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Reproductive Toxicity and Bioavailability of Arsenic in Contaminated Artificial and Natural Soils using the Earthworm

Stephen W. Wong

Department of Natural Resource Sciences

McGill University, Montreal

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Abstract

High concentrations of arsenic are found near gold-mine tailings. The most common form of arsenic found in soil is arsenate, which is a known toxicant. We used the standardised earthworm reproduction test for the species *Eisenia andrei* (*E. andrei*) to study the toxicity and bioavailability of arsenic-contaminated soil. Arsenic is toxic to earthworms as indicated by the decrease in survival and reproduction. Arsenic-spiked artificial soil was more toxic than arsenic-spiked field soil based on total arsenic concentration in soil. Moreover, soil from near mine tailings showed a reduced toxic effect despite its high soil arsenic concentration as compared to spiked field soil. Measurements of arsenic tissue concentrations in the earthworm indicated that uptake of arsenic into earthworm tissue was higher in spiked artificial soil as compared to spiked field soil and that the maximal body burden was 396 μg As/g dry tissue weight. However, when considering tissue arsenic concentration, spiked field soil is more toxic than spiked artificial soil. Therefore the tissue rather than soil content may better reflect the magnitude of arsenic toxicity to *E. andrei*.

Résumé

Des concentrations élevées d'arsenic sont trouvées à proximité des mines d'or. L'arsenate, un produit toxique connu, est la forme la plus commune d'arsenic trouvée dans le sol. Un test de reproduction a été effectué sur des vers de terre *Eisenia andrei* (*E. andrei*) en utilisant une procédure normalisée pour étudier la toxicité et la biodisponibilité de l'arsenic présent dans le sol contaminé. La diminution de la survie et de la reproduction prouve que l'arsenic est toxique pour les vers de terre. L'arsenic semble plus toxique lorsque l'exposition des vers est effectuée dans un sol artificiel que dans un sol forestier. De plus, le sol contaminé prélevé à proximité de mines a montré un effet toxique moindre que le sol forestier en dépit de sa teneur plus élevée en arsenic. Les mesures indiquent que la concentration de l'arsenic dans le tissu des vers de terre est plus élevée suite à une exposition dans un sol artificiel que dans le sol forestier et que la quantité maximale est de 396 µg/g de tissu sec. Cependant, quand on compare la concentration de l'arsenic dans le tissu, le sol forestier est plus toxique que le sol artificiel. Par conséquent, la concentration dans le tissu du ver, plutôt que la concentration dans le sol, reflète mieux la toxicité de l'arsenic pour *E. andrei.*

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Contribution of Authors

The thesis is presented in a manuscript format. Chapter 1 is a brief introduction to the content of the thesis. Chapter 2 includes a detailed review of the background information. Chapter 3 describes the details of the experiments and their findings. The co-authors of the manuscript (Chapter 3), Dr. Geoffrey I. Sunahara and Dr. Laurie H.M. Chan, contributed by supervising the research, helping me to interpret the results, and editing the document. Chapter 4 contains the summary and conclusions.

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List of Abbreviations

AAS	Atomic absorption spectroscopy
ASTM	American Society for Testing and Materials
ATSDR	Agency for Toxic Substances and Disease Registry
BRI	Biotechnology Research Institute
C	Celsius
CAS	Chemical Abstracts Service
CINE	Centre for Indigenous Peoples' Nutrition and Environment
cmol	Centimoles of positive charge
CPHA	Canadian Public Health Association
d	Days
DINA	Department of Indian and Northern Affairs
DORM	Dogfish muscle tissue
EC_{50}	Effective concentration at which there is 50% reduction
E_h	Reduction-oxidation potential
FCAR	*Les Fonds pour la Formation de Chercheurs et l'Aide à la Recherche*
g	Grams
h	Hours
ISO	International Organization for Standardization
kg	Kilograms
L	Litres
LC_{50}	Lethal concentration at which there is 50% mortality

LOEC	Lowest observed effect concentration
m	Meters
MESS	Marine sediment
mg	Milligrams
min	Minutes
mL	Milliliters
mM	Millimolar
mV	Millivolts
NOEC	No observed effect concentration
NRC	National Research Council
OECD	Organization for Economic Cooperation and Development
OT	Oyster tissue
P	Probability
ppb	Parts per billion
ppm	Parts per million
psi	Pounds per square inch
RSD	Relative standard deviation
SD	Standard deviation
TMG	Trace metal grade
UQAM	*Université du Québec à Montréal*
USEPA	United States Environmental Protection Agency
v/v	Volume per volume
w/w	Weight per weight

µg	Microgram

Chapter 1. Introduction

1.1. Introduction

Soil contamination by industrial waste is a persistent global problem that presents a risk to both human and ecological health (Callahan and Lindler 1992). The activity of two gold mining and milling operations located at Yellowknife, Northwest Territories, Canada is an example of mine waste contamination. Emissions from the mining and smelting operations, which began in 1938, have resulted in the accumulation of arsenic and other contaminants in the soil, and the pollution of water draining from the area (CPHA 1977). Arsenic is known to cause acute and chronic poisoning, external irritant effects, immune suppression, mutagenesis, teratogenesis, and carcinogenesis in humans (CPHA 1977, Squibb and Fowler 1983, ATSDR 2000, Vahter and Concha 2001).

The behaviour and availability of contaminants are influenced by their interactions with soil components. However, it is difficult to relate soil concentration with toxicity without taking into account the soil chemistry parameters. Ecotoxicity testing is designed to assess the effects of pollutants on organisms. These tests have the advantage of directly reflecting contaminant bioavailability and their associated hazardous effects (Callahan and Lindler 1992). With biological testing, effects such as reduced growth, reduced reproduction, and death are common endpoints (Callahan and Lindler 1992).

Soil organisms are exposed to soil toxicants mainly via the aqueous phase of the soil (Van Gestel 1997). As residents in soil, earthworms have great potential for use in assessing sublethal risks to public and environmental health from contaminated soils and hazardous waste sites and evaluating the toxicity of mixtures (Venables et al. 1992, Callahan and Lindler 1992). Hence, standardised tests have been established for assessing sublethal effects on earthworms (ASTM 1998, ISO 1998). A laboratory bioassay is used when it is not possible to find native earthworms in the contaminated soils (Van Gestel 1997).

1.2. Rationale

The rationale is that earthworms can be used as bioindicators to study the ecotoxicological effects of arsenic in soil.

1.3. Objectives

The objective of this research is to determine the effects of arsenic-contaminated artificial and natural soils on the reproduction of *E. andrei* and to examine the bioaccumulation of arsenic from these soils in the earthworm.

1.4. Hypothesis

The hypotheses to be tested are the following:

1) There is a difference in the bioavailability between three different types of arsenic-contaminated soil:
   - Arsenic-spiked artificial soil
   - Arsenic-spiked field soil
   - Soil found near mine tailings in Yellowknife
2) Arsenic-contaminated soils affect survival and growth of adult earthworms
3) Arsenic-contaminated soils lower the reproductive success of earthworms
4) The decrease in reproductive success is the result of:
   - Lethal acute effects
   - Effects on larval development
5) Effects on survival, growth and reproduction depend on tissue bioaccumulation levels of the toxicant in the earthworm

Chapter 2. Literature Review

2.1. Arsenic in the Environment

Arsenic (As) is a metalloid element that belongs to the V(A) group of elements. However, it exhibits both metallic and non-metallic properties (Chan and Huff 1997). Arsenic and its compounds occur naturally and are ubiquitous in the environment. It is the 52nd most common element in the earth's crust (Peters et al. 1996) and is present in at least 245 different minerals but occurs most commonly as arsenopyrite (FeAsS) (CPHA 1977; Hindmarsh and McCurdy 1986; Peters et al. 1996; Thornton 1996). Arsenic occurs in +5, +3, 0 and –3 oxidation states. Arsenic cannot be destroyed in the environment; it can, however, change forms or become attached to or separated from particles (ATSDR 2000). Oxidation-reduction, precipitation-dissolution, adsorption-desorption, and methylation and volatilisation are mechanisms that control the mobilisation of arsenic in the environment (Hindmarsh and McCurdy 1986, Bhumbla and Keefer 1994).

Arsenic in the environment is modified via reactions with molecules in air, water, or soil, and through the action of bacteria that are present in soil or sediment (ATSDR 2000). The As compounds normally found in the environment can be divided into six categories (Chan and Huff 1997):

1) inorganic water-soluble compounds (soluble arsenite and arsenate salts, arsenic trioxide and arsenic pentoxide),

2) inorganic compounds having low or virtually no water solubility (various arsenite and arsenate salts, arsenides, and arsenic sulphide),
3) organic arsenic compounds (biologically methylated arsenic compounds and pesticides),
4) organic arsenic compounds in marine organisms (arsenobetaine and arsenocholine),
5) organic compounds used as feed additives (arsanilic acid), and
6) gaseous inorganic and organic compounds (arsine).

Arsenic in the atmosphere is usually composed of a mixture of trivalent (+3) and pentavalent (+5) forms (ATSDR 2000). In the aquatic environment, arsenic is present predominantly in the inorganic water-soluble forms (ATSDR 2000). In the pH range of natural waters, the prevalent aqueous arsenate species are $H_2AsO_4^-$ and $HAsO_4^{-2}$, and the predominant aqueous arsenite species is H_3AsO_3 (Neff 1997, ATSDR 2000).

2.2. Arsenic in the Soil

The natural arsenic content in soils varies from 0.1 to 40 mg/kg with an average of about 5-6 mg/kg (CPHA 1977, Hindmarsh and McCurdy 1986, Peters et al. 1996). Soil is an important component of the environment. Soil can adsorb and release ions depending upon the types of minerals present, the proportion of organic matter, pH, redox potential, and moisture status (Oliver 1997). Adsorption of As onto soil components involves two mechanisms: non-specific adsorption and specific adsorption. Non-specific adsorption refers to coulombic interactions between the positive charges on oxides with

arsenate anions (Manful et al. 1989). Non-specific adsorption occurs in the order of seconds and is influenced by pH (Pierce and Moore 1982). Specific adsorption refers to the incorporation of arsenate anions as ligands in the co-ordinated shell of iron atoms (Manful et al. 1989). Specific adsorption occurs in the order of hours (Pierce and Moore 1982). The sequestration of arsenic from aqueous solution is dependent on the clay content of the underlying soil or sediment. Iron oxides are major components of clays. In soil, arsenic tends to form negatively charged oxyanions that coprecipitate and adsorb onto the cationic sites of clay particles, or that coprecipitate as metal-ion precipitates (Hindmarsh and McCurdy 1986, Peters et al. 1996). Arsenic-containing complexes are generally very stable and insoluble in water, therefore arsenic does not travel through soils rapidly even when soils are leached repeatedly. Because of the stability of these complexes, arsenic has a residence time of approximately 2,400 years in terrestrial soils (Peters et al. 1996).

