Investigations into the Uptake and Effects of Long-term Cadmium and Arsenic Exposure on the Earthworm *Eisenia fetida*

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Abstract

Cadmium (Cd) and arsenic (As) are trace elements that differ in their chemistries but are both highly toxic, and common soil contaminants in agricultural land and contaminated sites. Their potential impacts range from adverse effects on soil-dwelling organisms to uptake into food and leading to human exposures. This study examines the uptake and effects of Cd and As individually and in mixtures primarily on the earthworm *Eisenia fetida* on up to three consecutive generations, and the potential for recovery when exposure ceases. Exposure ranges were selected to minimise mortality and permit reproduction. Key variables examined were contaminant concentrations and associated trace elements in worm tissue, growth, reproduction, and levels of gene expression.

In worms exposed to Cd-spiked soils, Cd accumulation was rapid. Three key factors determining [Cd] in worm tissue were exposure level, time, and the bioconcentration factor (BCF), which increased with decreasing soil [Cd]. Results indicate that for all exposure conditions, and given enough time, Cd accumulation will continue until a lethal tissue level is reached. This point may be either before or after reproduction has occurred (depending on circumstances), but indicates a need to re-examine standardised approaches to toxicity testing for cumulative and biologically persistent contaminants such as Cd. The biological half-life for Cd loss was 6.5 months. This implies that worms that have been exposed to elevated Cd for more than a few weeks would unable to eliminate much of the accumulated burden over their normal lifetimes.

Worms exposed to Cd took longer to reach sexual maturation, and at higher exposures, cocoon production progressively decreased from generation to generation. However, there were differences depending on the exposure level. At the lowest level (30 mg/kg), first generation worms returned to clean soil showed a large rebound effect, and by the third generation there was a recovery in cocoon production. By contrast, for higher (90 and 270 mg/kg) and longer (56 d and 84 d) exposures worms performed more poorly, suggesting that there is a tissue Cd threshold beyond which recovery becomes challenging. Evidence from gene expression results are consistent with the idea that this threshold corresponds to a

point at which the Cd-sequestering protein metallothionein (MT) has reached saturation, as can also occur in human kidney tissue. Below this point, worms transferred to clean soils will recover. Above it, they will not.

As (spiked as arsenate, AsO_4^{3-}) also accumulated in worm tissue with exposure concentration and time, but showed some distinct differences compared to Cd. Modest As exposure extended for longer than 28 d had the unusual effect of stimulating growth and causing excessive cocoon production, an effect likely to be missed in most standardised tests. The effects are not thought to be related to parasite suppression, because they were accompanied by large-scale changes to gene expression. Despite appearing beneficial, by the second generation it was clear that effects of the As exposure were overwhelmingly negative, both in terms of extremely low survival rates and the delayed growth of surviving earthworms.

Perhaps more notably, results for both the lower exposure condition (10 mg/kg soil As) and As-exposed worms returned to clean soils, suggest there are circumstances where As may promote its own uptake in a positive feedback loop. If correct such an effect may be linked to an increase in uptake of phosphate (PO_4^{3-}) for cellular repair, with co-uptake of arsenate (AsO_4^{3-}, which is isomorphous). A parallel mechanism is known for marine fish. Remarkably, results suggest that Cd exposure may have also caused an increase in As uptake, from soils that contained only natural [As]. Though a tentative finding, such an effect would be consistent with the idea that any contaminant that causes cellular damage in an invertebrate may trigger a need for more soil phosphate, presumably with some As co-uptake. This would also imply that many (presumed) single contaminant exposures whether in the laboratory or the field may in fact be As co-exposures.

Relevant to this, the adverse impacts of As and Cd co-exposure were found to be more severe than effects of exposure to either contaminant alone; despite the fact the lower amounts of each contaminant were taken up under the co-exposure condition. This result supports an argument that soil guideline values derived from single contaminant toxicity experiments may be insufficiently protective for soil invertebrates in many real-life settings.

Gene expression results were useful as an interpretive tool, with numbers and overlaps of differentially expressed genes being more useful than knowledge of the subset of named genes and their putative functions. Exposure to Cd or/and As triggered large-scale changes in gene expression, indicating 'organism-wide' biochemical responses and providing circumstantial evidence that supported particular interpretations, such as existence of an MT saturation-threshold, and the existence of substantive biochemical changes between lower and higher As exposures. Analysis of differentially expressed genes in common between Cd-only, As-only and co-exposure suggests existence of both similar and different impacts of toxicity under the three conditions.

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Abbreviations

As – Arsenic

ATP – Adenosine triphosphate

BCF – Bio Concentration Factor

Ca – Calcium

Cd – Cadmium

EC10 – 10% effect concentration

EC50 – 50% effect concentration

Fe – Iron

MLD – minimum lethal dose

MT – Metallothionein

OECD - Organisation for Economic Co-operation and Development

Se – Selenium

Zn – Zinc

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Chapter 1: Introduction

1.1 Background

Cadmium (Cd) and arsenic (As) pollution can cause significant adverse effects on soil biota, as well as having implications for human health. The focus of this thesis was to study the uptake and effects of Cd, As and both elements together in earthworms, over the long-term and across up to three generations.

An Eisenia species was selected as a model organism. As these are widely used for ecotoxicology studies using standardize bioassay protocols. Annelids are key part of the soil ecosystems and one of the first phyla to be affected by soil toxicants. Cadmium and As are persistent contaminants and it is important to characterise their risks to exposed biota. Several researchers have investigated chronic Cd and As exposure of earth worms. However in most research, the exposure period was limited to 28 d or less (Liebeke et al., 2013, Burgos et al., 2005), the experiment was only on one generation, and the focus was solely or primarily on the classical phenotypic responses growth, maturation and cocoon production. As far as the author is aware, the research outlined in this work is the first to study uptake and effects on up to three consecutively-exposed generations, and explore relationships over those periods between phenotypic responses and patterns of gene expression. A longer exposure period also allows worms time to adapt to the new stressful environment. Shorter length chronic exposure experiments may lead to incomplete or misleading results because during the adaptation or acclimatising period the level of performance of worms such as growth and reproduction may both be reduced.

Metal exposure and gene expression

In the last few decades, scientists have commonly measured sub-lethal parameters such as survival, growth and reproduction to evaluate effects from contaminants. However, in recent years there has been more investigation of genetic effects of contaminant exposure. These can be characterised in terms of both changes in expression reflected in differential expression of RNA, and changes to the whole genome. Such work is costly and can yield ambiguous results. Despite a number of studies investigating genetic impacts, few focus on long-term exposures over two or

three generations or cases where the exposure is ongoing so that offspring are born into the contaminated environment. Effects seen in the first generation may or may not be mirrored in second or third generations. For example, there is a possibility that annelids may develop tolerance over time, and that the level of tolerance could increase with each generation. When exposure ceases, worms may or may not have the ability to recover and return to typical patterns of gene expression.

Cadmium has the ability to affect the transcription and expression of a number of genes. As examples Cd induces metallothioneins (MT) 1 and 2 genes (Karin et al., 1984, Shinkai et al., 2016), and has the ability to reduce *DMT1* gene expression (Tallkvist et al., 2001). Cadmium induced MT up-regulation in Cd exposed earthworms works as a protective detoxification mechanism (Stürzenbaum et al., 2004, Spurgeon et al., 2004a), because the MT protein binds strongly to the Cd. In most of molecular studies on Cd exposure in earthworms, the focus has been on one or more targeted genes which represent key biological functions. This is useful but can miss the complexity of the natural system in which many hundreds or thousands of genes are working in concert. In the current study the focus is on changes in gene expression that are caused by exposures to Cd, As and both elements together.

Several experiments have reported changes in gene expression after As exposure. Arsenic has the ability to change the level of expression by hypermethylation. Hypermethylation of DNA repair genes such as *p16, p53, MLH-1, RHBDF1, GMDS* and tumour suppressor genes causes down-regulation of their expression (Paul and Giri, 2015, Lu et al., 2014, Hossain et al., 2012, Gribble et al., 2014, Argos et al., 2006, Chanda et al., 2013). As for Cd exposure experiments, most As exposure experiments have targeted one or a few specific genes.

Questions about whether some of the toxic effects from Cd and As exposure can be transferred to progeny have not been studied. The transferrable properties of changes in gene expression of Cd and As have never been investigated until now. In a study on *C. elegans*, Wang an Peng (2007) reported that effects of Pb exposure transferred from parents to their progeny (Wang and Peng, 2007). However in real-life settings there are two aspects to cross-generational effects. One is whether some impacts of first generation exposures may transfer to subsequent offspring after

exposure ceases. The other is a reality that in many 'real-life' environmental contamination cases, the exposure will be ongoing, with offspring born into the same environment. Under these circumstances some toxic effects may simply compound between generations. The focus of this work has primarily been around the second aspect, to ensure the results are most applicable to real-life situations.

