will interact in mixtures. More research is needed to identify the active components of the execretory secretions as well as the active components of the cuticle. Specific *C. elegans* studies such as type performed by Vatamaniuk *et al*. (2001) and Liao and Freedman (1999) where metal-specific genes or enzymes are identified would be helpful to better understand the differing relationships between different metals.

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Table 1. Assessmnet of Toxic Unit and Additive Indices

Metal Pairs	Expected TU	Observed TU	Assessment	Expected Additive Value	Additive Index	Assessment
Cd w/ Hg LC10	1.2	1.8	antagonistic			
				0.0	-2.6	less than additive
Hg w/ Cd LC10	1.3	2.3	antagonistic			
Cd w/ Hg LC50	2.0	2.2	antagonistic			
				0.0	-2.2	less than additive
Hg w/ Cd LC50	2.0	2.0	antagonistic			
Pb w/ Hg LC10	1.2	0.4	synergistic			
				0.0	3.9	greater than additive
Hg w/ Pb LC10	1.5	0.5	synergistic			
Zn w/ Hg LC10	1.2	1.6	antagonistic			
				0.0	-1.4	less than additive
Hg w/ Zn LC10	1.6	1.6	additive			
Zn w/ Hg LC50	2.0	2.1	antagonistic			
				0.0	-1.7	less than additive
Hg w/ Zn LC50	2.0	2.6	antagonistic			
Cd w/ Zn LC10	1.6	2.6	antagonistic			
				0.0	-2.1	less than additive
Zn w/ Cd LC10	1.3	1.4	antagonistic			
Cd w/ Zn LC50	2.0	2.1	antagonistic			
				0.0	-1.0	less than additive
Zn w/ Cd LC50	2.0	1.9	greater than additive			
Zn w/ Pb LC10	1.5	1.3	greater than additive			
				0.0	-1.9	less than additive
Pb w/ Zn LC10	1.6	1.6	additive			
Pb w/ Zn LC50	2.0	1.9	greater than additive		n/a	
Pb w/ Cd LC10	1.3	0.5	synergistic			
				0.0	4.7	greater than additive
Cd w/ Pb LC10	1.5	0.5	synergistic			