The degree of arsenic adsorption depends on the type of soil. Arsenic binds less to sandy or low clay soils than to organic, silty or clay soils. Hence, arsenic would be more mobile in the former types of soil (Walsh et al. 1977, Peters et al. 1996). Available As is controlled by adsorption reactions in soil instead of precipitation reactions (Livesey and Huang 1981). Biomethylation, which can occur under aerobic or anaerobic conditions, results in the loss of arsenic from soil. The methylated arsenic species are volatile, and arsenic is lost from soil into the atmosphere (Peters et al. 1996).

The speciation of arsenic in soil is dependent on soil pH and levels of iron. Elevated levels of iron favours a high redox potential (E_h > 300 mV), which in turn will favour the formation of the pentavalent arsenate species (Hindmarsh and McCurdy 1986, Peters et al. 1996, Thornton 1996). Arsenate (AsO_4^{-3}) is the most common form of arsenic in soil followed by arsenite (AsO_2^-). Arsenites are theoretically of greater environmental concern than arsenates since they have a greater toxicity and higher mobility in soil (ATSDR 2000).

2.3. Yellowknife: Gold Mining and Arsenic

Arsenic is present in lead, zinc, copper, silver and gold ores. Mining has the potential to increase soil As levels because it produces waste tailings that are rich in arsenic. The mine tailings are left near mining sites to be weathered, which leads to leaching of arsenic into the soil and ground water (Peters et al. 1996). Smelting of the ores results in the emission of arsenic as gaseous or solid waste by-products (Hindmarsh and McCurdy 1986, Peters et al. 1996, Thornton 1996). This process releases arsenic trioxide (As_2O_3), which may react with basic oxides in air to form arsenates that can deposit onto soil (Bérubé et al. 1972, Thornton 1996). Smelting accounts for 50 to 60 % of the total global emissions of arsenic (Peters et al. 1996). Of the total arsenic added to soils, 10 % comes from mine tailings and 7 % from smelters (Bhumbla and Keefer 1994).

The city of Yellowknife, Northwest Territories, Canada is located at 62°27'N, 114°W. Gold was discovered in the area in 1936 (CPHA 1977). One year later, the town

was established; gold mining and smelting operations began in 1938, leading to soil contamination by arsenic (CPHA 1977). Most of the current arsenic contamination in the Yellowknife area results from the past gold mining activities of the Giant Yellowknife Mine (located 3.5 miles north of Yellowknife) and the Cominco Mine, now known as Miramar Con Mine (located 1.5 miles south of the city). The Giant Yellowknife Mine has operated a gold mine and smelter since 1948 (CPHA 1977). The Cominco mill began operation in 1938; roasting started in the late 1940s and eventually stopped in 1970 (CPHA 1977).

Gold ore contains arsenopyrite. The recovery of gold from gold ore changes arsenopyrite to more toxic forms. Both the roasting of gold ores and the weathering of mine tailings have resulted in arsenic leaching into the soils and groundwater (Peters et al. 1996, Thornton 1996). It is estimated that approximately 50,000 tons of arsenic are contained within the two storage ponds on the Cominco property (CPHA 1977).

Soils in the city of Yellowknife contain arsenic ranging from 1 to 600 mg/kg; in the proximity of the mines, levels of greater than 4,000 mg/kg have been reported (CPHA 1977). The regulatory limits established by the Ministry of Environment Canada for arsenic cleanup in agricultural, industrial, and residential soils are 25, 50, and 25 mg/kg, respectively (Chen et al. 2001).

2.4. Ecotoxicity Testing

Ecotoxicity testing involves experiments designed to assess the fate of chemical pollutants at different levels of biological organisation (Forbes and Forbes 1994). Ecotoxicity tests include chemical analyses of contaminant levels in soil and soil properties, and toxicity analyses using laboratory and/or field tests (Forbes and Forbes 1994). Laboratory tests examine the impact on individuals and populations whereas field tests concern populations and ecosystems (Römbke and Moltmann 1996). Laboratory tests provide relatively precise information regarding the toxic effects of a contaminant, but the relevance of the data is limited; field tests yield more valid assessments on actual risk, however, there is a greater uncertainty associated with them (Römbke and Moltmann 1996).

Although it may be useful to assess ecotoxicity under field conditions, there are a number of disadvantages associated with this approach. The disadvantage of field tests include: complexity of the system; difficulties in obtaining a dose-response relationship; duration of the experiment in order to elicit an adequate response; and difficulty in determining the biological significance of contaminant levels in the presence of multiple contaminants since interactions can obscure the interpretation of results (Forbes and Forbes 1994, Römbke and Moltmann 1996, Van Gestel 1997). Field tests do not allow for standardisation, therefore results are only applicable to a case-by-case basis (Römbke and Moltmann 1996).

The advantages associated with field tests are that they represent real conditions and allow for the detection of direct toxic effects and indirect effects of contaminants resulting from species interactions (Forbes and Forbes 1994, Römbke and Moltmann 1996). In the field, ecological compensation and regulation mechanisms can operate (Van Gestel 1997).

In the laboratory, organisms are tested under optimal growth conditions. Laboratory tests may be too sensitive for predicting the effects in field soils, as the availability of the contaminant is often higher in laboratory than field studies (Spurgeon 1997, Van Gestel 1997). For laboratory tests, chemicals are usually freshly added to the substrate, while in the field, equilibrium is established over a long period (Van Gestel 1997). In addition, in the field, both pollutants and soil organisms are heterogeneously distributed in the soil, thereby limiting a proper prediction of exposure levels (Van Gestel 1997). The disadvantages associated with laboratory tests on single species are: the influence of physical environmental conditions are not considered; a particular stage is selected to represent the entire lifecycle; a selected number of species which are easy to breed and control are used to represent a large number of species in the environment; interactions with other species are not considered; and the toxicity data for a single chemical is usually inadequate since contaminated soils often contain a mixture of pollutants (Römbke and Moltmann 1996, Van Gestel 1997).

In spite of these limitations, laboratory tests have the obvious practical advantages of simplicity and cost-efficiency. Laboratory tests also have the advantage that the

procedure can be standardised—the test results can be reproduced and replicated, the test results are easy to manage for statistics, and comparative data are available for the evaluation of test results (Römbke and Moltmann 1996).

Soil ecotoxicity studies most commonly employ soil microorganisms and macroorganisms, plants, and higher vertebrates (Römbke and Moltmann 1996). However, the number of standardised test procedures available for the terrestrial medium is limited (Römbke and Moltmann 1996). There are relatively few test guidelines for assays with plants (Römbke and Moltmann 1996). The acute earthworm test is the most common test using soil animals (Römbke and Moltmann 1996). However, one of the major drawbacks is that the test uses *Eisenia fetida* or *Eisenia Andrei*, which are not found in field soils. Earthworms commonly found in the field, such as *Lumbricus terrestris* or *Aporrectodea caliginosa*, are not used because of their longer generation time and the difficulty in maintaining cultures (Römbke and Moltmann 1996).

To establish the effects of a contaminant on survival, the LC_{50} is used, which indicates the concentration that causes 50% mortality in the test organism (Forbes and Forbes 1994, Van Gestel et al. 1995). There are three categories of sublethal effects, the NOEC (no-observed effect concentration), LOEC (lowest-observed effect concentration) and EC_{50}, which indicates that a 50% inhibition occurs compared to the control (Forbes and Forbes 1994, Van Gestel et al. 1995). The LC_{50} and EC_{50} are obtained from concentration-response curves while the NOEC and LOEC are obtained from comparing

the endpoint (e.g. survival, growth, or reproduction) at different concentrations with the control (Van Gestel et al. 1995).

2.5. Arsenic Toxicity

Arsenic is present in all living organisms. Most organisms, with the exception of coelenterates and some molluscs and crustaceans, contain between 0.1 and 1.0 μg As/g dry weight (Hindmarsh and McCurdy 1986). However, when an organism resides in an arsenic-contaminated environment, there is the potential for bioaccumulation of the toxicant.

The similarity of arsenic to phosphorus in terms of oxidation state and electron orbital, and its ability to form covalent bonds with sulphur makes it bioavailable to many organisms. Arsenate is an analogue of phosphate, an essential mineral, and can be taken up by the phosphate transport system of some organisms (Tamaki and Frankenberger 1992). Arsenate is thought to replace phosphate in energy transfer phosphorylation reactions and interferes with phosphate transport and metabolic processes (Tamaki and Frankenberger 1992, Jonnalagadda and Prasada Rao 1993, Morgan et al. 1994, Chan and Huff 1997, ATSDR 2000). Arsenite has a high affinity for thiol groups in proteins and can therefore inactivate enzymes (CPHA 1977, Tamaki and Frankenberger 1992, Jonnalagadda and Prasada Rao 1993, Chan and Huff 1997, Simeonova and Luster 2000, Vahter and Concha 2001). It is possible that arsenate is reduced to arsenite in cells, in order to exert a greater toxic effect (Jonnalagadda and Prasada Rao 1993).

2.5.1. Animal and Human Studies

Indications of arsenic toxicity come from epidemiological evidence in humans and laboratory studies with animals. Arsenic is thought to be well absorbed via inhalation and oral routes, while absorption through the dermal route is thought to be relatively low (ATSDR 2000). Concentrations as low as 3-10 ppm arsine gas produced symptoms in humans after several hours of exposure (Morse and Setterlind 1950). In mice, the LC_{50} for arsine by inhalation is approximately 0.5 mg/L after 2.4 min (Levvy 1947). From published observations, the lethal dose of inorganic arsenic in humans ranged from 22 to 121 mg As/kg body weight (Levin-Scherz et al. 1987, Quatrehomme et al. 1992, Civantos et al. 1995, Hantson et al. 1996). The oral LD_{50} for arsenite in rats and mice is about 10 mg/kg body weight and that for arsenate is about 100 mg/kg body weight (Schroeder and Balassa 1966).

There is limited evidence that arsenic is a reproductive and developmental toxicant. In human case studies, prenatal exposure to high acute doses of arsenic resulted in miscarriage and early neonatal death (Shalat et al. 1996). Chronic low-dose exposure has caused spontaneous abortion, congenital malformations, and developmental impairment (Shalat et al. 1996, ATSDR 2000). Inhalation exposure in mice to 22 mg As/m^3, as arsenic trioxide during gestation decreased the number of live foetuses and impaired foetal development while those exposed to 0.20 mg As/m^3 showed only a decrease in foetal weight (Nagymajténji et al. 1985). In intraperitoneal injection studies with rats, the developmental NOEC for arsenite and arsenate was approximately 3 mg

As/kg body weight; the LOEC was 7.6 mg/kg body weight and 8.4 mg/kg body weight for arsenite and arsenate, respectively (Stump et al. 1999). Following oral administration of arsenite by gavage to rats, a NOEC of 15.2 mg/kg body weight and a LOEC of 22.7 mg/kg body weight was determined (Stump et al. 1999). Mice given a single gavage dose of 23 mg/kg body weight during gestation also had increased foetal mortality and decreased foetal body weight, with no effect at 11 mg As/kg body weight (Baxley et al. 1981). Burk and Beaudoin (1977) found embryotoxic effects—an increase in the number of resorptions and malformations and a decrease in foetal weight—at an interperitoneal dose of 15 mg/kg body weight arsenate. With a dose of 12 mg/kg body weight arsenate, only a decrease in foetal weight was observed (Burk and Beaudoin 1977). Increased foetal mortality and decreased foetal body weight were found in hamsters treated with a single gavage doses of 14 mg/kg body weight of arsenite during gestation, with no effects at 11 mg/kg body weight (Hood and Harrison 1982).