Arsenic and cadmium

Arsenic (As) and cadmium (Cd) are two common environmental contaminants for which non-fatal chronic poisoning cases are well known, toxicity may include reproductive effects, and potential for inter-generational transfer of some impacts may exist.

Contamination in terrestrial and aquatic environments can cause significant adverse effects on humans and other organisms. Although both elements occur naturally, Cd and inorganic As are also highly toxic. Since industrialisation, anthropogenic activity coupled with expansion of the human population has caused an increase in the liberation of Cd and As to surface environments and the biosphere.

Cadmium is regarded as a highly toxic metal and is capable of causing both acute and chronic toxicity (ATSDR, 2012). Cadmium and Cd compounds are ranked as a Group 1 carcinogen (carcinogenic to humans) by the International Agency for Research on Cancer (IARC, 2018).

Cadmium is listed as a seventh hazardous substance by the Environmental Protection Agency (EPA) of the United States. Anthropogenic contamination of the environment with Cd has been occurring ever since the advent of metal mining and refining (Nordberg, 1974) but has increased significantly since the industrial revolution (Nriagu and Pacyna, 1988, Friberg, 2018). This is both through its presence as an unwanted contaminant element (for example, Cd in phosphate fertilisers), and through use of Cd and its compounds in industrial processes and commercial products, such as in nickel-cadmium batteries, for Cd plating of aircraft parts, and as a pigment in paints and plastics. Potential for excess soil Cd to cause significant harm to human health became a major focus after the *Itai Itai* case in Toyama Prefecture in Japan, where a farming community were exposed to elevated

dietary Cd from Zn mine wastes contaminated rice paddy fields (Kasuya et al., 1992). After this is appreciated that excess Cd could enter the food chain resulting in excessive long-term absorption by humans and other organisms. Absorbed Cd accumulates in the human body because the biological half-life of Cd is 10-30 years. In humans, about half of this accumulates in the kidney and liver, and the other half is distributed among other tissues (ATSDR, 2012). The protein MT is induced upon Cd exposure and plays a prominent role in binding Cd in liver and kidney tissue, causing its detoxification but also its retention (Klaassen et al., 2009). Irreversible kidney and liver damage can occur once their capacity to retain Cd is exceeded (Satarug, 2018). At lower levels, cancer and more subtle effects such as interference in hormonal signalling are possible (Zang et al., 2009).

As is a metalloid found in air water and soil. Arsenic enrichment can come about through both natural and anthropogenic causes. In some area, higher levels of natural As cause significant health problems. Prominent examples are areas of West Bengal (Mukherjee et al., 2005, Chakraborti et al., 2001) and Bangladesh (Cherry et al., 2008, Smith et al., 2000) where a significant segment of the population is affected from As contaminated groundwater. Different types of As compounds are introduced to the environment everyday through geological processes such as weathering of rocks and minerals and also through human utilisation of water and geothermal resources. Anthropogenic sources of As contamination include or have included mining and smelting, As based pesticides and herbicides, use in timber treatment chemicals (particularly CCA: copper chrome arsenic), electroplating, and use As containing paints (Mahimairaja et al., 2005).

Earthworm models

In this research *Eisenia fetida* (phylum Annelida, family Lumbricidae, genus Eisenia) (with a amall propotion of the closely related species *E. Andrei* (Section 3.1.2))was used as a test and model organism. *E. fetida,* commonly called the tiger worm, is commonly found in New Zealand soils. This long tube-like worm is a hermaphroditic; however, two worms are required for the reproduction. *E. fetida* is a surface dwelling worm which is widely used in vermin-composting. *E. fetida* is also a well-established model organism for soil toxicology experiments for which

standardized methods exist (OECD, 2016). An advantage of using *E. fetida* in this work is that it is sexually mature within a short period of time and therefore a good model organism for examining potential effects of toxicity across three generations.

1.2 Aims and structure of this thesis

Aims

This research is divided into five main aims and these are as follows:

1. To quantify uptake and effects of chronic Cd, As and combined (Cd+As) exposures on *E. fetida* in up to three generations;
2. To examine changes in gene expression and assess their potential significance;
3. To explore the changes that occurs when exposure ceases;
4. To assess whether sub-lethal ongoing exposure causes effects that compound across subsequent (second or third) generations;
5. To examine whether relationships may exist between Cd and As exposures and the behaviour of selected other trace elements.

Structure

This thesis is structured in the following way.

Chapters 1-3 are the background and methodology chapters.

- **Chapter 1: Introduction** includes a brief introduction to the research and the purpose of the thesis. This is followed by thesis structure and general research aims.
- **Chapter 2: Literature review** provides an overview of Cd and As pollution, exposure, and toxicity including reproductive toxicity; the use of earthworms as model organisms, and effects of Cd and As exposure on gene expression. At the end of this thesis (**Appendix 1**) a related publication from this work is reproduced (with the publisher's permission). This explores maternal

cadmium exposure and impact on foetal gene expression through methylation changes, and was published in *Food and Chemical Toxicology*.

- **Chapter 3: Material and methods** includes details of all experimental methodologies used in this thesis

Chapters 4-7 are the results and discussion chapters.

- **Chapter 4: Uptake and effect of Cd exposure to *E. fetida* over three generations** includes results and discussion for all Cd exposure experiments on *E. fetida.* These include its impacts on growth, reproduction and gene expression, recovery when Cd exposure ceases.

- **Chapter 5: Uptake and effects of As exposure to *E. fetida* over two generations** includes results and discussion for all As exposure experiments on *E. fetida.*

- **Chapter 6: Co-exposure of *E. fetida* to both As and Cd** includes results and discussion for the co-exposure experiments.

- **Chapter 7: Behaviour of other trace elements** explores behaviour of other selected elements (including Ca, Cu, Fe, Mg, P, Se, Zn, as well as Cd and As themselves) during exposure experiments and the potential significance of some results.

Chapter 8: Conclusion summarises the key findings of the research in a synthesis of results, and provides recommendations for possible future research directions.

Chapter 2: Literature review

This chapter is to provide background context for various aspects of the work that were carried out in this thesis. It includes:

- an overview of cadmium (Cd) and arsenic (As), the two contaminants that were studied: focusing on their sources, exposures and human toxicity;
- a review of their reproductive toxicology (this relates to the fact that the earthworm experiments extended to three generations);
- an overview of the use of earthworms as model organisms, and patterns among reported phenotypic impacts of Cd or As exposures; and
- an overview of gene expression responses of earthworms to metal exposure, identifying some general research gaps.

A paper relating to the second area that was produced during the course of this work entitled 'Maternal cadmium exposure and impact on foetal gene expression through methylation changes' is reproduced in the **Appendix 1**.

2.1 Cadmium sources, exposures and human toxicity

2.1.1 Overview

Cadmium (Cd) is a metallic element in the 'd' block of the Periodic Table (Group 12) and atomic number 48 (Byrne et al., 2009). It occurs naturally and so is present in the environment at low levels, where it mainly exists as the divalent cation Cd^{2+} (whether dissolved, adsorbed, or as part of a compound). However, anthropogenic activities have caused substantial mobilization and release of more Cd to surface environments in particular, through both direct deposition and release to air followed by atmospheric transport. Cadmium released to the atmosphere has the ability to travel long distances (WHO, 2016). Cadmium enrichment of the biosphere has been accompanied by increasing environmental and human exposures (Nriagu, 1990).

2.1.2 Sources

Agricultural soils and foods

Increasing productivity in the agricultural industry during the last century has led to higher levels of Cd in farm soils in many countries worldwide. Application of phosphate fertilizers which contain elevated natural Cd is the main reason for this phenomenon (Järup and Åkesson, 2009). Phosphate fertilizer is the largest input of Cd to the pastoral and horticultural farming industry in New Zealand (Loganathan et al., 1995, Bramley, 1990, Rothbaum et al., 1986). Enrichment of Cd in farm soils causes higher levels of Cd to accumulate in livers and kidneys of older grazing animals, which may increase cadmium intakes for people eating offal products. Cadmium uptake into grains and vegetables also increases, which is significant because the majority of ingested cadmium is attributable to the grain and vegetable components of the diet (Al Mamun et al., 2016).

Other industries

Cd has been used as pigment, stabilizer and catalyst in plastic polymerization and production (Tamaddon and Hogland, 1993). Where used as a chemical stabilizer in PVC products it increases resistance to adverse weather, light and temperature

(Tamaddon and Hogland, 1993). Cadmium pigments have the ability to provide a wide range of colours from the greenish, lemon-yellow through orange to maroon. The main pigments are cadmium sulphide (CdS) (yellow) and cadmium selenide (CdSe) (maroon), and cadmium thioselenide (oranges and reds). Yellow colours are often solid solutions of CdS and zinc sulphide (ZnS) (Tamaddon and Hogland, 1993). Cadmium pigments have been used in artists' paints, car paints, ceramic glazes, and plastics. In the laboratory, yellow and red markings on glass pipettes are commonly made with cadmium paint (Jansen and Letschert, 2000), and can be a significant source of laboratory contamination.