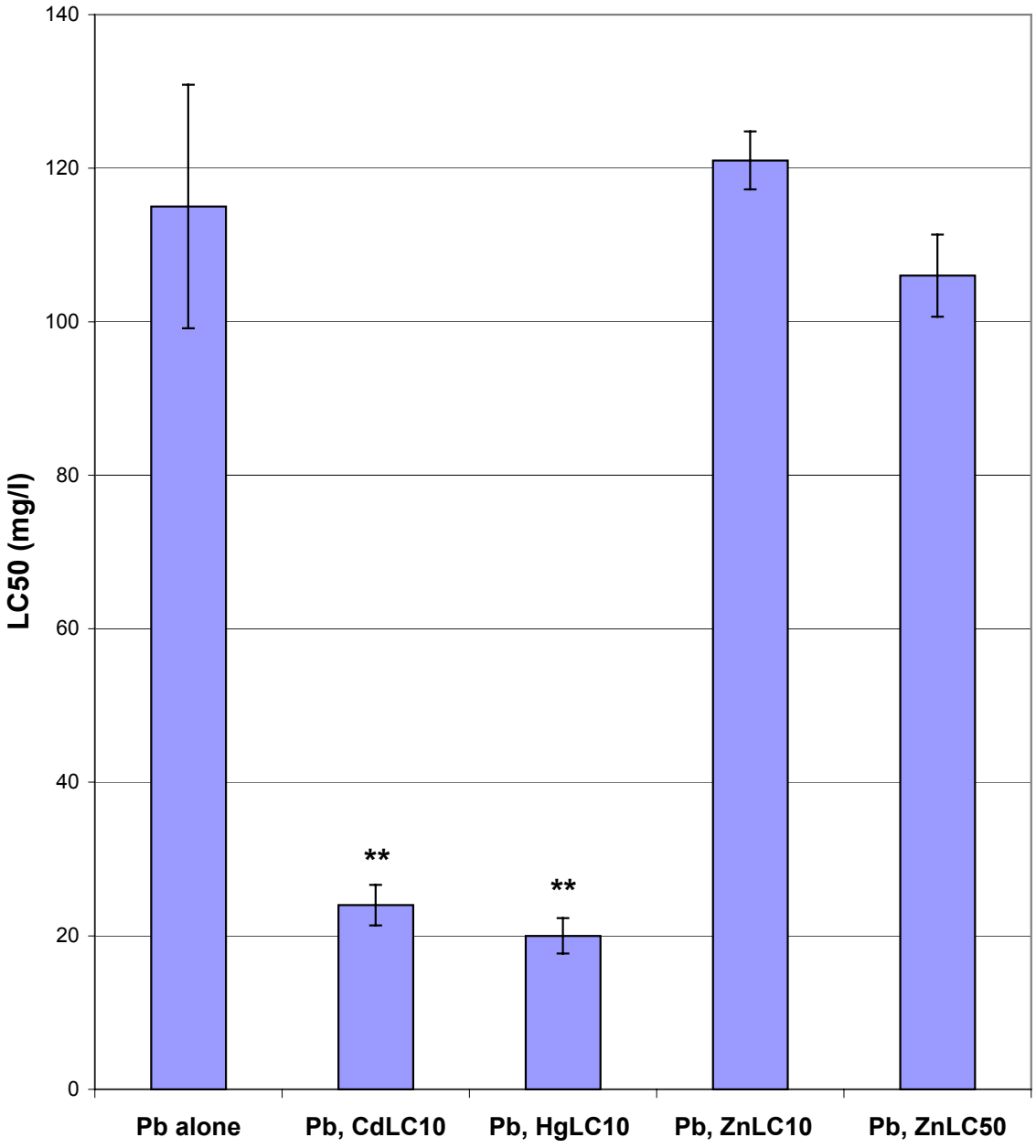

Fig. 1 24 h Pb single and mixture data. LC50 values are means for three replicate tests. Significant differences ($p<0.05$) from the single metal LC50 values are indicated with (**)