2.5.2. Marine Toxicology

The average concentration of total arsenic in the ocean water is about 1.7 μg As/L (Neff 1997). Marine sediments usually contain between 5 to 40 mg As/kg dry weight (Neff 1997). Arsenate and arsenite are the dominant forms of inorganic arsenic in the marine ecosystem (Neff 1997). Among these two forms, arsenate is thermodynamically more stable and comprises ~80% of the inorganic arsenic found in waters (Neff 1997). Marine algae convert arsenate into organoarsenic compounds, mainly arsenobetaine and arsenocholine, both of which are relatively non-toxic (Neff 1997, ATSDR 2000). Tissue

concentrations in marine invertebrates and fish are usually between 1 to 100 μg As/g dry weight.

Ecotoxicity studies with arsenic have been conducted mostly in marine environments. Among marine organisms, the growth of the diatom *Skeletonema costatum* was inhibited at a concentration of 20 μg arsenite/L or 13 μg arsenate/L (Sanders 1979). Cytocarps maturation, an indicator of sexual reproduction, in the macroalga *Champia parvula* was inhibited upon exposure to 95 μg arsenite/L (Thursby and Steele 1984). Growth was inhibited at 145 μg arsenite/L and mortality occurred at 300 μg arsenite/L (Thursby and Steele 1984). In contrast, a concentration of 10,000 μg arsenate/L in seawater did not kill *C. parvula* (Thursby and Steele 1984). Exposure (12 d) of *Dunaliella* sp. to arsenate concentrations greater than 3000 μg/L inhibited growth (Yamaoka and Takimura 1986).

For the toxicity of arsenite and arsenate on marine animals, the 96 h LC_{50} of arsenite to juvenile bay scallops, *Argopecten irradians*, was 3,490 μg/L (Nelson et al. 1976). Arsenite at 961 μg/L significantly decreased the survival of the amphipod *Gammarus pseudolimnaeus* to 20% after 7 d and elicited 100% mortality after 14 d (Spehar et al. 1980). The 96 h LC_{50} of arsenite to the amphipod *Corophium insidiosum* and *Elasmopus bampo* were 1,100 μg/L and 2,750 μg/L, respectively (Reish 1993). The 96 h LC_{50} of arsenic trioxide to the crab *Scylla serrata* was 17,000 μg/L; the NOEC was 7,500 μg/L (Krishnaja et al. 1987). Larvae of Dungeness crab *Cancer magister* had a 96 h LC_{50} of 232 μg arsenite/L (Martin et al. 1981). Embryos of the Pacific oyster *Crassostrea*

gigas had a 96 h LC_{50} of 326 μg arsenite/L (Martin et al. 1981). The acute lethal concentration of arsenate to the mysid, *Mysidopsis bahia*, was 2,319 μg/L (USEPA 1984). The LC_{50} for arsenate upon 96 h and 384 h exposure of adult shrimp *Crangon crangon* was 96,000 μg/L and 47,000 μg/L, respectively (Madsen 1992). Rainbow trout, *Salmo gairdneri*, exposed to 961 μg arsenite/L and 973 μg arsenate/L accumulated 3.0 μg As/g dry weight of arsenic, this accumulation level did not differ from the controls (Spehar et al. 1980).

2.6. Soil Toxicology

In the soil matrix, only arsenic that is bioavailable can exert toxic effects (Pantsar-Kallio and Manninen 1997, Alexander 2000). Bioavailability refers to the accessibility of a chemical for uptake and possible toxicity. The toxicity of As varies widely, depending on the species involved. Generally, trivalent arsenic compounds are more toxic than the pentavalent form, and inorganic arsenic compounds are more toxic than organoarsenicals. Elemental arsenic is one of the least toxic forms of the element (Peters et al. 1996); arsine gas (AsH_3) is generally considered the most toxic form of arsenic (Squibb and Fowler 1983).

2.6.1. Plant studies

Most soil ecotoxicity tests with arsenic have been performed with plants. Crop yields in ryegrass, *Lolium perenne*, and barley, *Hordeum vulgare*, were reduced

following arsenic application of 50 and 250 mg As/kg soil as either arsenite or arsenate (Jiang and Singh 1994). At an arsenic rate of 2.0 mg/L as arsenite or arsenate, a decrease in total dry weight was observed for the marsh grasses *Spartina patens* and *Spartina alterniflora* (Carbonell-Barrachina et al. 1998). In rice, *Oryza sativa*, arsenite at a rate of 0.8 mg/L caused a decrease in biomass accumulation (Marin et al. 1992). Tissue accumulation at this rate was 466.5 μg As/g dry weight in the root and 48.0 μg As/g dry weight in the shoot (Marin et al. 1992). Arsenate, which was not phytotoxic at the concentrations tested, resulted in arsenic tissue accumulation of 248.2 μg/g dry weight in the root and 25.2 μg/g dry weight in the shoot at a rate of 0.8 mg As/L (Marin et al. 1992). A soil arsenate concentration of 3.0 mg/kg decreased the fresh and dry weight of the shoot and the fresh weight of the root in the plant *Pisum sativum* (Päivöke and Simola 2001). Accumulated levels of arsenic in the plant at the highest concentration tested (5.0 mg As/kg soil) was 308.0 μg As/g dry weight in the cotyledons, 289.0 μg As/g dry weight in the root, and 4.0 μg As/g dry weight in the shoot (Päivöke and Simola 2001). Woolson (1973) examined the arsenic dry tissue levels that reduced growth in different plants grown in soil contaminated with arsenate. The EC_{50} was 43.8 mg/kg in radish, 10.0 mg/kg in spinach, 4.5 mg/kg for tomato, 3.7 mg/kg for green beans, 3.4 mg/kg for cabbage, and 1.7 mg/kg for lima beans (Woolson 1973).

2.6.2. Animal and Human Studies

Studies examining the toxicity of arsenic in contaminated soils using flies, *Drosophila melanogaster*, gave LC_{50} values of 0.54 mM for arsenite and 0.79 mM for

arsenate (Goldstein and Babich 1989). Stoneflies, *Pteronarcys dorsata*, and snails, *Helisoma campanulata* and *Stagnicola emarginata*, exposed to 89 µg arsenate/L accumulated 12 µg As/g, 8.8 µg As/g and 8.2 µg As/g, respectively (Spehar et al. 1980).

2.7. Earthworms and the Species *Eisenia andrei*

Earthworms can be used to assess the risks to environmental and public health arising from contaminated soils and waste sites. Laboratory bioassays can be used when it is not possible to find sufficient native worms in the contaminated soil (Van Gestel 1997). Earthworms (phylum *Annelida*, class *Oligochaeta*) can be cultured and used in chronic exposure tests to measure effects of contaminants on growth and reproduction. A standardised test is available to assess the sublethal toxic effect on *Eisenia andrei* (*E. andrei*) which gives reproducible results (ASTM 1998, ISO 1998). Body weight change yields information regarding sublethal effects, however, changes in reproduction parameters may be more sensitive (Neuhauser and Callahan 1990, Van Gestel 1992).

Some researchers consider the *Eisenia fetida* complex to consist of two subspecies, *Eisenia fetida fetida* and *Eisenia fetida andrei*, while others consider the complex to consist of two separate species, *Eisenia fetida* and *Eisenia andrei* (ASTM 1998). The latter set of designations will be used throughout this thesis. The species *Eisenia fetida* (*E. fetida*) has a high genetic variability because it is a species complex (Bouché 1992). Hence, this may lead to variability in the toxicity test results. It has been recommended that *E. andrei* replace *E. fetida* for testing because the former has a lower

genetic variability (Bouché 1992). *E. andrei* is an epigeic species that naturally lives in soil of very high organic matter, such as composts or manure piles. In general, the species is neither less nor more sensitive than indigenous earthworms and is therefore considered as a representative test species (Kula and Larink 1998).

E. andrei is selected primarily because: 1) it is robust and can be cultured at high densities in the laboratory; 2) it has a short generation time allowing for full life-cycle studies; 3) there are several juveniles per cocoon to give adequate reproduction parameters 4) it is genetically homogenous; and 5) there exists extensive knowledge on the toxicity and bioaccumulation of various chemicals to this species (Bouché 1992, Kokta 1992, Kula 1994, Gibbs et al. 1996, Kula and Larink 1998, Spurgeon and Weeks 1998).

The life cycle for *E. fetida* has a mean of about 51.5 days at 25°C, which refers to the timeframe from deposition of a cocoon by clitellated worms to deposition of the next generation of cocoons (Tomlin and Miller 1980). *E. fetida* has a maximum life expectancy of 4 to 5 years, although between 1 to 2 years is more common (Reynolds 1977). The life cycle of *E. andrei* can be divided into three phases: 1) the cocoon phase, 2) the young phase, and 3) the adult phase (Jeffries and Audsley 1988). The cocoon phase consists of an egg cocoon that can produce juveniles. The young phase is that in which the juveniles grow but cannot produce cocoons. The adult phase is that in which the worms have a fully developed clitellum and are capable of producing cocoons (Jeffries and Audsley 1988).

Earthworms are more susceptible to heavy metal pollution than many other soil invertebrates (Spurgeon and Hopkin 1996a). Earthworms take up xenobiotics through the skin and through the gut after ingestion (Viswanathan 1994). These invertebrates also tend to concentrate certain heavy metals in their body tissue (Reinecke 1992). The concentration of a metal in earthworm tissues is determined by the metal concentration in the soil, the rate of bioaccumulation, and the tolerance of the earthworm to the metal (Ma 1982). Studies have shown that high concentrations of cadmium, copper, lead, and zinc in soils affect the population density, viability, cocoon production, growth and sexual development of earthworms (Spurgeon and Hopkin 1996a, Reinecke and Reinecke 1998). Toxicity tests have shown that there is an increased sensitivity for juveniles, indicating that differences in sensitivity may exist between life stages (Spurgeon and Hopkin 1996b, Spurgeon and Weeks 1998).

Sublethal and lethal effects on the earthworm *E. fetida* have been found when they are exposed to arsenic, in the form of potassium arsenate (KH_2AsO_4). *E. fetida* tolerated 87 mg arsenate/kg dry soil without lethal effect, but juvenile mass gain and adult cocoon production were decreased significantly (Fischer and Koszorus 1992). A maximum sublethal concentration of 902 µg As/g tissue dry weight accumulated in the earthworms (Fischer and Koszorus 1992). No decrease in arsenic content within the tissue was observed after an 8-week period (Fischer and Koszorus 1992).