Electroplating is one of the major industries that releases Cd to the environment via wastewater. Cadmium has been used for metal plating and coating operations, including transportation equipment, machinery and baking enamels, photography, and television phosphors (Islamoglu et al., 2006). Cadmium in wastewater may first pollute soil and groundwater and subsequently enter vegetables and poultry, to eventually end up with human body via food and water (Dai et al., 2008).

Nickel-cadmium rechargeable batteries have been used worldwide. Presence of Cd electrodes in these batteries means that special care is needed in their disposal (Bernardes et al., 2004). These batteries have potential to be a substantial local source of Cd effect to environment depending on fate, e.g. land filling or incineration.

2.1.3 Human exposures

General population (non-occupational) exposures

Food is the main source of Cd exposure in non-smokers (Järup and Åkesson, 2009). Cadmium is present in every food but its concentrations vary to a great extent. Cadmium levels of foods depend on several factors such as the type of food, and levels of environmental contamination. Higher concentrations are commonly present in molluscs and crustaceans such as bivalves and crabs (Järup and Åkesson, 2009). Brown meat and offal products with liver and kidney (particularly from older animals) also contain higher levels of Cd. Several plant food products contain more Cd than meat, eggs and dairy products. Grains such as rice and wheat, green leafy vegetables, potatoes and root vegetables such as carrot and celeriac contain higher

concentrations than other plants (Järup and Åkesson, 2009). Cadmium is readily absorbed by plants via the roots, with availability and uptake increasing as pH decreases (Olsson et al., 2002).

Occupational exposures

Cd is well known for its occupational risks, especially among people working in industries that involve use of Cd-containing products or substances (such as agriculture, battery manufacture, construction, plastic and soldering activities) (Järup, 2002). Cadmium exposure through the respiratory system is the main route of exposure in the occupational environment (Järup et al., 1998). Cadmium fumes or dust in the working environment are readily inhaled. Absorption through the oral route (the gastrointestinal tract) is also involved but to a lesser degree (Järup, 2002).

2.1.4 General toxicity to humans

Cd is regarded as a highly toxic heavy metal and is non-essential in humans (Wong et al., 2017). As with other trace elements there are three main routes by which Cd may be absorbed into the bloodstream: through ingestion (via the gastrointestinal tract), inhalation/pulmonary (through the lung surface) and dermal absorption (through the skin), and Cd is capable of causing both acute and chronic toxicity. As noted above food is the main source of Cd intake in the non-smoking non-occupationally exposed population (Järup and Åkesson, 2009). The digestive tract absorbs approximately 5% of ingested Cd. For smokers and in occupational exposure cases, inhalation becomes a major route. The human lung has the ability to absorb 40-60% of the Cd in tobacco smoke (Godt et al., 2006). Few studies quantify Cd absorption via skin, but absorption through this route is less efficient because the skin primarily acts as a barrier. There are two mechanisms that would facilitate Cd absorption through skin: binding of a free cadmium ion to sulfhydryl radicals of cysteine in epidermal keratins, or induction and complexation with metallothionein (Godt et al., 2006). Absorption of Cd into the human body depends on the solubility of the compound. A significant proportion of absorbed Cd is retained in the body, with approximately 50% being distributed between liver and kidneys where it is usually complex with metallothionein (WHO, 2011). Cd has long biological life (10-

35 years) and for this reason the body burden of Cd increases with age (WHO, 2011). This capacity of Cd to accumulate is also the main reason that low doses of moderately elevated Cd are capable of eventually causing serious chronic toxicity over the longer-term, as was seen in the case of *itai-itai* disease (reviewed below).

2.1.5 Reproductive toxicity

Hormonal activity

A number of environmental contaminants including some heavy metals have ability to mimic the effects of oestrogen. This process can disrupt the reproduction of humans and other animals. Cadmium has been found to have potent oestrogen-like activity *in vivo* (Johnson et al., 2003).

Exposure to Cd was found to increase uterine weight, promote growth and development of the mammary glands, and induce hormone-regulated genes in ovariectomized animals (Johnson et al., 2003). Cadmium affects the production of progesterone and testosterone. Under *in vivo* conditions Cd exposure highly affected production of progesterone and testosterone in proestrus rats, and Cd interferes with the ovarian steroidogenic pathway in rats at more than one site (Piasek and Laskey, 1999).

Testes, spermatogenesis and male fertility

Deniz and co-workers (1991) administered 10 mg/L Cd in drinking water to male and female Wistar rats for 52 weeks, using tap water as a control. After this time all the Cd-treated male rats showed pathological testicular alterations and reproductive capacity was lost in 39.9% of the animals (Deniz et al., 1991). Men occupationally exposed to Cd fumes at levels that caused renal glomerular and tubular damage showed lack of testicular damage (Mason, 1990).

Several animal studies have examined the effect of Cd on the male reproductive system. Leoni *et al* (2002) examined acrosome integrity and sperm viability of sheep exposed to drinking water at two different Cd concentrations (2 and 20 μM as $CdCl_2$). Spermatozoa viability was reduced to 57.6% at the lower concentration, and decreased further to only 35.6% at the higher concentration (Leoni et al., 2002). In

another study, adult male rats were injected with Cd chloride at doses from 1.6-150 µmol Cd/kg body weight, and after 14 days showed significant weight loss of the testis, seminal vesicles and epididymides. Weights were reduced by 40% to 50% in groups receiving 16 or 33 µmol Cd/kg which also showed that vas deferens sperm concentrations and hCG-stimulated serum concentrations were essentially zero (Laskey et al., 1984). Under zinc deficiency conditions Cd has the ability to cause prostatic proliferation lesions (Waalkes and Rehm, 1992). In an experiment conducted in 1992, male Wistar rats were used to assess the effect of dietary zinc deficiency on the carcinogenic potential of dietary Cd. Rats were fed diets that were either adequate or marginally deficient in zinc, along with various levels of Cd (0, 25, 100 and 200 mg/kg). At the highest doses (100 and 200 mg/kg) of Cd the rats showed bodyweight reduction and a positive incidence of prostatic proliferation lesions (Waalkes and Rehm, 1992).

Ovaries and oogenesis

Studies on reproductive toxicity of Cd in women are limited. However there are numerous studies on rats, mice, hamsters, pigs and sheep. In sheep, Leoni et al. (2002) conducted a study to determine the effect of Cd on oocyte maturation and fertilization, and found that oocyte maturation is significantly altered by both of the concentrations of Cd that were used (2 and 20 µM $CdCl_2$). In the fertilization process the presence of fertilised oocytes was decreased in both culture systems containing Cd (compared with the control) and polyspermy was increased in the 2 µM samples (Leoni et al., 2002). Effects of Cd on ovulation, egg transport and early pregnancy in golden hamsters were examined by Saksena and Salmonsen (1983). The hamsters experienced periods of sterility ranging from 11-59 days and 46-71 days for 5 mg/kg and 10mg/kg doses, respectively. Injection of $CdCl_2$ also induced ovulation inhibition which was dose and time dependent. The minimum dose required to inhibit ovulation was 5mg/kg (Saksena and Salmonsen, 1983).

Cd has potent oestrogen-like activity *in vivo*(Johnson et al., 2003). Exposure of Cd induces hormone regulating genes, increased uterine wet weight and development of the mammary gland in ovariectomized animals (Johnson et al., 2003). An increase of the uterine wet weight was accompanied by proliferation of the endometrium and

induction of progesterone receptors (Johnson et al., 2003). Cadmium can also up regulate the progesterone receptors. Because of the above factors Cd is regarded as a likely causative agent of oestrogen dependent diseases, such as endometrium and breast cancers, endometriosis and spontaneous abortions (Rzymski et al., 2014, Johnson et al., 2003). Nasiadek *et al.* (2011) showed a significant relationship between oestrogen receptors and concentrations of Cd in human uterine fibroids and surrounding tissues (Nasiadek et al., 2011).

Success of implantation

Sutou *et al*. (1980) administered increasing doses of Cd (0, 0.1, 1.0, and 10.0 mg/kg/day) to Sprague-Dawley rats to examine the effect of Cd on implantation, and reported that Cd toxicity had a significant negative effect, particularly at higher doses. Implantation was decreased slightly at the 0.1 mg/kg dose but significantly deceased at the 10 mg/kg dose (Sutou et al., 1980). Trophoblastic invasiveness was also effected by Cd toxicity. At higher administered concentrations of Cd (5-10 μg/mL) poisoned embryos showed trophoblastic outgrowth in variable areas (Yu and Chan, 1987). In a study done using human choriocarcinoma cells it was found that Cd (20 and 40 μM) inhibits human trophoblast cell proliferation (Powlin et al., 1997). Cadmium can replace calcium and interferes with cell proliferation (Powlin et al., 1997).