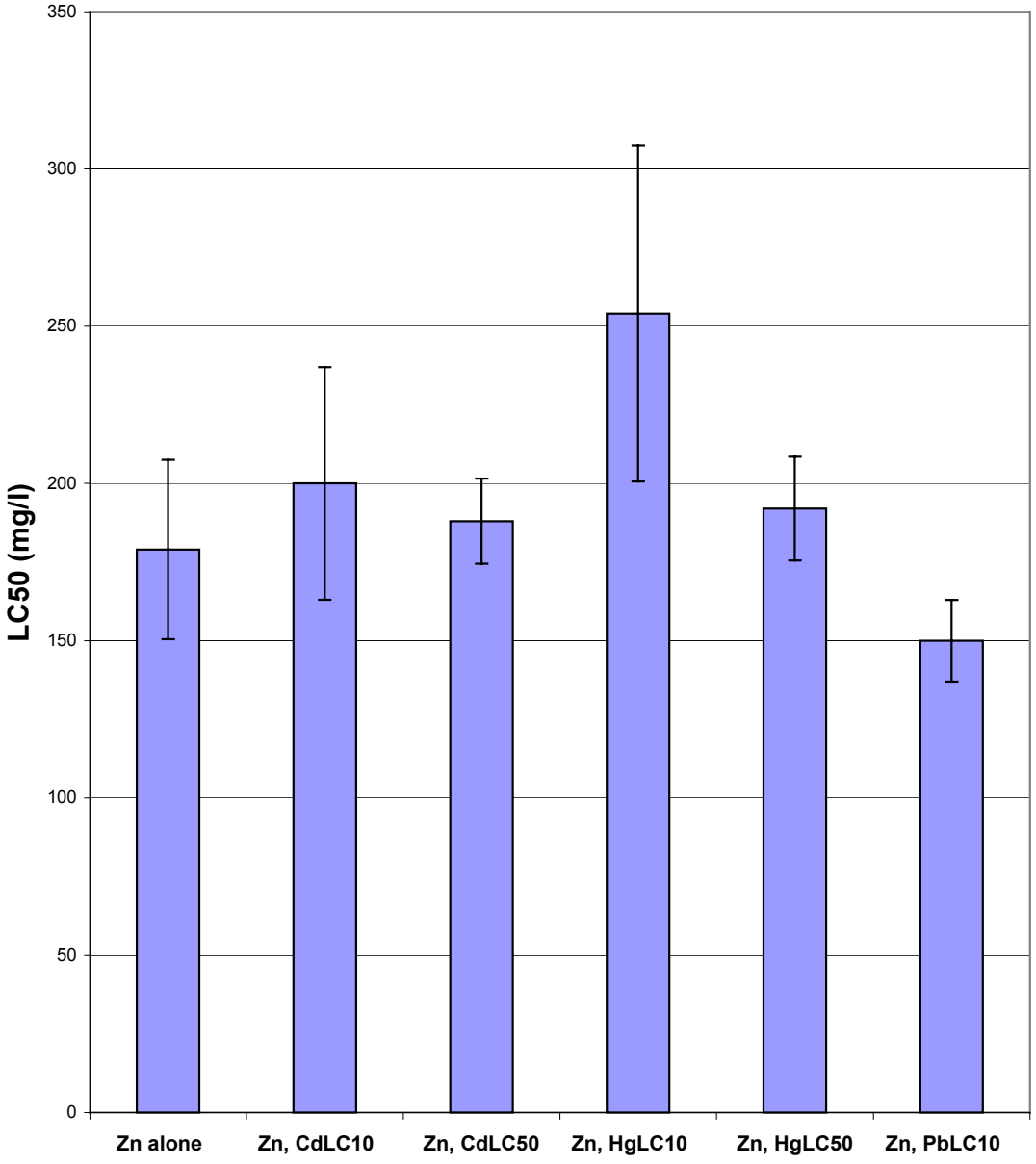

Fig. 2 24h Zn single metal and mixture data. No significant differences were noted between single and mixture data