Chapter 3. Reproductive Toxicity and Bioavailability of Arsenic in Contaminated Artificial and Natural Soils using the Earthworm (Manuscript)

3.1. Title Page

Reproductive Toxicity and Bioavailability of Arsenic in Contaminated Artificial and Natural Soils using the Earthworm

Stephen W. Wong,[†,§]

Laurie H.M. Chan,[‡,†]

Geoffrey I. Sunahara*,[§]

[†] Department of Natural Resource Sciences, Macdonald Campus of McGill University, 21,111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada, H9X 3V9

[§] Applied Ecotoxicology Group, Biotechnology Research Institute, 6100 Royalmount Avenue, Montreal, Quebec, Canada, H4P 2R2

[‡] Center for Indigenous Peoples' Nutrition and Environment, McGill University, 21,111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec, Canada, H9X 3V9

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*Corresponding author e-mail: geoffrey.sunahara@nrc.ca; phone: (514) 496-8030; fax: (514) 496-6265

3.2. Abstract

Anthropogenic activities such as gold mining and milling have contributed to soil contamination by arsenic, a known toxicant. The earthworm reproduction test for the species *Eisenia andrei* (*E. andrei*) was used to examine the bioavailability and toxicity of arsenic-contaminated soil. Results indicate that arsenic is toxic to earthworms as evidenced by decreased survival, growth, and reproductive capacity. These findings indicate that arsenic-spiked artificial soil was more toxic than spiked field soil. Despite its high soil arsenic concentration, mine soil was less toxic than the spiked field soil used in this study. Uptake of arsenic into *E. andrei* tissue was higher in earthworms exposed to spiked artificial soil than those exposed to spiked field soil, leading to a maximal accumulation of 396 μg/g dry tissue. Based on tissue arsenic content, effects on adult mortality and juvenile survival are less influenced by the differences in soil characteristics than on the reproductive responses such as cocoon production. Therefore, soil contamination by arsenic is toxic as shown by its effects on *E. andrei* using different types of arsenic-containing soils; however, the magnitude of its toxicity may be better reflected by tissue rather than soil content.

3.3. Introduction

Gold mining and smelting operations around Yellowknife (Northwest Territories, Canada) generate soil contamination by arsenic (As) that presents a risk to ecological health. Gold ore contains arsenopyrite (FeAsS). Roasting of the ore releases arsenic trioxide, which reacts with basic oxides in air to form arsenates that deposit onto the soil *(1, 2)*. Large mine tailing deposits from the gold extraction process, which are rich in arsenic, are often left to be weathered, releasing arsenic into the soil *(2, 3)*.

Arsenic, a known toxicant, is a metalloid but is often grouped as a metal. It occurs in +5, +3, 0 and –3 oxidation states. The toxicity of As varies widely, depending on the valencies involved. Generally, trivalent arsenic compounds are more toxic than pentavalent forms, and inorganic arsenic compounds are more toxic than organoarsenicals *(3)*. The reaction of arsenic compounds with sulfhydryl groups of proteins and enzymes are believed to be responsible for its toxicity to animals *(4)*.

Arsenic speciation in soil is dependent on soil pH and redox potential *(5, 6)*. In aerobic soils, the predominant form of arsenic is the pentavalent species arsenate (AsO_4^{-3}) whereas in anaerobic soil, the predominant form is the trivalent species arsenite (AsO_2^-) *(2, 3)*. Organic forms of arsenic do not accumulate in soil *(7, 8)*. Arsenic availability in soils is determined by adsorption-desorption mechanisms, which are controlled by factors such as soil pH, the amount and type of clay and iron oxides *(5)*. However, it is only the mobile and bioavailable fraction of a toxic metal that causes toxicity and leads to an

environmental risk *(7)*. Arsenic also forms negatively charged oxyanions that coprecipitate and adsorb onto clay particles or that coprecipitate as metal-ion precipitates *(3, 6)*. These complexes are very stable and insoluble in water, which accounts for the extended residence time of arsenic (approximately 2,400 years) in soil *(3)*. During soil aging, arsenic that is initially adsorbed on the clay surfaces can diffuse into internal pores of the clay aggregates leading to a decrease in available arsenic *(9, 10)*.

Earthworms (phylum *Annelida*, class *Oligochaeta*) are soil dwelling organisms that can be used to determine the ecological risks associated with contaminated soils. A standardized test is available to assess the sublethal effects on *Eisenia andrei* (*E. andrei*) *(11)*. This test allows for interlaboratory comparisons of the effects of toxic chemicals on earthworm survival, growth and reproductive capacity. Effects on growth and reproduction are thought to be more sensitive to toxicants than survival *(12)*.

The rate of bioaccumulation, or the accumulation of chemicals into the tissue of living organisms, depends on abiotic factors, such as soil characteristics, and biotic factors, such as routes of uptake *(13)*. For earthworms, this soil ingestion and dermal uptake are two ways that contaminants can bioaccumulate in their tissues *(14, 15)*. Chemical toxicity ensues after the tissue concentration (internal dose) surpasses a certain toxic threshold. Toxicity may be better predicted from the tissue concentration in an organism rather than from the concentration in its surrounding environment since the former accounts for bioavailability *(16, 17)*.

Currently, there is little information regarding the sublethal toxicity of arsenic-contaminated soil on soil invertebrates. Fischer and Koszorus *(18)* studied the lethal and sublethal effects of arsenic on the earthworm *Eisenia fetida* (*E. fetida*) in a vermicomposting system. *E. fetida* survived 50 mg As/kg dry soil without lethal effects, but relative mass gain and adult cocoon production were decreased at 87 mg As/kg dry soil *(18)*. These authors did not use the standardized earthworm toxicity test so it is difficult to compare their data with those reported in the literature. In addition, the test soil was not adequately described in their study and it contained a high organic matter content, so it is difficult to relate their results to any naturally occurring soil types.

In the present study, the standardized *E. andrei* test was used to examine the toxicity of arsenic-contaminated artificial, field, and mine soils. Total arsenic concentrations in soil were measured and correlated with arsenic bioaccumulation in the adult earthworms. This study compares the toxicological effects of arsenic with its bioaccumulation in earthworms.

3.4. Experimental Section

3.4.1. Chemicals and Reagents

Potassium arsenate (KH_2AsO_4; CAS 7784-41-0) was obtained from Sigma Chemical Company. The pesticide 2-chloroacetamide ($ClCH_2CONH_2$; CAS 79-07-2), used as a reference toxicant, was of the highest purity available (98%), and obtained from

Aldrich Chemical Company. Other chemicals were of the highest purity available and were obtained from commercial suppliers. Deionized water (ASTM, type II) was obtained using the Zenopure® Mega-90 (Zenon Environmental, Burlington, ON, Canada) or Nanopure D4741 (Barnstead/Thermolyne, Dubuque, IA, USA) water purification systems and was used throughout the study. Glassware was rinsed with distilled water, washed with a phosphate-free detergent, followed by rinses with acetone, 10% (v/v) nitric acid or 20% (v/v) hydrochloric acid, and deionized water.

3.4.2. Soil

Artificial soil was prepared according to the Organization for Economic Cooperation and Development (OECD) method *(19)* and contained 70% (w/w) grade 4010 silica sand (Unimin Canada, Jérôme, ON, Canada), 20% (w/w) colloidal kaolinite clay (CAS 1332-58-7) and 10% 2-mm screened Canadian sphagnum peat moss. Each ingredient was obtained from local suppliers. Calcium carbonate (1%, w/w) was used to adjust the pH of the wetted substrate to 6.0 ± 0.5. The field soil was sampled approximately 25 km northwest of Yellowknife (Northwest Territories, Canada) and the mine soil was taken from near the Miramar Con Mine (N 62°25.781' W 114°24.652') located near Yellowknife. The field and mine soil samples were homogenized by passing through a 2-mm mesh sieve.

3.4.3. Soil Characteristics

Soil pH values were measured with a Corning 320 pH meter using a 1:10 (v/v) suspension of soil in water *(20)*. The soil redox potential (E_h) was determined using a Fischer Scientific Accumet Ar15 pH/redox meter. E_h values were measured after 5 min of contact with the soil, ensuring that the change in E_h was also less than 5 mV/min *(21, 22)*. The soil cation exchange capacity was measured in triplicate for each soil type, using the method of Hendershot et al. *(23)*. The soil particle size distribution was determined using the method developed by Sheldrick and Wang *(24)*. The organic matter content of the soils was determined in triplicate for each soil type, according to Tissen and Moir *(25)*. The water content of the soil was determined gravimetrically by drying 5–10 g of soil for 18 h at 103 ± 2 °C *(26)*. The water holding capacity was determined after adding 50 mL of water to approximately 10 g of soil in a Whatman No. 1 (Whatman International, Maidstone, England) filter paper-lined funnel and allowing the water to drain for 3 h *(27)*. Between 5–10 g of the wetted soil was dried for 18 h at 103 ± 2 °C. The water-holding capacity was calculated from the difference in weights.

3.4.4. Earthworm Reproduction Test

Eisenia andrei, obtained from Carolina Biological Supply (Burlington, NC, USA), were initially used to establish the laboratory cultures. Earthworms were maintained in earthworm bedding (Magic Products, Amherst Junction, WI) at 20±1 °C,

70–80% water content, with a 16:8 light/dark cycle, and were fed dry cereal (Magic Worm Food, Magic Products).

The earthworm reproduction test using *E. andrei* was carried out according to the International Organization for Standardization (ISO) method *(11)*. A range of 2-chloroacetamide concentrations (from 2 to 35 mg/kg dry soil) was used for quality assurance purposes (positive control). Artificial and field soils were spiked with potassium arsenate at nominal concentrations ranging from 10 to 1000 mg/kg dry soil (corresponding with 4.2 to 416.2 mg As/kg dry soil). At the concentrations tested in these soil spiking experiments, potassium, as potassium chloride (KCl), is not toxic to *E. andrei* (Robidoux, P.Y. unpublished data). Four replicates were tested for each concentration with ten adult earthworms per replicate. Due to the limited quantity available, the mine soil sample was only tested without dilution. About 500 g dry soil was moistened to 50% of its water-holding capacity with the test compound dissolved in water. For the negative controls (uncontaminated artificial and field soils) and contaminated mine soil, water was used for hydration. Soil subsamples were conserved in plastic tubes at –20 °C for chemical analysis.

Earthworms were fed weekly by adding 1-2 g of dry cereal to the soil surface. Survival and growth of mature adult earthworms were determined after 28 d exposure. Surviving *E. andrei* were allowed to depurate their gut contents on moistened filter paper for 24 h, rinsed with water, and stored at –20 °C for chemical analysis *(28)*. The

following measurements were taken on day 56 of the test: cocoon production (the number of hatched and unhatched cocoons), and the number of juveniles and their biomass.