Embryonic effects

Introduction of Cd to pregnant female rats resulted insignificant accumulation of Cd in the foetal kidneys (Kuriwaki et al., 2005). Toxicity of Cd has been found to decrease the weight of the mouse embryo and increase the weight of the placenta (Sutou et al., 1980). Ingestion of Cd (10 mg/kg/day) by pregnant female rats during the first twenty days of gestation period was also found to reduce ossification (alter bone modelling and development) in the foetus (Sutou et al., 1980). Soukupova and Dostal (1990) examined "embryo-lethal" effects of $CdCl_2$ in Institute of Cancer Research (ICR) mice. Death of the embryos was highest after treatment with 6.0 mg/kg $CdCl_2$ on the 12th and 13th day of pregnancy (Soukupova and Dostal, 1990). Although fetal toxicity by Cd to rodents is well-defined, there is little information

about its potential effects of fetal viability on humans. It is known that maternal exposure to Cd results in low birth-weight and an increase in spontaneous abortions (Frery et al., 1993).

2.2 Arsenic sources, exposure and toxicity

2.2.1 Overview

Arsenic (As), a metalloid with atomic number 33, is a natural component of the Earth's crust that is distributed throughout air, water, and land. It ranks 20th in abundance in the Earth's crust, 14th in seawater, and 12th in the human body (Mandal and Suzuki, 2002). Arsenic can bind with many elements and occurs naturally in over 2000 different mineral forms. In the majority (approximately 60%) of these, it exists as arsenate (AsO_4^{3-}) which is isomorphous with phosphate (PO_4^{3-}). In another 20% of As minerals it exists as sulphide and sulfo salts, and in the remaining 20% its forms include arsenides (AsO_2^-), oxides, silicates, and elemental As (Mandal and Suzuki, 2002). Both inorganic and organic As species can exist in one of four oxidation states (-III, 0, +III, and +V), with this being dependent on the type and amounts of sorbents, pH, redox potential, and microbial activity (Wang and Mulligan, 2006). In general, inorganic As is of higher toxicity than organic forms (Cullen and Reimer, 1989, Sakurai et al., 2004).

2.2.2 Natural and anthropogenic sources

Where its levels are elevated, As often represents a significant threat to public health. Sources can be natural or industrial. Of these, release from natural sources, but often through human activity, is of the greatest significance to human health.

Groundwater sources and exposures

Natural exposures to elevated As tend to be strongly linked to human activity. Contaminated drinking water, in particular, is a major source of exposure to humans (Butts et al., 2015). Arsenic is naturally present at high levels in groundwater of often widespread areas of a number of countries including Bangladesh, China, Argentina, India, Chile, Mexico, USA (Butts et al., 2015, Mahanta et al., 2016, Wang et

al., 2006). In New Zealand, high As in groundwater is found throughout the Taupo Volcanic Zone (Robinson et al., 2006).

Elevated As in groundwater can become a problem when As-enriched water is used for drinking and irrigation. Large-scale exposure of human populations have occurred in South East Asia following the installation of millions of tube wells (Sharma et al., 2014).

Many studies have focussed on bioaccumulation of As in seafood (Cullen and Reimer, 1989, Goessler et al., 1997). However most of these As species in marine organisms are organo-arsenicals, which are much less toxic than inorganic As (Lunde, 1972).

Exposure to As results in a drastic decrease of plant growth in some plant species. Barrachina et al (1995) reported the effect of As exposure on yield and plant growth of tomato plants. The experiment was performed at three levels of As: 2, 5 and 10 mg/L (added as sodium arsenite ($NaAsO_2$) in a nutrient solution), together with corresponding control plants. Arsenic uptake depended on the As concentration in solution and the As content in the roots increased as the time of treatment increased. Arsenic exposure resulted in a decrease in plant growth parameters (maximum decrease of 76.8% in leaf fresh weight) and in tomato fruit yield (maximum reduction of 79.6%) (Barrachina et al., 1995). In general the chances of poisoning of animals grazing As contaminated plants is very low because plant injuries occurs before toxic concentrations are reached (Mandal and Suzuki, 2002). This may not be the case when As has accumulated on the surface of the plant, as occurs in As-enriched watercress and other aquatic plants growing in the Waikato River system (Robinson et al., 2003).

Industrial sources

Production of energy from fossil fuels and smelting of non-ferrous metals are major industrial processes that release As to the environment (Järup, 2003). Compounds of As are also used as alkylation agents, in processing glass, as part of pesticide formulations including wood preservatives, in some animal feed additives, as

pigments, in the textile industry and in paper processing. Some of these uses are outlined in further detail below.

Activities like mining, smelting, combustion of fossil fuels, and pesticide use can result in contamination of soil and freshwater by As (He and Charlet, 2013). Effluents from mining sites usually contain high As concentrations. As an example from Canada, it was reported that the annual total fluvial input of As to the Moira Lake in Ontario was approximately 3.5 tonnes due to local mining and mineral processing (Wang and Mulligan, 2006). As is also released during refining and production, with (for example) the sum of Canadian smelter and base-metal refineries releasing an estimated 15 tonnes of As in liquid effluent, 310 tonnes into the atmosphere, and 770 tonnes as solid waste in one study year (1992) (Wang and Mulligan, 2006). Arsenic is released to environment during coal pyrolysis and combustion, for example in thermal power stations (Zeng et al., 2001). Like a number of other trace elements, As bound to coal is mobilized during coal combustion and may be released associated both with the particles and in vapour form (Xie et al., 2006). Wu et al (2016) estimated that global emissions of As to the atmosphere were 30.7 Gt per year, with 15.8 Gt (51%) of this originating from East Asia and 4.4 Gt (14%) from South America (Wu et al., 2016a).

Arsenic has a long history of use in pesticide formulations. Most As pesticides are now used in cotton production (Mandal and Suzuki, 2002). In 1892, Mexican boll weevil spread across the cotton belt of USA. In 1917 entomologists in the U.S Agriculture Department found that calcium arsenate was effective to killing the boll weevil. Along with lead arsenate it became a leading insecticide worldwide from 1917 to 1945 (Lever, 2002). In New Zealand and many other countries, As contamination of old orchard soils also resulted from a long-term build-up of As over time caused by the annual use of lead arsenate as an insecticide (Gaw et al., 2008).

Both inorganic and organic arsenical compounds have been extensively used as herbicides. For example, use of sodium arsenite as a weed killer and non-selective soil sterilant started in 1890 (Mandal and Suzuki, 2002, Barrachina et al., 1995). From 1953 to 1969 the Commonwealth of Massachusetts funded a program to

evaluate the effectiveness of sodium arsenite for treating algae and macrophyte infestations in lakes. More than 30,000 kg of sodium arsenite was applied to 12 lakes and ponds (Lattanzi et al., 2007). Since that time they have experienced several problems because of the high persistence of As compounds in lake sediments. In New Zealand, bed sediments of Hamilton Lake (Lake Rotorua) were similarly contaminated with 5.5 tonnes of As in 1959 (Tanner and Clayton, 1990).

Organoarsenic compounds are used as feed additives in the poultry industry for disease control and enhanced feed efficiency. The high As concentration in poultry litter eventually increases As levels in soil in cases where poultry litter is repeatedly applied in agriculture (Morrison, 1969, Jackson and Bertsch, 2001).

2.2.3 As toxicity

Like Cd, As is capable of causing both acute poisoning at high doses, and chronic poisoning through longer-term lower dose exposures. Symptoms of acute poisoning normally occur within 30 minutes (Wang and Mulligan, 2006). Acute As poisoning is associated initially with nausea, vomiting, abdominal pain, and severe diarrhoea (Ratnaike, 2003).

At the As concentrations normally involved, exposure via drinking water causes chronic effects. Arsenic does cause incremental genetic and other damage that lead to chronic toxicity. Arsenic is a well-documented human carcinogen affecting numerous organs. Long term exposure of toxic inorganic As cause arsenicosis, which is the common term used for As-related diseases (Saha, 2003, Sharma et al., 2014). Inorganic arsenicals have been classified as Group I carcinogens based on human epidemiological data. Exposure to As in drinking water causes an increased incidence of cancers in lungs, kidney, bladder and skin, and reproductive disorders (Mandal and Suzuki, 2002).

As contaminated water is odourless, colourless and has no special taste, and so exposure is hard to detect and avoid by members of a normal community (Bhattacharya et al., 2012). The World Health Organization has referred to poisoning by As contaminated drinking water in Bangladesh as “the largest poisoning of a population in history” (Smith et al., 2000). Contaminated drinking

water leads to poverty by increasing health cost and it is considered as major health hazard in developing countries (Mahanta et al., 2016).

2.2.4 Reproductive toxicity

A summary of human exposure studies reviewed as part of this work is provided in **Table 2.1**. Toxicity studies (including those that examine reproductive toxicity) are commonly carried out in rodents. These have shown that prolonged exposure to As can cause developmental toxicity at maternally non-toxic levels. For example inorganic As exposure throughout the gestation period affects fetal brain development and newborn behaviour of rats. Arsenate teratogenicity has been tested in golden hamsters and rats when given at high doses. In these species malformations consisted mainly of exencephaly, eye defects, renal agenesis, and gonadal agenesis (Domingo, 1994). In the case of As, wide-scale exposure of human populations has provided another avenue for studying the impact on As exposure on humans. The following section provides a review of these effects.