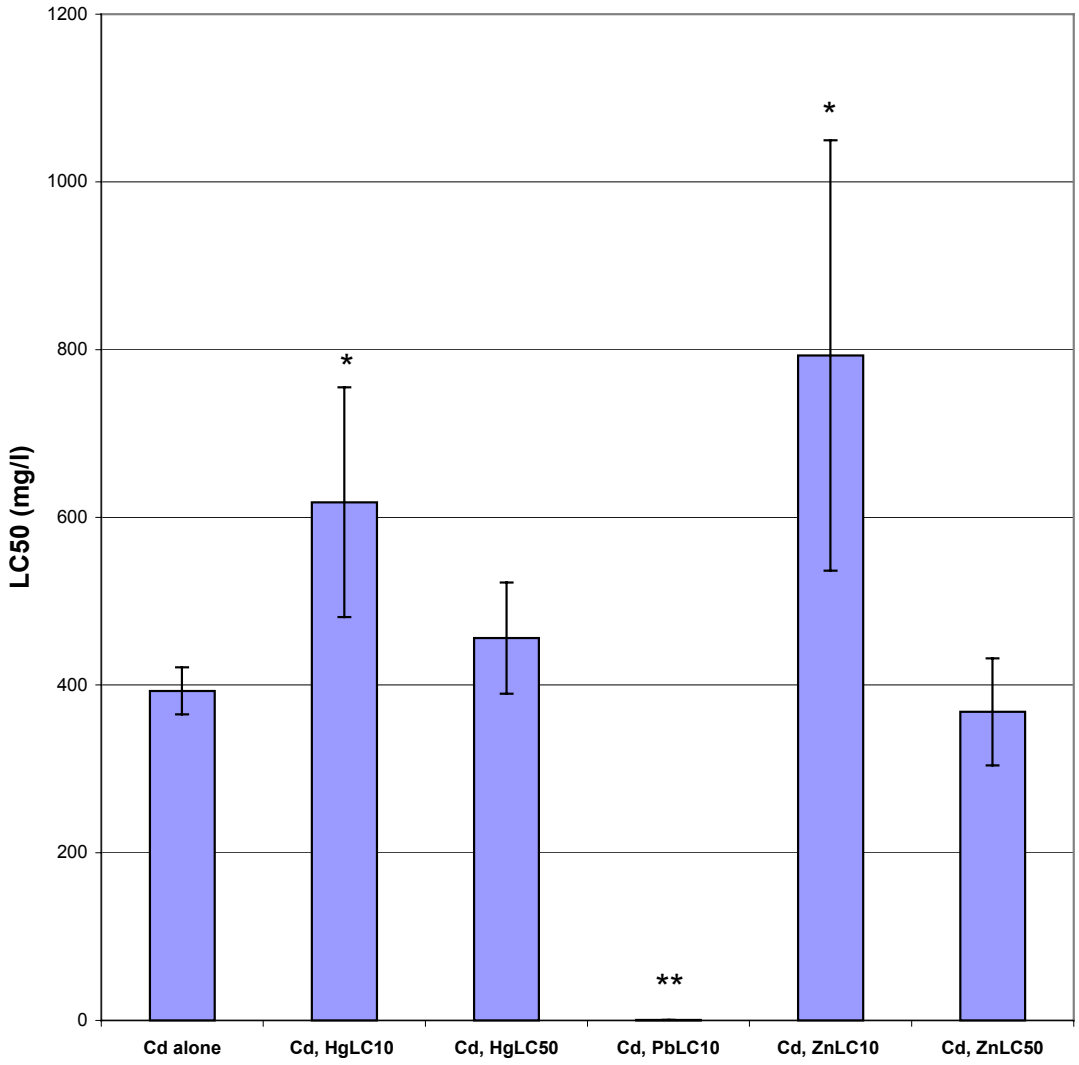

Fig. 3 24h Cd single and mixture data. LC50 values are means for three replicate tests. Significant differences ($p \leq 0.05$) from the single metal LC50 values are indicated with (**). Marginal differences($0.05 > p < 0.10$) are indicated with (*)