The effects of 2-chloroacetamide on adult mortality, growth, and reproduction were verified with laboratory in-house control data. The observed NOEC and the LOEC were 25 mg/kg dry soil and 35 mg/kg dry soil (nominal concentrations) respectively, and were consistent with reported values for reproductive effects *(29-31)*.

3.4.5. Determination of Total Arsenic in Soil and Adult Earthworms

Subsamples of soil for each replicate test unit were dried at 60 °C under vacuum (15- 25 psi) for 48 h. Dry soil (0.5 g) was added to a Teflon digestion vessel along with 9 mL of concentrated nitric acid (trace metal grade HNO_3) and 3 mL of concentrated hydrochloric acid (trace metal grade HCl). The digestion was carried out following the United States Environmental Protection Agency (USEPA) Method 3052 *(32)* in an ETHOS-Plus microwave system. After cooling, the contents of each vessel were diluted to 50 mL with water and stored in plastic vials until analyzed for total arsenic.

From each replicate test unit, two to five (depending on the number of surviving earthworms) adult *E. andrei* were randomly selected for arsenic analysis. Earthworms were placed in a glass boiling tube and dried at 60 °C under vacuum (15- 25 psi) for 48 h. Tissue was digested using 4 mL of concentrated nitric acid (TMG) at room temperature (20 ± 2°C) for 16-20 h, then heated at approximately 110 °C for 4-6 h *(33, 34)*. After

cooling, the contents from each tube was diluted to 12.5 mL with water and stored in plastic vials until analyzed for total As.

The arsenic content of soil and earthworm tissue digests was determined using a graphite furnace atomic absorption spectrophotometry (Hitatchi Z8200 Polarized Zeeman Atomic Absorption Spectrophotometer). Calibration standards from 5 to 100 μg As/kg in 12.6% HNO_3 and 2.2% HCl were used for the soil digest studies, and from 2.5 to 50 μg As/kg in 22.4% HNO_3 were used for the earthworm tissue digest studies. Data was collected using the software provided by the manufacturer.

The second highest calibration standard was reanalyzed after every ten samples and the calibration curve was adjusted accordingly. The R-value for the calibration curve was > 0.995. Each sample was measured in duplicate and was reanalyzed if the relative standard deviation (RSD) was greater than 15%. Standard reference materials were used to determine arsenic recovery during the digestion process. Dogfish muscle (DORM-2; National Research Council of Canada; Ottawa, Canada) and oyster tissue (OT; National Institute of Standards and Technology; Gaithersburg, Maryland) were used for the earthworm tissue analyses (recoveries from 61.6 to 98.7%) and marine sediment (MESS-2; National Research Council of Canada; Ottawa, Canada) was used for the soil analyses (recoveries from 57.7 to 82.9%).

3.4.6. Statistical Analysis

For the earthworm reproduction test, results of the treatment groups were statistically compared to their respective negative control. The toxicity point estimates (lethal concentration at which there is 50% mortality - LC_{50}, effective concentration at which there is a 50% reduction - EC_{50}), no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were determined using the ToxCalc program (version 5.0; Tidepool Scientific Software, McKinleyville, CA, USA). Shapiro-Wilk's test was used to assess the normality of distribution. Bartlett's test was used to determine equality of variances. Statistical methods for point estimates included maximum likelihood regression, Spearman-Karber methods and linear interpolation with bootstrapping. Wilcoxon's two-sample test, Dunnett's multiple comparison test and Steel's many-one rank test were used to determine the NOEC and LOEC ($P \leq 0.05$).

The SPSS program (version 8.0; SPSS Inc., Chicago, IL, USA) was used for statistical analysis of the arsenic tissue concentrations. Shapiro-Wilk's test was used to assess the normality of distribution. Equality of variances was tested using the Levene statistic. The Games-Howell test was used to determine the statistical significance for multiple comparisons ($P \leq 0.05$).

3.5. Results and Discussion

3.5.1. Soil Characterization

Selected properties of each of the soil types are summarized in Table 1. The most significant factors determining arsenic speciation in soil are the pH and redox potential values *(5, 6)*. Arsenate is the dominant arsenic species ($\geq$ 50%) that would exist in each of the soil samples tested based on their pH and E_h (Figure 1), which was why the soils were spiked with arsenate. Masscheleyn et al. *(5)* found that for contaminated field soils with soil redox levels of 200 and 500 mV and pH between 5.0 and 8.0, 65-98% of the arsenic exists as arsenate. Most of the soluble arsenic in soils (> 95%) is in the arsenate form.

Mine soil has a higher total arsenic content than artificial and field soils spiked with arsenic (Tables 2 & 3).

3.5.2. Arsenic Tissue Concentration in Adult Earthworms

The arsenic tissue concentration in *E. andrei* that were exposed to arsenic-spiked artificial and field soil samples for 4 weeks, increased as a function of arsenic soil concentration (Figure 2). The concentration of a metal in earthworm tissue depends on the metal concentration in soil, the rate of bioaccumulation, and the tolerance of the earthworm to the element *(35)*. It seems likely that the As levels would be at steady state

in the earthworms used in our study. According to Neuhauser et al. *(13)*, an exposure period of a few weeks is sufficient for metal concentrations to reach equilibrium in earthworms. Meharg et al. *(36)* found that arsenic residues are homeostatically maintained in living *Lumbricus terrestris* (*L. terrestris*). In this study, tissue data suggests that arsenic is more bioavailable in the artificial soil than in the field soil. For earthworms in the spiked artificial soil, the maximal bioaccumulation of arsenic occurred at 22.3 ± 3.7 mg As/kg dry soil, whereas in the spiked field soil, the maximal bioaccumulation occurred at 65.6 ± 9.1 mg As/kg dry soil (Figure 2). The maximal bioaccumulation in the spiked-artificial soil was not statistically different from that for earthworms in the spiked-field soil, giving an average maximal bioaccumulation of 396 μg As/g tissue dry weight (Figure 2). Tissue levels of arsenic in worms exposed to the mine soil sample were within the range of this value considering the biological variability. The maximum bioaccumulation value reported in this article differs with those of other reports. Fischer and Koszorus *(18)* found a maximum accumulation of 902 μg As/g dry weight for arsenic in *E. fetida*, whereas Meharg et al. (*36*) found tissue levels of 120 μg As/g in living *L. terrestris*. Data presented here suggests that the accumulation of arsenic in the tissue may depend on a number of factors including the type of earthworm species and exposure matrix used for the study.

Bioaccumulation of arsenic in earthworms may be due to the sequestration of arsenic in tissues in forms that cannot be readily eliminated *(18,36)*. Fischer and Koszorus *(18)* found no elimination of arsenic after transfer to a clean substrate for 8 weeks. Spurgeon and Hopkin *(37)* postulated three pathways of elimination from

earthworm tissue including: excretion from the body; binding to inorganic granules and attachment to proteins. For metals that are detoxified by excretion, body concentrations should decrease when exposed worms are transferred to a clean environment, as seen for essential metals like copper and zinc *(37)*. For metals that are bound to an inorganic matrix or organic ligand, metal levels should remain constant even after exposure has stopped, as is found for xenobiotic metals such as cadmium and lead *(37)*. Morgan et al. *(38)* found that arsenic is bound to sulfur-rich enzymes in earthworm tissue. Regardless of whether As is taken up as arsenate or arsenite, it was distributed in the discrete chloragocytic compartment according to the sulphydryl-attaching trivalent form *(38)*. The major arsenic compounds found in the extracts of *Lumbricidae* earthworms were arsenous acid ($As(OH)_3$) and arsenic acid ($OAs(OH)_3$) *(39)*. It is plausible that the latter compounds are also present in *E. andrei* suggesting that these may be the forms of arsenic that exert toxic effects in the earthworm. To test whether the soil-specific bioaccumulation of arsenic in *E. andrei* is associated with toxicological effects, survival, growth, and reproduction tests were conducted.

3.5.3. Toxic Responses of Earthworms to Soil Contaminated with Arsenic

The effects of arsenic-spiked artificial and field soils, and mine soil on the reproduction and development of *E. andrei* were assessed using the earthworm reproduction test. Arsenic, in the form of potassium arsenate, significantly decreased *E. andrei* survival, growth, and reproduction compared to the negative control groups in both artificial and field soil tests (Tables 2, 3). In addition, arsenic decreased the number

of cocoons and number of juveniles at lower soil concentrations than those decreasing adult survival and growth, as indicated by the lower LOEC and toxicity endpoints (LC_{50} and EC_{50}) (Table 4). Fischer and Koszorus *(18)* observed significant effects on relative worm mass and number of cocoons per worm at 87 mg As/kg dry soil. The latter value is higher than the results obtained in the present study (reproduction LOEC using artificial soil = 22.3 ± 3.7 mg As/kg dry soil, LOEC for adult growth = 34.2 ± 1.5 mg As/kg dry soil). Hence, our results indicate that arsenic is more toxic in artificial soil than in the vermicomposting soil used by Fischer and Koszorus *(18)*.

Among the reproduction endpoints, the total number of cocoons produced seems to be the most sensitive response to arsenic (Tables 2, 3). Interestingly, the effect of arsenic on earthworm reproduction occurred at concentrations where bioaccumulation reached a maximal value (artificial: 22.3 ± 3.7 mg As/kg dry soil, field: 65.6 ± 9.1 mg As/kg dry soil). This data indicates that the toxic effects of arsenic on earthworm reproduction are occurring at concentrations that approximate the maximal bioaccumulation (i.e., a toxicity threshold at which the uptake and elimination mechanisms become saturated and homeostasis is compromised). This arsenic-mediated decrease in reproduction may be due to detrimental effects on earthworm reproductive processes such as oogenesis, spermatogenesis, sperm counts, sperm viability, or cocoon production *(29, 40, 41)*. It is also known that xenobiotic metals induce stress *(42, 43)*. Stress-resisting mechanisms, such as avoidance, exclusion, removal or complexation, consume much energy in the earthworm *(42, 43)*. Therefore, the amount of energy required to maintain normal physiological processes such as somatic growth or

reproduction, may be reduced when earthworms are exposed to xenobiotics *(42, 43)*. Further research needs to be done to investigate these possibilities.

Literature provides little evidence that organic matter can bind arsenic since As anions cannot accumulate on the predominantly negative surfaces of organic matter *(44)*. When comparing the clay content in artificial soil (16.9 %) with that in field soil (43.0 %) (Table 1), one would expect arsenic in field soil to be less bioavailable, and less toxic. Our results support this hypothesis since the toxicity of the arsenate-spiked artificial soil was lower than that for arsenate-spiked field soil (Table 4). Spurgeon and Weeks *(45)* concluded that soil properties strongly modulate zinc toxicity along with aging time of the contaminant; however, biotic factors such as the test species and its life stage, as well as certain environmental parameters (such as temperature) were of minor significance. The soil factors known to affect arsenic bioavailability include: oxidation-reduction; adsorption-desorption; and organic and biochemical methylation *(6, 46)*. For the mine soil samples, there was a significant decrease in earthworm survival, growth, and reproduction compared to the field soil negative control (Table 3). Mine soil contained a greater amount of total measured arsenic compared to those of the spiked artificial and field soil samples (Tables 2, 3). However, the exposure effects of mine soil samples on earthworm survival, growth and reproduction are not as severe as in some of the spiked soils having less total measured arsenic content (Tables 2, 3). This is likely due to the effect of soil aging on arsenic bioavailability *(9, 10, 17)*. Soil aging is an important factor because the toxicity of soil-borne toxicants declines as these chemicals become

increasingly sequestered with time *(47)*. Decreased toxicity following aging in soil was found for other metals such as zinc *(45, 48)*.