Table 2.1: Studies examining the relationship between As and reproduction

Type of the study (human subject and country)	Arsenical exposure	Type of analysis	Major finding	Reference
Lanyang Basin, Taiwan N= 18,259	Exposure via drinking water Undetectable levels (<0.15 ppb) to 3.59ppm (mg/L)	Compared the risk of adverse pregnancy outcomes between areas with high well water As levels and non-As exposed areas	As in drinking water is associated with, 1. Risk of low birth weight(babies were lighter by 30g on average in As exposed areas compared with non-exposed areas) 2.Preterm delivery (not significantly)	(Yang et al., 2003)
Village of Samta, Bangladesh N=192	Out of 192 people, 98% - drinking water containing ≥ 0.10 mg/L As 43.8% - drinking As containing water for 5-10 years. 22.9% - skin manifestation due to chronic As exposure	Compared adverse pregnancy outcomes of the women of reproductive age who were exposed to As to those not exposed to As.	Following factors were significantly increased with As exposure. 1.Spontaneous abortions (p= 0.008) 2.Stillbirth(p=0.046) 3.Preterm birth rates(p=0.018)	(Ahmad et al., 2001)
Bangladesh N=533	As concentration of drinking water greater than 50µg/L	Cross sectional study. Interviewed women about sociodemographic characteristics, drinking water use, and adverse pregnancy outcomes	Spontaneous abortions and stillbirths were higher among participants who chronically exposed to higher concentration of As.	(Milton et al., 2005)
Sweden N=662	Exposure to As by inhalation	Study on spontaneous abortions among female employees in smelter.	Increased increment of spontaneous abortions and low birth weight among pregnant mothers who were employed in smelter(frequency was higher again if they also lived near to the smelter). Birth weight was decreased mainly in late pregnancy	(Nordström et al., 1979)

WestBengal, India N=202	Exposure via drinking water (≥200µg/L)	As exposure during pregnancy period measured using all water sources used. Odds ratios for spontaneous abortion, stillbirth, neonatal mortality, and infant mortality were estimated with logistic regression based on the method of generalized estimating equations.	Exposure to high concentrations of As (≥200 µg/L) during pregnancy was associated with a six-fold increased risk of stillbirth. After adjustment for potential confounders (odds ratio (OR) 6.07, 95% confidence interval (CI): 1.54, 24.0; p <0.01). As related skin lesions were found in 12 women who had a substantially increased risk of stillbirth (OR 13.1, 95% CI: 3.17, 54.0; p <0.002). The odds ratio for neonatal death was 2.81 (95% CI: 0.73, 10.8). No association was found between As exposure and spontaneous abortion (OR 1.01, 95% CI: 0.38, 2.70) or overall infant mortality (OR 1.33, 95% CI: 0.43, 4.04).	(Von Ehrenstein et al., 2006)
USA N= 1667		Investigated the relationship between community drinking water quality and spontaneous abortions	Increased frequency of spontaneous abortion was associated with detectable levels of Hg, and high level of As, potassium and silica.	(Aschengrau et al., 1989)
Bangladesh N=29,134	More than 50 µg/L	Evaluated the effect of As using health and demographic surveillance system in Matlab.	Significantly increased risk of fetal loss and infant death.	(Rahman et al., 2007)

OR - odd ratio, CI - confidence interval

Hormonal activity

At extremely low concentrations As can act as an endocrine disruptor and alter hormone-mediated cell signalling processes in living organisms (Kaltreider et al., 2001). When As(III) (AsO_2^-) is given through drinking water or injection, it can interfere with spermatogenesis and alter activities of spermatogenetic enzymes. Furthermore, it can reduce the levels of testosterone and gonadotrophin in male mice and rats (Wang et al., 2006).

Embryonic effects and pregnancy failure

It is well established that As metal is toxic to embryonic and fetal tissues and has the ability to induce teratogenicity in mammals (Domingo, 1994).

As discussed above, water contamination by As in Bangladesh and India is a major water crisis in Southeast Asia. Higher spontaneous abortions and stillbirths were experienced in Bangladesh due to this phenomenon. Ahmad *et al*. (2001) investigated two groups of reproductive-age women, classified as exposed and non-exposed to As via their drinking water. Of the respondents in the exposed group, 98% had been drinking water containing ≥0.10 mg/L As and 43.8% had been drinking As-contaminated water for 5–10 years. Adverse pregnancy outcomes such as spontaneous abortion, stillbirth and preterm birth rates were significantly higher among the exposed group than in the non-exposed group (Ahmad et al., 2001).

The weight of evidence is that As causes developmental disorders and increases the occurrence of spontaneous abortions and stillbirths in humans.

Studies on other animals show parallel results, usually with additional information relating to the quantification of endpoints and identification of mechanisms of toxicity. Arsenic is not teratogenic in ducklings, however it reduced whole egg weight and caused eggshell thinning. Arsenic did not affect hatching success in ducklings, but it had the ability to reduce body weight and liver weight and reduce overall duckling production (Stanley Jr et al., 1994). One study examined the relationship between repeated sodium arsenate injection and neoplastic response in male and female Swiss mice. Groups (n = 25) of mice received sodium arsenate

(0.5 mg/kg, iv) or saline (control) once/week for 20 weeks and were observed for a total of 96 weeks. In females, As induced marked increases in the incidence and severity of cystic hyperplasia a condition of excessive proliferation of cells of the uterus compared against controls. Further, As-exposed animals showed hyperplastic uterine epithelium with a strong positive immunostaining response for the proliferating cell nuclear antigen, and also an upregulation of oestrogen receptor immune reactive protein in the early lesions of uterine luminal and glandular hyperplasia (Waalkes et al., 2004).

Testes, spermatogenesis and male fertility

Studies on the effects of As on the male reproductive system fall into those that directly relate to humans and those carried out on other animals, with fewer in the first category.

Among the human studies, a significantly increased risk of low sperm motility was linked to low-level exposure to environmental As. In the cross sectional study done in Michigan US, the Odds Ratio for low sperm motility with the highest As quartile was 3.80 (1.38–10.4). Arsenic has also been implicated as a reason for low semen volume (Wirth and Mijal, 2010).

In mice, chronic oral exposure to As has been found to have effects on male sex organ weight, sperm parameters and testicular marker enzymes (Pant et al., 2001, Pant et al., 2004). Sodium arsenate was given to male mice at doses of 53.4, 133.47, 267 and 534 µmol/L. At the highest concentration of 534 µmol/L a significant decrease occurred in sperm count and motility, along with an increase in sperm abnormality. However, no reproductive effects were observed at the lowest concentration of 53.4 µmol/L (Pant et al., 2001). Pant *et al.* (2004) introduced sodium arsenate to male mice *via* drinking water at a dose of 4 mg/L for 365 days. Exposed mice showed decrements in absolute and relative testicular weights. The activity of marker testicular enzymes such as sorbitoldehydrogenase, acid phosphatase and 17β-hydroxysteroid dehydrogenase (17β-HSD) were significantly decreased. Sperm counts and sperm motility were decreased and abnormal sperm morphologies were reported. Significant accumulation of As was found to occur in testes, epididymis, seminal vesicles and the prostate gland (Pant et al., 2004).

Eight week old male ICR mice showed decreased epididymal sperm counts and testicular weights after they were poisoned by sodium arsenate (20 or 40 mg/L) with drinking water for five weeks (Im Chang et al., 2007). In another study, As (as arsenic trioxide) was given to Wistar rats at a dose of 3 mg/kg body weight per day throughout 28 days. This treatment caused an increase in seminiferous tubular luminal size, reduced accumulation of spermatozoa and signs of necrotic changes with disarray of cellular organization (Mukherjee and Mukhopadhyay, 2009).

2.3 Worms as a model organism

2.3.1 Introduction

In terms of biomass and activity annelids are the most important part of the soil invertebrate community (Hendrix et al., 1986). The fact that they inhabit the soil medium and tend to bioaccumulate contaminants makes them good model organisms for assessing soil contamination. They are one of the first organisms affected by heavy metal contamination in soils, but in many cases can also tolerate reasonably high levels. Toxicity and bioaccumulation of soil toxicants have been observed in different species of earthworms such as *E. fetida* (Neuhauser et al., 1985, Spurgeon and Hopkin, 1999), *Eisenia Andrei* (Van Gestel et al., 1993a, Peijnenburg et al., 1999) and *Lumbricus terrestris* (Šrut et al., 2017, Kennette et al., 2002). Several studies have proven that earthworms can live under heavily contaminated conditions (Becquer et al., 2005, Maity et al., 2008, Dai et al., 2004) and accumulate heavy metals up to high levels (Dai et al., 2004, Ireland, 1979).