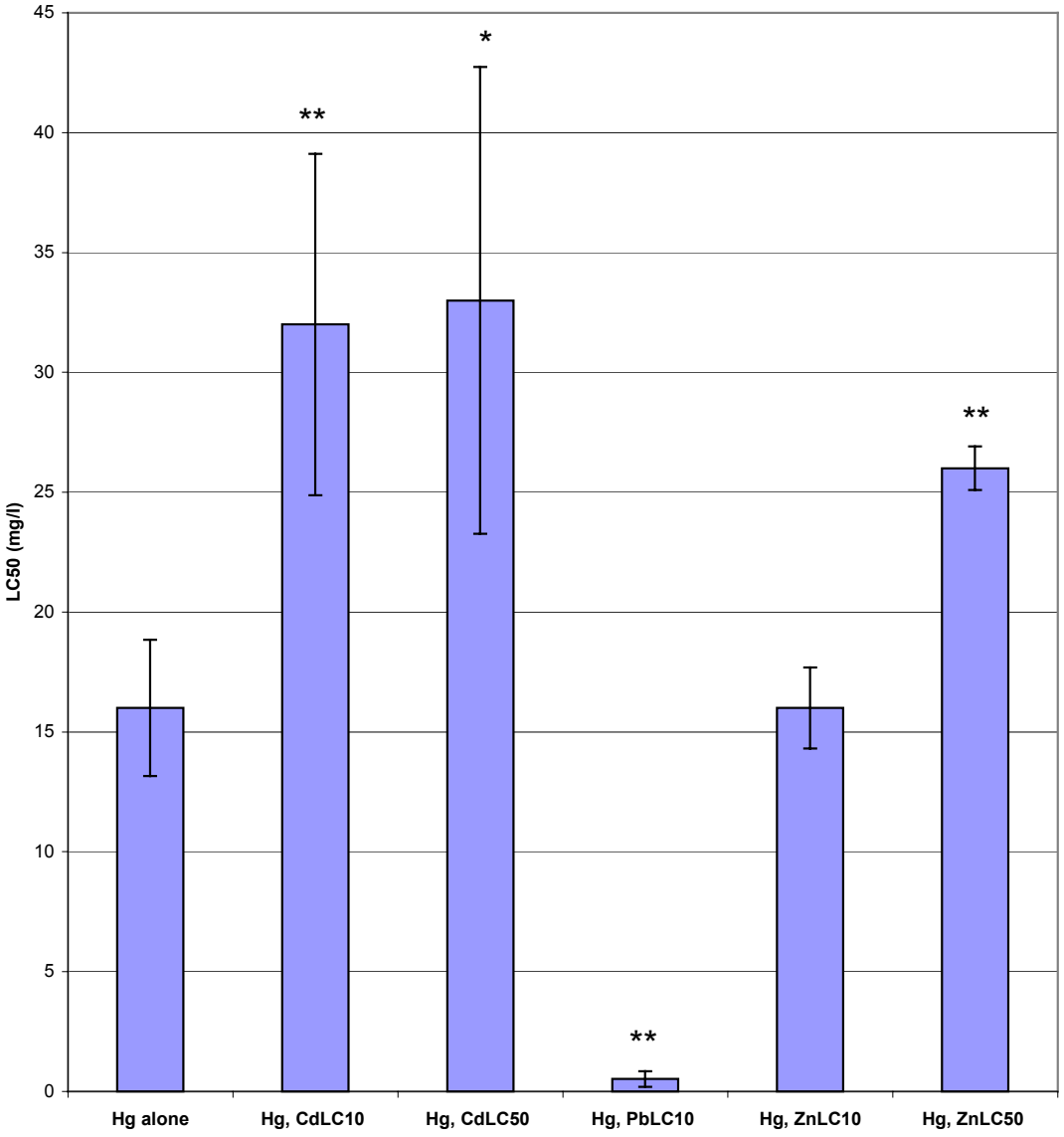

Fig. 4 24h Hg single and mixture data. LC50 values are means for three replicate tests. Significant differences ($p<0.05$) from the single metal LC50 values are indicated with (**). Marginal differences($0.05>p<0.10$) are indicated with (*)

CHAPTER 4

CONCLUSIONS

Clearly, there are profound effects form multiple metal exposure, which differ from the single metal exposure data. Apparently these effects can be greatly enhanced or reduced not only depending on the presence of other metals, but also by the levels of the metals present. This is important because many regulations see metals contamination below the set criteria standards or Maximum Contaminanat Limits (MCLs), as negligible. Consequently, the toxicity of low concentrations of metal mixtures is not always considered in the development of chemical exposure standards. The conclusion that metals can be more toxic to aquatic organisms at lower concentrations in combination then as a single exposure is in agreement with the literature on other aquatic organisms (Keller et al., 1991, Hemelraad et al., 1987, Schubert et al, 1978, Khalil et al., 1996). As our study showed, the levels of one metal present, compared to the others present, is important in the overall effect. The results showed considerable variations. The results ranged from strong anatgonism with the relationship between Zn and Hg as well as between Zn and Cd to greater than additive as with the relationship between Cd and Pb. The combinations of Hg and Pb also showed greater than additive effects.

Since aquatic organisms occupy an important niche in the ecosystem and food chain, it would seem beneficial to the health of aquatic organisms and ultimately humans, to use mixture data to reassess existing laws and regulations. More paired metal research

using *C. elegans* or other standardized test organisms is necessary to understand these relationships first, before any solid and concrete conclusions can be drawn concerning the real effects from multiple metal or other multiple chemical exposure. Considering the variations between metals and between species, it is essential to understand these relationships on the simplest level possible.

Perhaps understanding a mechanism of uptake and detoxification in *C. elegans* first, would permit a more accurate assessment of interactions and allow knowledge to be accumulated into a working foundation upon which real conclusions can be drawn. A metals tissue distribution project using *C. elegans* could shed light on specific points of uptake and mechanisms of action. The possibility of performing dissection and metal analysis using scanning electron microscopy are currently being discussed in our laboratory. If uptake in *C. elegans* is accomplished through the gut, then a project comparing gut surface area to surface areas of uptake (gills, gut, membranes, etc.) in other aquatic test organisms, would allow better understanding and comparability between species. Perhaps a correction factor could be integrated into some of the methods for assessing mixtures and permit more readily comparable data between species. A detailed *C. elegans* project designed to qualify the gut components (disulphides, sulphydryl groups, cysteine residues, integral proteins, ionopores, etc.) and ratios of these components could further clarify the mechanism of uptake and detoxification. More research concerning the role of the excretory gland secretions in metal detoxification is also needed. This could be accomplished by research to isolate the secretions and performing analysis to identify and quantify the various components. Other projects such

as identifying metal-specific enzyme induction systems would be useful in explaining why metals behave differently when paired in certain combinations.

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