A goodness of fit (R^2) of the data using a second order polynomial equation was carried out at different arsenic tissue concentrations in earthworms for adult survival (Figure 3A), growth (Figure 3B), total number of cocoons (Figure 4A), number of hatched cocoons (Figure 4B), and number of juveniles (Figure 4C). For the reproduction parameters (Figure 4), the R^2 was ≥ 0.96 for both artificial- and field-spiked soils. Interestingly, despite the high arsenic concentration of arsenic in mine soil, the tissue concentration-response of the mine soil closely resembled to those of the other two soils (Figures 4A-C). These data support the possibility that a cause-effect relation exists between arsenic body concentrations in adult *E. andrei* and the observed toxic effects on their reproduction. A similar observation was made by Lock and Janssen *(17)* using other heavy metals. They found that cocoon production for *E. fetida* decreased with increasing internal cadmium concentrations. However, cocoon production was not related to the internal zinc concentration of *E. fetida* as the earthworm was able to regulate the internal zinc concentration within a narrow range *(17)*.

Since the fitted curves for adult survival and growth for both spiked-artificial and field soil were almost identical (Figures 3A, 3B), it appears that these two responses are related to arsenic tissue content and are independent of the soil matrix. However, other factors (such as soil type) in addition to tissue concentration, may be determining the effects of As on certain reproduction responses. If one considers the tissue concentration

of arsenic, and the total number of cocoons (Figure 4A) or the number of hatched cocoons (Figure 4B), the exposure to As in spiked field soil appears to be more toxic than artificial soil. These data indicate that arsenic toxicity is greatly over-estimated if one considers only the total arsenic concentrations in soil (Table 4). A possible reason for the higher toxicity of arsenic-spiked field soil relative to arsenic-spiked artificial soil may be due to the contribution of other contaminants which may lead to a synergistic effect. Van Gestel et al. *(49)* have shown that high pH ($\geq$ 7.0) causes a significant reduction in cocoon production of *E. andrei* in artificial soil. However, in the present study, there is no difference between the total number of cocoons produced in the artificial (pH 6.2) and the field soil (pH 7.9) control groups. Therefore, pH does not directly impact the number of cocoons produced, although synergy between pH and As exposure can not be presently excluded. It is normally viewed that a decrease in the number of cocoons leads to a decrease in number of juveniles. However, the decreased number of juveniles (Figure 4C) caused by arsenic exposure can be explained by the decreased survival of the adult earthworms (Figure 3A). Normalizing the NOEC, LOEC, and LC_{50} or EC_{50} for arsenic tissue content (Table 5) indicates that arsenate is more toxic in spiked field soil than in spiked artificial soil. Although tissue concentration of arsenic was considered in the interpretation of our toxicological data, it should be emphasized that the total tissue content may not necessarily reflect the amount of metal that is available to disrupt metabolic pathways *(43)*.

Soils contaminated with arsenic, like other heavy metals such as Zn and Cd, represent environmental hazards as demonstrated by the sublethal toxic effects on *E.*

andrei reproduction. However, the extent of arsenic toxicity depends on biotic factors such as the species at risk and arsenic tissue content, as well as abiotic considerations such as soil pH, redox potential, clay content, and aging. The results presented in this article suggest a possible relation between maximal arsenic bioaccumulation in *E. andrei* and the impact on reproduction (number of cocoons, number of hatched cocoons, and number of juveniles).

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3.8. Tables and Figures

Table 1. Soil properties of the artificial, field and mine soil samples.

Soil Property	Artificial Soil	Field Soil	Mine Soil
Water Content (% ± SD)[a]	1.3 ± 0.1	9.6 ± 0.3	20.5 ± 0.9
Water Holding Capacity (% ± SD)[a]	58.3 ± 7.4	27.4 ± 4.0	71.1 ± 4.9
pH[a]	6.2 ± 0.2	7.9 ± 0.1	8.3 ± 0.1
Redox (mV ± SD)[b]	233.9 ± 8.3	336.4 ± 5.6	356.3 ± 0.8
Cation Exchange Capacity (cmol/mg ± SD)[a]	13.6 ± 0.6	20.2 ± 0.2	34.3 ± 0.5
Organic Matter Content (% ± SD)[a]	4.5 ± 0.8	2.1 ± 0.2	7.3 ± 0.3
Particle Size Distribution	68.4 % sand 16.9 % clay 14.7 % silt	18.0 % sand 43.0 % clay 39.0 % silt	24.8 % sand 16.3 % clay 58.9 % silt
Texture	sandy loam	silty clay	silt loam

[a] n = 3 replicates; SD = standard deviation.

[b] n = 2 replicates; SD = standard deviation.

Table 2. Responses of earthworm (*E. andrei*) exposed to potassium arsenate-spiked artificial soil samples[a]

Parameter	Nominal soil arsenic concentrations (mg/kg dry soil)							
	Negative control	4.2	10.4	20.8	41.6	104.0	208.1	416.2
Measured arsenic concentration (mg As/kg dry soil ± SD)	0.4 ± 0.0	3.5 ± 0.2	6.5 ± 0.5	22.3 ± 3.7	34.2 ± 1.5	94.5 ± 4.9	196.4 ± 6.9	364.8 ± 43.9
Adult survival / replicate (% ± SD; 28 d)	100	100	100	92.5 ± 5.0	80 ± 8.2[c]	87.5 ± 9.6	80.0 ± 8.2[c]	45.0 ± 33.2[c]
Adult growth / worm[b] (mg ± SD; 28 d)	74.7 ± 58.4	101.8 ± 40.3	108.5 ± 75.6	-5.7 ± 38.7	-70.7 ± 45.1[c]	-116.0 ± 30.2[c]	-85.1 ± 46.2[c]	-176.6 ± 225.3
[% change ± SD]	14.4 ± 11.2	19.9 ± 7.9	21.6 ± 14.9	-0.9 ± 7.1	-13.6 ± 8.5	-21.8 ± 4.3	-17.6 ± 8.3	-34.5 ± 46.7
No. cocoons / replicate (± SD; 56 d)	70.3 ± 5.1	66.5 ± 13.8	69.3 ± 6.3	35.0 ± 10.1[c]	8.8 ± 4.7[c]	3.3 ± 2.1[c]	1.0 ± 1.2[c]	0[c]
No. hatched cocoons / replicate (± SD; 56 d)	64.0 ± 8.0	62.5 ± 14.3	62.5 ± 5.7	30.5 ± 9.6[c]	5.8 ± 4.4[c]	0[c]	0[c]	0[c]
Hatchability (% ± SD, 56 d)	90.8 ± 5.6	93.6 ± 5.1	90.4 ± 4.5	86.7 ± 6.9	60.4 ± 22.9[d]	0[d]	0[d]	-
No. of juveniles / replicate (± SD; 56 d)	154.0 ± 52.5	131.8 ± 41.2	141.0 ± 26.0	60.5 ± 19.7	10.5 ± 9.3[c]	0[c]	0[c]	0[c]
Biomass of juveniles / replicate (mg ± SD; 56 d)	579.6 ± 333.0	804.3 ± 221.8	643.1 ± 122.4	351.8 ± 66.4	8.0 ± 3.3[d]	-	-	-
Biomass / juvenile (mg ± SD; 56 d)	3.6 ± 0.7	6.4 ± 1.9	4.8 ± 1.5	6.3 ± 2.3	1.5 ± 1.5	-	-	-

No. juveniles / hatched cocoons (± SD; 56 d)	2.4 ± 0.7	2.1 ± 0.2	2.3 ± 0.5	2.0 ± 0.2	1.7 ± 0.5	-	-	-

[a] n = 4 replicates; rehydrated (50 % water holding capacity) artificial soil, negative control: no contaminant added; SD = standard deviation.

[b] Mean change in body weight during the experiment (average of initial minus average of final body weight).

[c] Significant effect on survival, growth, or reproduction compared to controls ($P \leq 0.05$; Wilcoxon's two sample test).

[d] Significant effect on reproduction compared to controls ($P \leq 0.05$: Steel's many-one rank test)

Table 3. Responses of earthworm (*E. andrei*) exposed to potassium arsenate-spiked field soil and mine soil samples[a]

Parameter	Nominal soil arsenic concentrations (mg/kg dry soil)								
	Neg. Control	4.2	10.4	20.8	41.6	104.0	208.1	416.2	Mine soil
Measured arsenic concentrations (mg As/kg dry soil ± SD)	9.5 ± 0.5	15.1 ± 1.5	18.8 ± 0.5	39.4 ± 2.4	65.6 ± 9.1	109.4 ± 9.6	221.0 ± 15.0	571.8 ± 15.6	2038.7 ± 149.2
Adult survival / replicate (% ± SD; 28 d)	100	100	100	100	97.5 ± 5.0	77.5 ± 17.1	55.0 ± 20.8[c]	57.5 ± 32.0[c]	72.5 ± 9.6[c]
Adult growth / worm[b]	71.6 ± 67.8	107.7 ± 29.9	99.5 ± 31.8	162.4 ± 48.4	129.1 ± 25.7	-0.6 ± 49.3	-63.3 ± 18.7[c]	-109.6 ± 25.8[c]	-23.9 ± 41.8[c]
(mg ± SD; 28 d)	15.0 ± 14.2	23.1 ± 6.5	23.1 ± 7.6	41.0 ± 10.8	31.1 ± 5.2	0.3 ± 12.4	-14.2 ± 3.1	-22.8 ± 4.5	-4.6 ± 9.2
[% change ± SD]									
No. cocoons / replicate (± SD; 56 d)	68.8 ± 11.6	60.3 ± 13.9	56.3 ± 15.2	51.3 ± 28.3	37.3 ± 4.6[c]	1.5 ± 1.9[c]	0[c]	0[c]	9.8 ± 4.3[c]
No. hatched cocoons / replicate (± SD; 56 d)	43.8 ± 16.1	44.8 ± 9.4	37.3 ± 11.1	36.8 ± 21.3	26.0 ± 5.0	0.3 ± 0.5[c]	0[c]	0[c]	6.3 ± 4.3[c]
Hatchability (% ± SD, 56 d)	62.2 ± 17.1	75.3 ± 11.5	68.0 ± 16.4	70.8 ± 2.9	69.5 ± 6.0	12.5 ± 17.7[d]	-	-	58.4 ± 17.4
No. of juveniles / replicate (± SD; 56 d)	199.0 ± 77.8	176.5 ± 29.3	160.3 ± 82.3	130.5 ± 72.2	104.0 ± 16.1	0.5 ± 1.0[c]	0[c]	0[c]	21.5 ± 11.7[c]
Biomass of juveniles / replicate (mg ± SD; 56 d)	1312.4 ± 628.4	939.3 ± 107.3	1120.6 ± 701.6	811.9 ± 483.7	489.7 ± 100.6	2.4 ± 4.7[e]	-	-	79.2 ± 59.4[e]
Biomass / juvenile (mg ± SD; 56 d)	6.3 ± 1.1	5.4 ± 1.1	6.6 ± 1.3	6.3 ± 2.0	4.7 ± 0.6	4.7[d]	-	-	3.3 ± 1.0[e]

No. juveniles / hatched cocoons (± SD; 56 d)	4.5 ± 0.4	4.0 ± 0.7	4.2 ± 1.3	3.7 ± 1.1	4.0 ± 0.5	-	-	-	3.8 ± 0.9

[a] n = 4 replicates; rehydrated (50 % water holding capacity) field soil, negative control: no contaminant added; SD = standard deviation.