Earthworms have been widely used as a model species in Cd and As toxicology experiments (Fischer and Koszorus, 1992, Lee and Kim, 2009a), including standardization of acute and chronic eco-toxicological assays. The measurement of biomarkers in earthworms has been increased over the last few years. A dominant focus on phenotypic responses (Fischer and Koszorus, 1992) has expanded to include measurement of biochemical responses including changes in the transcriptome (Anderson et al., 2013, Lee and Kim, 2009a).

2.3.2 Uptake and impacts of Cd

Growth, survival and reproduction of earthworms are all affected by Cd exposure.

Growth and survival

Results of four studies that have examined the impacts of Cd exposure on worm growth in artificial soils are summarised in **Table 2.2**. Some authors have reported significant effects of Cd on growth (Žaltauskaitė and Sodienė, 2014, Burgos et al., 2005, Spurgeon et al., 2004b), whereas others have not (Van Gestel et al., 1993a, Spurgeon et al., 1994b, Žaltauskaitė and Sodienė, 2010a). The degree of impact can depend on the worm species and adaptation but is also dose-dependent. Generally, growth is negatively correlated with Cd at high Cd concentrations in soil. At low Cd concentrations in soil worms do not show a significant reduction in growth compared with worms in control soils. However, under higher Cd concentrations worms show a relative loss in weight. Another factor relates to experimental design specifically, whether the worms were provided with additional food.

Table 2.2: Studies examining the effect of Cd on the growth of worms

Reference	Cd concentrations	Organism	Food	Results	Soil type
(Van Gestel et al., 1993a)	0, 100, 180, 320, 560 mg/kg	*E. andrei*	Treated cow dung	Growth was not significantly affected. Showed dose related increase	Artificial soil
(Žaltauskaitė and Sodienė, 2014)	1, 2, 5, 10, 40, 100, 250, and 500 mg/kg	*E. fetida*	Oatmeal	Significant effect of Cd to growth ($p < 0.001$). Under low concentrations of Cd worms were gain weight and under higher concentrations loss weight.	Artificial soil (OECD)
(Burgos et al., 2005)	5, 25, 125 and 200 mg/kg	*Lumbricus rubellus*	Sheep manure	Under low Cd concentration (5,25,125) worms gain weight and under high concentration (200mg/kg) worm lost weight	Artificial soil (OECD)
(Žaltauskaitė and Sodienė, 2010a)	1, 2, 5, 10, and 100 mg/kg	*E. fetida*	None given	Weight loss was reported in all concentration	Artificial soil (OECD)

Spurgeon *et al* exposed *E.fetida* to Cd (5, 20, 80, 300 mg/kg) and reported increased weights during the first week for except in worms exposed to the highest concentration

300 mg/kg. After that there was a reduction in weights to 56 days, but this also occurred in controls. This could be seen as a methodological problem because the worms were not fed during the experiment and had to rely on organic matter in the OECD soils. After consuming that, the worms started to lose weight (Spurgeon et al., 1994b). For exposure trials in this thesis the approach taken was to provide the worms with food in the form of oats. According to Gestel *et al*. (1993) growth was not significantly reduced by Cd exposure (10, 18, 32, 56, 100 mgCd/kg). Instead, growth showed a positive correlation with dose of Cd (Van Gestel et al., 1993a). Žaltauskaitė and Sodienė (2014) exposed worms (*E. fetida*) to 1, 2, 5, 10, 40, 100, 250, and 500 mg/kg of Cd contaminated soil and found a significant ($p < 0.001$) effect of increasing Cd on juvenile growth. Worms exposed to the lowest concentrations of Cd (1-2 mg/kg) showed very similar growth to the control group. Those treated with 1-40 mg/kg Cd gained absolute weight during 14 weeks. However, worms exposed to 250 and 500 mg/kg Cd showed absolute and relative weight loss (Žaltauskaitė and Sodienė, 2014). In previous work, Žaltauskaitė and Sodienė (2010) had exposed *E. fetida* to 1-100 mgCd/kg over 28 d and reported weight loss in worms at all concentrations. However the reason for this would again be that they had not supplemented with additional food in that case (Žaltauskaitė and Sodienė, 2010a). Burgos *et al*(2005) exposed *Lumbricus rubellus* to 5, 25, 125 and 200 mg/kg of Cd contaminated soil and reported similar results to those of Žaltauskaitė *et al* (2014). After 21 d of exposure under lower (5, 25, 125 mg/kg) Cd concentrations worms showed positive growth and under higher concentrations they showed weight loss. Worms at the highest concentration (200 mg/kg) lost nearly 3.5% of their original weight ($p<0.01$) (Burgos et al., 2005).

Higher Cd exposures can also have an impact on earthworm survival (Žaltauskaitė and Sodienė, 2014, Novais et al., 2011). Mortality increases with increasing Cd concentrations, and is also typically higher in juvenile worms than in adults (Žaltauskaitė and Sodienė, 2014, Novais et al., 2011). In some studies Cd was found to have no significant effect on survival of worms (Žaltauskaitė and Sodienė, 2010a, Spurgeon et al., 1994b). The outcome can depend on the worm species and bioavailability of Cd from the soil being investigated; as in real soils, Cd bioavailability will vary substantially. For example, Spurgeon *et al* (1994) mentioned there was no significant effect ($p>0.05$) on mortality of *E. fetida* even at the highest test concentration (300 mg/kg). When the concentration was raised to

800 mg/kg in a follow-up study (Spurgeon et al., 2004b), mortality became significant.

Sexual maturation and reproductive success

Sexual maturity and cocoon production of annelids are sensitive parameters for Cd contamination (Žaltauskaitė and Sodienė, 2010a) in soil and are severely affected by Cd. Žaltauskaitė*et al*. (2014) report that the proportion of mature earthworms in control groups was significantly higher than in Cd treated groups. After 14 weeks 86% of worms in control soils reached sexual maturity, compared with only 23% in 10 mgCd/kg samples (Žaltauskaitė and Sodienė, 2014). The time needed for earthworm sexual maturation is positively correlated with the concentration of Cd in soil ($p<0.001$); as an example, earthworms incubated in 10 mgCd/kg soils reached sexual maturity after 11.3 weeks while those in 40 mgCd/kg soils took 12.1 weeks (Žaltauskaitė and Sodienė, 2014). The results of same study indicated a significant correlation between the percentage of the earthworms that reached maturity and the weight of the earthworms ($p<0.001$) (Žaltauskaitė and Sodienė, 2014).

Cocoon production is more sensitive to Cd poisoning than mortality (Spurgeon et al., 1994b), and decreases with increasing Cd (Žaltauskaitė and Sodienė, 2014). Spurgeon (2004) showed Cd significantly affects mean daily cocoon production of *Lumbricus rubellus*. However, there was no significant impact on the viability of cocoons or number of juveniles per cocoon (Spurgeon et al., 1994b, Spurgeon et al., 2004b). Van Gestel *et al.* reported there was a significant ($p<0.05$) reduction of cocoon production at soil Cd of 10 mg/kg (Van Gestel et al., 1993a). In another experiment with Cd-spiked soil (1, 2, 5, 10, 100 mg/kg) cocoon production of *E. fetida* exposed to Cd was inhibited 18-65% in comparison with controls (Žaltauskaitė and Sodienė, 2010a). Spurgeon *et al* (2004) also calculated cocoon production as a rate per day, and found this to be negatively correlated with Cd concentrations of the soil (Spurgeon et al., 2004b) (**Table 2.3**).

Table 2.3: Impact of Cd on cocoon production of *Lumbricus rubellus* as reported by Spurgeon (2004).

Cd concentration in soil (mg/kg)	Cocoon production rate (cocoons per worm per day)
0	0.12±0.01
12.5	0.09±0.02
50	0.09±0.02
200	0.06±0.02
800	0

The impact of Cd exposure on sperm maturation has also been examined. Cikutovic et al. (1993) reported results of experiments in which *Lumbricus terrestris* was exposed to 100, 200, and 300 mgCd/kg spiked artificial soil. After 16 d exposure there was a highly significant ($p=0.001$) depression of sperm counts relative to controls in all cases. Significance ($p<0.05$) was reached after 9 days of exposure to 300 mgCd/kg and after 16 days at 100 and 200 mgCd/kg. Once initial depression occurred no further changes happened with time (Cikutovic et al., 1993).

Recovery of cocoon production

Some researchers have investigated the recovery of Cd-exposed worms after they are introduced back to clean soil. Under low and medium Cd concentrations worms showed complete recovery of reproduction. Van Gestel *et al* (1993) reported *Eisenia andrei* reproduction completely recovered in worms exposed to 10-100 mgCd/kg for 3 weeks, after 3 weeks in clean soil (Van Gestel et al., 1993a). However, Cd concentrations in the worm tissues remained significantly elevated even after the recovery period (Van Gestel et al., 1993a).