[b] Mean change in body weight during the experiment (average of initial minus average of final body weight).

[c] Significant effect on survival, growth, or reproduction compared to controls ($P \leq 0.05$; Wilcoxon's two sample test).

[d] Significant effect on reproduction compared to controls ($P \leq 0.05$; Dunnett's multiple comparison test).

[e] Significant effect on reproduction compared to controls ($P \leq 0.05$: Steel's many-one rank test)

Table 4. Toxicity endpoints of potassium arsenate spiked-artificial and field soils on earthworm reproduction using soil concentrations

Parameter	NOEC (mg As/kg dry soil)		LOEC (mg As/kg dry soil)		LC_{50} or EC_{50}[a] (mg As/kg dry soil)	
	Artificial	Field	Artificial	Field	Artificial	Field
Adult survival / replicate	22.3	109.4	34.2	221.0	322.3[b]	540.1[c]
Adult growth / worm	22.3	109.4	34.2	221.0	15.6[d]	91.7[d]
No. cocoons / replicate	6.5	39.4	22.3	65.6	22.1[d]	69.1[d]
No. hatched cocoons / replicate	6.5	65.6	22.3	109.4	21.5[d]	72.2[d]
Hatchability	22.3	65.6	34.2	109.4	39.0[c]	86.9[b]
No. of juveniles / replicate	6.5	65.6	22.3	109.4	18.9[b]	67.5[d]
Biomass of juveniles / replicate	22.3	65.6	34.2	109.4	22.5[d]	52.1[d]
Biomass / juvenile	-	65.6	-	109.4	-	84.7[d]
No. juveniles / hatched cocoons	-	65.6	-	109.4	-	83.7[d]

[a] LC_{50} for survival or EC_{50} for growth or reproduction

[b] LC_{50} or EC_{50} determined using trimmed Spearman-Karber

[c] LC_{50} determined using maximum likelihood regression (probit analysis)

[d] EC_{50} determined using linear interpolation with bootstrapping

Table 5. Toxicity endpoints of potassium arsenate spiked-artificial and field soils on earthworm reproduction using tissue concentrations in adult earthworms

Parameter	NOEC (µg As/g dry tissue)		LOEC (µg As/g dry tissue)		LC_{50} or EC_{50}[a] (µg As/g dry tissue)	
	Artificial	Field	Artificial	Field	Artificial	Field
Adult survival / replicate	337.5	218.3	396.7	350.6	523.8[b]	719.4[b]
Adult growth / worm	396.7	218.3	403.6	350.6	271.3[c]	290.3[c]
No. cocoons / replicate	177.5	102.7	337.5	218.3	335.7[c]	229.3[c]
No. hatched cocoons / replicate	177.5	218.3	337.5	350.6	330.0[c]	239.3[c]
Hatchability	-	218.3	-	411.8	426.7[d]	396.1[d]
No. of juveniles / replicate	177.5	218.3	337.5	350.6	302.7[c]	224.3[c]
Biomass of juveniles / replicate	337.5	218.3	403.6	411.8	338.6[c]	158.6[c]
Biomass / juvenile	-	218.3	-	411.8	384.0[c]	339.3[c]
No. juveniles / hatched cocoons	-	218.3	-	411.8	>403.6[c]	374.7[c]

[a] LC_{50} for survival or EC_{50} for growth or reproduction

[b] LC_{50} determined using maximum likelihood regression (probit analysis)

[c] EC_{50} determined using linear interpolation with bootstrapping

[d] EC_{50} determined using trimmed Spearman-Karber

3.8.1. Legend to Figures

Figure 1. pH – E_h diagram for arsenic speciation in artificial, field, and mine soil. The illustration represents a predominance-area diagram since the respective areas indicate species that constitute more than 50% of the total composition. Diagram is modified from (*5*).

Figure 2. Arsenic tissue concentrations in adult *E. andrei* after 28 d exposure to potassium arsenate-spiked artificial and field soil, and mine soil. * indicates a statistically significant ($P \leq 0.05$) difference for multiple comparisons using the Games-Howell test.

Figure 3. A) Adult survival and B) growth (28 d exposure) related to adult *E. andrei* arsenic tissue concentrations following 28 d exposure to potassium arsenate-spiked artificial and field soils, and mine soil.

Figure 4. Reproductive parameters (56 d exposure) A) total number of cocoons, B) number of hatched cocoons, and C) number of juveniles, related to adult *E. andrei* arsenic tissue concentrations following 28 d exposure to potassium arsenate-spiked artificial and field soils, and mine soil.

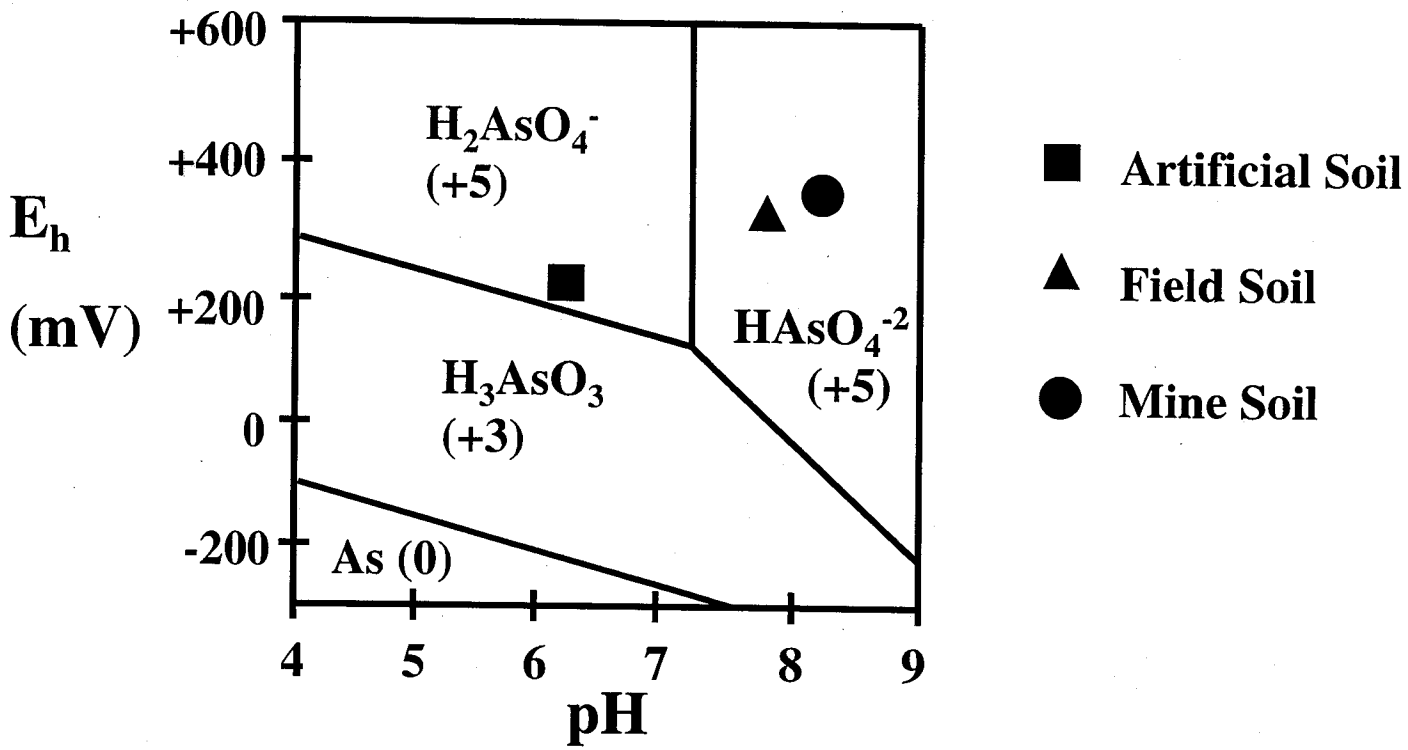

Figure 1.

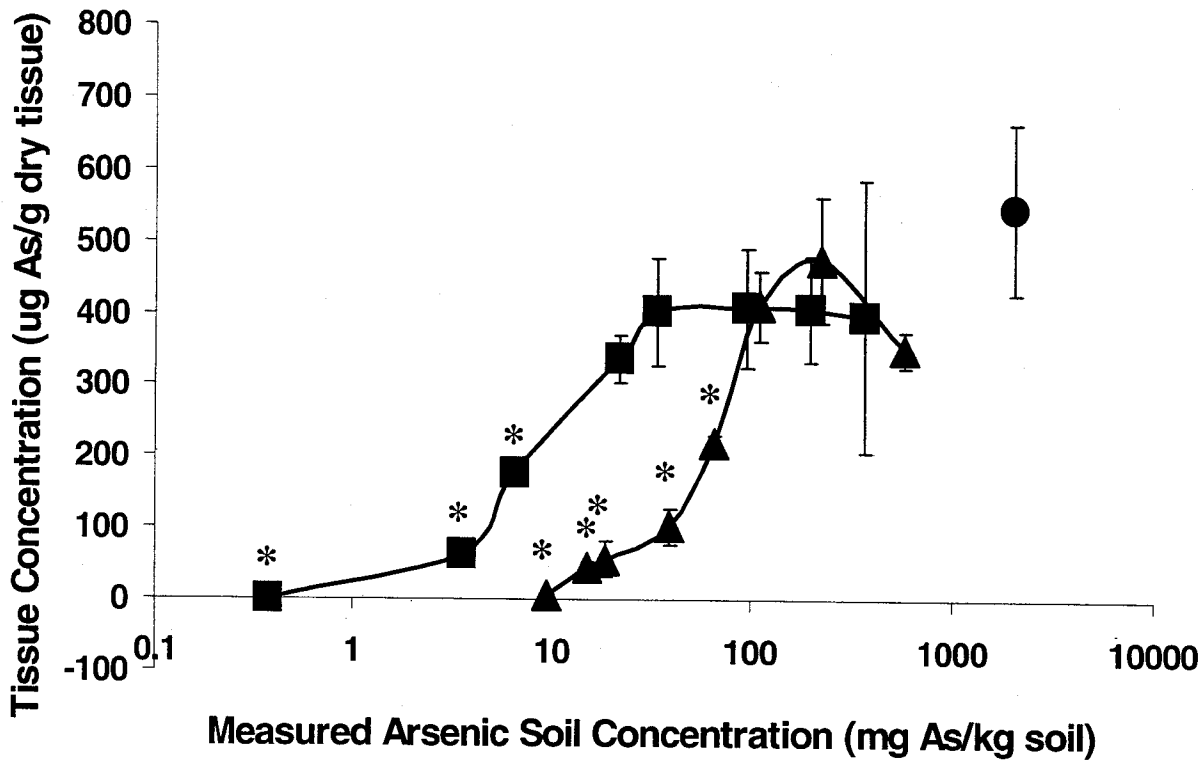

Figure 2.

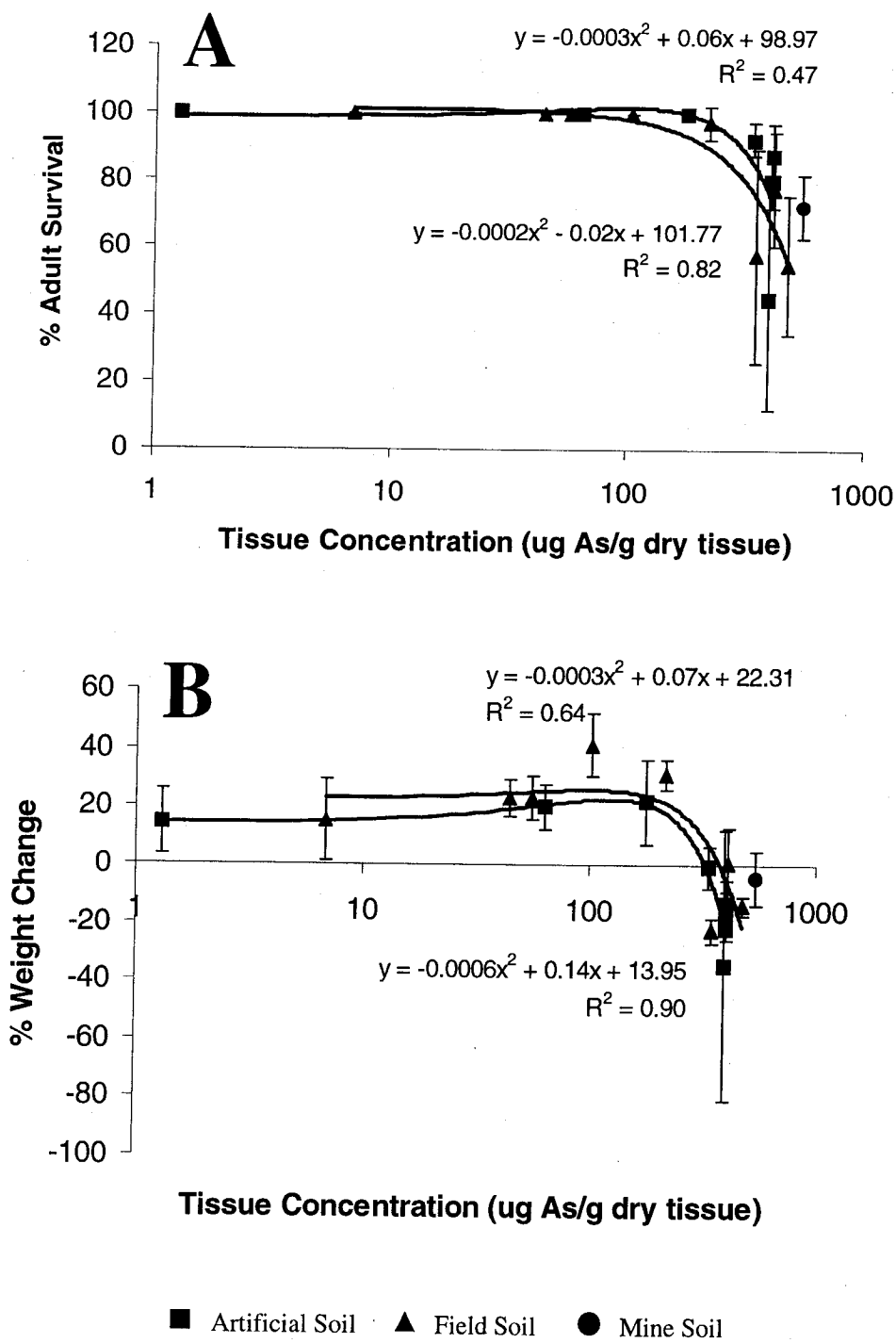

Figure 3.

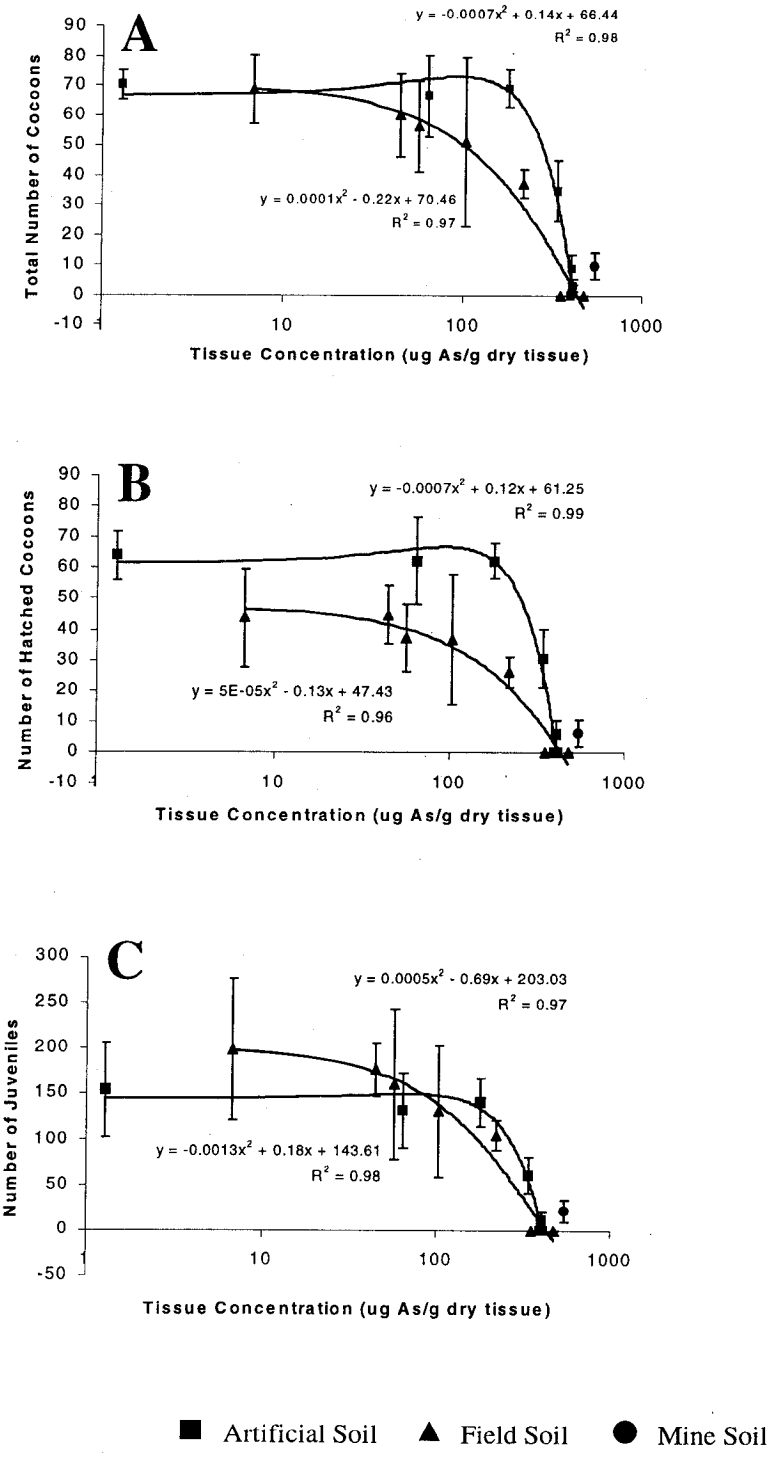

Figure 4.

Chapter 4. Summary and Conclusions

4.1. Summary

Summary of results:

1) arsenic significantly decreased *E. andrei* survival (LOEC artificial soil 34.2 mg As/kg dry soil; field soil 221.0 mg As/kg dry soil)
2) arsenic significantly decreased *E. andrei* adult growth (LOEC artificial soil 34.2 mg As/kg dry soil; field soil 221.0 mg As/kg dry soil)
3) arsenic significantly decreased *E. andrei* reproduction (LOEC artificial soil 22.3 mg As/kg dry soil; field soil 65.6 mg As/kg dry soil)
4) the decrease in total number of cocoons appears to be the most sensitive endpoint
5) arsenic tissue concentration in *E. andrei* is a function of arsenic soil concentration
6) the average maximal bioaccumulation was 396 μg/g tissue dry weight
7) the results suggest a possible relation between maximal arsenic bioaccumulation and reproductive toxicity in *E. andrei*

4.2. Conclusions

The experiments were successful in testing the hypotheses described in section 1.4 of this thesis. Arsenic contaminated soil (whether spiked artificial soil, spiked field soil, or contaminated mine soil from near mine tailings in Yellowknife) lowered the

reproductive capacity of the earthworm *E. andrei*. When looking solely at soil concentrations, arsenic appeared to be more toxic in the artificial soil. In all cases, the reproduction parameters (total number of cocoons, number of hatched cocoons, and number of juveniles) were more sensitive to the effects of arsenic as compared to other endpoints such as adult survival and growth.

In spite of the fact that mortality occurred in the adult earthworms, this acute effect could not directly account for the decrease in reproductive output. The decrease could not be directly attributed to a decrease in cocoon hatchability or the number of juveniles produced per cocoon. However, the results seem to indicate that reproductive toxicity is directly related to the bioaccumulation of arsenic in the tissue of *E. andrei*. Hence, the body concentration of arsenic in the earthworm may be a more appropriate indicator of toxicity.

Future studies should be carried out to improve the experimental design, including the use of aged spiked soils to allow for metals to reach equilibrium in the soil matrix (Lock and Janssen 2001). In addition, it will be necessary to characterise the speciation of the arsenic compounds in the soils since arsenic toxicity and bioavailability are dependent on the oxidation state.

The effects of arsenic on reproductive processes such as spermatogenesis, egg production, and egg laying should be examined in order to elucidate the mechanism of reproductive toxicity of arsenic in the *E. andrei*.

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