2.3.3 Uptake and impacts of As

Growth and survival

Most literature reports a reduction of growth at higher As concentrations (Anderson et al., 2013) and fewer effects at lower exposures. However, at the lowest exposures As work may work to promote growth. Lee and Kim (2009)

reported that growth was significantly ($p<0.05$) reduced at 14.9 mg/kg and 3.7 mg/kg. However, it was not affected at 0.74 mg/kg and increased at 1.8 mg/kg. In a follow-up study these authors examined the speciation of As and found that both inorganic forms were associated with an increase in growth compared with controls at the lowest concentrations, but at different exposure thresholds (Lee and Kim, 2013). For arsenite (As(III)) growth after 28 days increased at 7.49 mg/kg and 1.8 mg/kg. For arsenate (As(V)) the growth also increased at 1.8 mg/kg but showed no difference at 7.4 mg/kg. These concentrations are all relatively low given that the natural concentration of As in soils is typically ~5 mg/kg (Lee and Kim, 2013).

At higher level exposures, decrements in growth are more consistently observed. Fischer and Koszorus (1992) reported that *E. fetida* grown in 50 mgAs/kg soils showed significant loss ($p<0.01$) in growth compared with worms in control soils (Fischer and Koszorus, 1992). Similarly, Anderson*et al* (2013) reported that growth of *Lumbricus rubellus* exposed to higher As concentrations (36 and 125 mgAs/kg) was significantly reduced ($p<0.001$); in fact the worms reached an average weight of only 0.084 g by the end of the exposure period (280 days) compared to a 1.8 g weight average for control worms. Anderson *et al* (2013) continued the research and measured the juvenile growth under As exposure conditions. According to the results, juvenile growth rate was also decreased with increasing As concentration in the soil.

Survival

At higher concentrations, As also adversely affects earthworm survival (Meharg et al., 1998, Shin et al., 2007a). Lee and Kim (2009) reported that a significant ($p<0.05$) effect on survival at highest level of As (22.47 mg/kg) after 14 days. Fischer and Koszorus (1992) reported that worms living in 25 mgAs/kg soils showed no mortality, and at 50 mgAs/kg the mortality remained low (10%). However, by 100 mgAs/kg it had drastically increased to 55%, and by 250 mgAs/kg it was nearly total (Fischer and Koszorus, 1992). Critical concentrations will vary depending on the soil type and worm species. Soil type might be expected to modify the bioavailability of As. Anderson et al (2013) found no significant

($p<0.05$) increase in worm mortality to 280 d in soils containing up to 125 mgAs/kg (Anderson et al., 2013). Speciation of As can also play a part. Lee and Kim (2013) reported that As (III) was more toxic than As (V); in fact after 14 days of exposure only 20% of worms survived in 14.98 mg/kg As (III), compared with 95% of worms exposed to the same concentration of As (V) (Lee and Kim, 2013).

Sexual maturation and reproductive success

Varied outcomes have been reported for the effects of As on cocoon production, but in general, the pattern is that cocoon production decreases among worms grown in As contaminated soil, and can cease at higher concentrations. Lee and Kim (2013) reported cocoon production is low in As spiked soil, but with considerable variations. Anderson *et al*. (2013) also reported that cocoon production was significantly affected at the highest As concentration studied which was 125 mgAs/kg. However, it was not significantly different from control at the lower concentrations (3, 12, 36 mgAs/kg) (Anderson et al., 2013).

In most research the effects of As on growth, survival and cocoon production in earthworms has only been observed over relatively short time periods. However, the earthworms need time to acclimatized to the new soil and activate survival and growth mechanisms. Therefore, there is a need for more research into effects of exposures over longer time periods.

2.3.4 Effect of metal exposure on gene expression of earthworms

Annelids are often used as model organisms in toxicological studies. In the past it was common to measure eco-toxicological parameters such as survival, growth and cocoon count. With the development of genomics techniques researchers have increasingly investigated genetic effects of contaminant exposures on earthworms. Both whole-organism methods (such as survival, growth and cocoon production) and molecular methods have specific limitations, and each has roles to play in determining biological responses to metals. Therefore, the best way is to use both whole genome and molecular methods to develop a more complete picture and conclusions.

Some annelids have the ability to adapt to toxic environments, and mechanisms to reduce the toxic effects of toxicants. Worms in metal-polluted environments may adapt to toxic environments and develop greater metal tolerance than worms living in control conditions (Mouneyrac et al., 2003). Some of these effects might be achieved through changes in gene expression. On the other hand, annelids may not thrive or grow in contaminated environments and with the time the population size may decrease.

Despite the fact that annelids are often being used in eco-toxicological experiments, the molecular mechanisms of their resistance remain largely unknown (Neave et al., 2012). This might be the reason why the focus of most research is on characterizing general pathways such as genes involved in oxidative stress (Spurgeon et al., 2004b), stress protein responses, and antioxidant generation (Fujino et al., 1999, Mo et al., 2012, Santoyo et al., 2011). Even though they do not provide enough information to provide a detailed picture of transcriptome changes, such research projects can provide valuable information about those specific processes.

There is little research that reports use of whole genome analysis to study effects of metal exposure on gene expression of annelids (Neave et al., 2012, Agbo et al., 2013).

Cd and gene expression of worms

Cd is a metal that can substantially alter gene expression in earthworms (Roh et al., 2006). In research done on toxicological cellular and gene expression responses in *Lumbricus rubellus,* changes of gene expression after exposure to Cd were assessed. The research mainly focused on the four genes: *β-act, l-rRNA, mt-2* and *lgp*. After exposure to Cd for 28 d, a significant effect on expression of *β-act* was found. Further experiments confirmed that this expression was significantly higher in 50 mg/kg than the control condition (12.36 mg/kg). Cadmium causes increased the expression of mt-2 gene compared to controls. This was a 7-fold increase at 11.12 mg/kg and an over 40-fold increase at 1.86 μMCd/g. *Lgp* gene was down-regulated after Cd exposure and there was no differential expression in *l-rRNA* after Cd exposure (Spurgeon et al., 2004b).

The most prominent research on effects on gene expression from heavy metals in earthworms is on MT gene expression. Production of MT increases on exposure to many metals and it has been considered as a biomarker of metal pollution in the environment (Stürzenbaum et al., 1998). MT binds metals like Cd and Hg and thereby participates in detoxification (Dabrio et al., 2002). Cadmium upregulates MT gene expression (Asensio et al., 2007, Burgos et al., 2005), with some differences between MT variants. Burgos *et al.* (2005) researched how 5, 25, 125, and 200 mg/kg Cd affected *MT1* and *MT2* gene expression in *Lumbricus rubellus*. After exposure, expression of *MT1* remained more or less consistent throughout the different concentrations of Cd levels and *MT2* showed increasing increments in expression with increasing Cd concentrations (Burgos et al., 2005).

Agbo *et al* (2013) examined transcriptional changes in *Lumbriculus variegatus* exposed to Cd using DNA microarrays, with results indicating numbers of differentially expressed genes after certain time periods. Numbers of genes showing more than 2-fold differential expression are shown in **Table 2.4** (Agbo et al., 2013).

Table 2.4: Numbers of >2-fold differentially expressed genes after 2, 6, 24, 56 h periods in the study of (Agbo et al., 2013).

Time (hours)	Up-regulated	Down-regulated
2	93	42
6	1025	558
24	1030	479
56	0	1

In Cd exposure experiments expression changes occurred during the early phases (up to 24 h) rather than later in exposure. Out of differentially expressed genes, many are known to play important roles in locomotion of worms; further muscular contraction and microfilament structure (Agbo et al., 2013).

As and gene expression

As with Cd exposure experiments, studies involving the effect of As on gene expression have included both whole genome experiments and target gene expression studies. Roh*et al*. (2006) investigated on the effect of As exposure on phytochelatin production in *Lumbricus rubellus*. Phytochelatins are chelating agents that have an important role in heavy metal detoxification. Arsenic exposure (28 d) was found to induce phytochelatin production in a dose dependent manner (Liebeke et al., 2013). These authors also reported that after 24 h As exposure, the *MT-1* gene was down regulated, and the *-2* gene was up regulated in *Caenorhabditis elegans* (Roh et al., 2006).

2.4 Cadmium and arsenic effect on the environment

2.4.1 Cadmium effect on the environment

Elevated exposures to heavy metals can directly and indirectly disrupt ecological systems. Cadmium inputs can come about through both industrial discharges and agricultural sources (Järup et al., 2009), as outlined in Section 2.1.2.Compared to other metals, Cd and its compounds are relatively water-soluble.

Cd is readily accumulated in many organisms across different trophic levels. In microorganisms (Ledin et al., 1996) and molluscs (Dovzhenko et al., 2005), Cd bioconcentration factors are in the order of thousands. Soil invertebrates including annelids also concentrate Cd markedly (Ramseier et al., 1989).Cadmium is also toxic to a wide range of organisms, from microorganisms up. However, the chemical composition in the living environment changes the level of toxicity (Ledin et al., 1996). For example the presence of sediments, high concentrations of dissolved salts, or organic matter can reduce the toxic impacts of Cd (De Vries et al., 2007). In many cases this is through adsorption processes reducing the availability of free unbound Cd for uptake.

Cadmium can bind to a wide range of biomolecules, *e.g.* by forming bonds with oxygen or sulphur groups; and can also displace other divalent cations, such as Ca^{2+} in bone and Cu^{2+} or Zn^{2+} in enzymes. For these reasons its effects can extend

to all tissues in a system-wide (systemic) way, and come about through a range of mechanisms including (a) disruption of biomolecular structures, (b) inhibition of enzymatic activity, and (c) Cd-induced oxidative stress causing major disturbances to cell metabolism (Dovzhenko et al., 2005). Cadmium tends to accumulate in some tissues more than others, especially where the organism up regulates metallothionein (MT) expression in one or more organs as a protective response.

Effects of Cd on survival, growth, reproduction and gene expression of soil dwelling organisms are extensive. Cadmium inhibition of growth has the potential to occur at all trophic levels of an ecosystem, from lower hierarchical levels to higher organisms living in that environment. A number of authors have shown that Cd has the ability to reduce the growth of microorganisms including bacteria (Vig et al., 2003) and fungi (Babich and Stotzky, 1977, Płaza et al., 1998) living in the soil. Of soil invertebrates, earthworms have the potential to experience significant Cd uptake, because they are exposed to the soil via both the skin and the gastrointestinal tract. According to the literature, significant reductions in growth of annelids can occur even at low Cd concentrations (Van Gestel et al., 1991).In experiments using the nematode *C. elegans* as a model organism it was shown that modest Cd decreased growth by 20% (Höss et al., 2009). Cadmium also has negative effects on growth and reproduction of other soil-dwelling organisms such as snails (Gomot, 1997), and at higher concentrations, plants. Apart from direct toxicity, the capacity for Cd to bioaccumulate in plants and other lower organisms means that it can be transferred to higher trophic levels via consumption(Mortensen et al., 2018). This capacity, known as biomagnification, occurs for some metals with longer biological residence times (notably Hg, Pb and Cd) and not others that are homeostatically regulated (*e.g.* Cu, Zn). Arsenic noted by (Croteau et al., 2005): "the greatest toxic effects of Cd are likely to occur with increasing trophic positions, where animals are ingesting Cd-rich prey (or food)."

As for growth, the toxic effects of Cd on reproduction are well-recognized (Thompson and Bannigan, 2008, Spurgeon et al., 1994a, Van Gestel et al., 1991). When considering earthworms, Cd exerts a negative impact on their production of both nematodes and annelids. For example Hoss *et al* reported Cd exposure inhibits reproduction by 40% and fertility 20% in *C. Elegans* (Höss et al., 2009).

proved range of other researchers have reported significant negative effects of Cd exposure on the reproduction of annelids (Van Gestel et al., 1993b, Žaltauskaitė and Sodienė, 2010b), as elaborated in Section 2.3.

Changes in gene expression brought about through exposure to a toxicant are a potential tool for pollution bio-monitoring. Because Cd pollution can cause upregulation of MT and glutathione to protect against oxidative stress, direct or indirect measures of these can be used as biomarkers (Demuynck et al., 2007b, Sandrini et al., 2006). Indirect measures focus on changes in gene expression for the upregulated biomolecules (Brulle et al., 2008).

At higher doses of toxicants, toxic effects typically show a linear dose-response relationship. At lower or initial exposures, however, the dose-response relationship may not be linear but sometimes shows a biphasic curve, or other more complex relationships. Hormetic dose-response relationships are those characterized by stimulation or beneficial effects at low-dose and inhibition or toxic effect a higher doses (Mattson, 2008). Hormetic responses may come about through a range of mechanisms (Zhang et al., 2009)that are still the subject of ongoing research and speculation(e.g. (Kim et al., 2018). The possible role of hormetic effects in toxicological responses has been the subject of some controversy and debate, partly due to attempts to create links with proposed mechanisms for homeopathy (Calabrese, 2019, Calabrese, 2017). However, biphasic dose-response curves consistent with stimulation at low dose and inhibition at high dose have been universally observed. According to (Shi et al., 2016):

"The universality of hormesis has been verified, but the degree to which hormesis should be taken into risk assessments and risk management plans remains controversial…mechanistic understanding of hormesis has come a long way but still lacks strong experimental support, which leads to uncertainty as to the exact underlying causes of hormesis."

As examples of such responses for Cd, it has been shown that a low concentration of Cd causes a stimulatory effect of some sort on the growth of plants (Jia et al., 2015, Jia et al., 2013) and animals (Calabrese et al., 1999). Hormetic-type activity

for Cd has also been observed in the earthworm *E. fetida*, where low concentrations induced an increase in the activity of catalase and superoxide dismutase, but higher concentrations inhibited these enzymes (Zhang et al., 2009). A criticism of viewing this type of response as *beneficial*, and of methods for classifying the degree of hormesis (e.g. as used by (Chen et al., 2017)could be that upregulation of such protective enzymes would come at an energy cost. The idea that a low doses of toxicant *stimulate* protective mechanisms has existed well before the concept of hormesis was developed. This emphasis a need to better define types of mechanistic responses that should be fairly classified as hormesis, to distinguish between situations where a biphasic dose-response relationship should be viewed as being primarily driven by a uniquely hormetic mechanism, and those where it could be seen as a predictable outcome of the first phases of a toxic response.

Responses to toxicants can also be modified by environment and genotype when organisms are constantly exposed to a particular toxicant. This increases the ability to tolerate toxicants, and resistance to the toxicants. Tolerance is normally seen as a shorter-term ability to reduce toxicity due to an innate capacity of the organism to upregulate its own responses. Resistance is seen as a longer-term effect caused through evolutionary pressure, where weaker individuals show lower reproduction while hardy individual survive and reproduce. In the area of microbial antibiotic resistance, it has been argued that tolerance invites resistance (Lewis and Shan, 2017) However, the two words may also be used interchangeably by some authors. The resistance to the heavy metals may be acquired either by genetic adaptation (Klerks and Weis, 1987) or by physical acclimation(Howell, 1985). As examples, *Gammarus pulex* was found to be significantly more *tolerant* of acute Cd toxicity after pre-exposure to sub-lethal concentrations of Cd (Stuhlbacher et al., 1992). Reinecke *at el* reported that multi-generational exposure of *E. fetida* to Cd caused development of resistance in the exposed worm population. In the experiment *E. fetida* was exposed to sublethal levels of $CdSO_4$ for more than 10 generations. Changes in growth, cocoon production, and hatchling success were observed. The results showed pre-exposed worms performed better than the unexposed worms (Reinecke et al., 1999b). In this thesis worms were exposed to Cd for up to three generations. Over this timeframe results did not

show any evidence for development of population resistance in offspring of exposed worms (Chapter 4).

Most assumptions about the likely effects of Cd in ecosystems are based on short-term experiments carried out in the laboratory. Such information may be insufficient to determine real damage to an ecosystem. On the other hand in natural environments the real effects of Cd can be hard to distinguish from effects caused by other contaminants and uncontrolled factors. However, an experiment carried out using *Tisbe holothurie* (a coastal and brackish-water copepod) showed interesting results after exposure to Cd for 20 consecutive generations. At lower concentrations (148 ppb and 222 ppb) compared to controls, exposed copepods took on a larger population size at the beginning. This was attributed to adaptation. However, after 20 generations the mean population density of the contaminated population had become similar to that of controls. . This observation was probably due to adaptation to the contaminated population within 20 generations (Hoppenheit, 1977). The ability of low dose Cd to cause an early population increase could represent a mixture of tolerance and hormesis. However in a natural environment the result might equally be due to Cd improving an external factor, e.g. by reduction in numbers of a more sensitive organism that competes for the same food sources. Such an organism might be external or internal, e.g. as a parasite. With this interpretation, the resumption of a more normal *Tisbe holothurie* population after 20 generations may be due to the other competing species being the organism that developed a resistance to Cd.

2.4.2 Arsenic effect on the environment

Arsenic is a metalloid that is present in water, soil, and plants due to natural biogeochemical cycling and anthropogenic activities. Arsenic compounds can cause short-term and long-term negative effects on the soil ecosystem. In the natural environment As can exist in both organic and inorganic forms with different mobility and toxicities (Quaghebeur and Rengel, 2005). Natural As concentrations in soil average between 5 and 6 mg/kg (Hindmarsh et al., 1986).

Arsenic can exert impacts on both plant and animal life in the soil. Its effects on growth of soil-dwelling organisms is not completely consistent because some

studies have reported that As accelerated growth(Lee and Kim, 2009b) and others reported a decrease (Shin et al., 2007b). However, most researchers reported a reduction of growth at higher As concentrations and fewer effects at the lower concentrations. Leading from the discussion in the previous Cd section: in this work, low-dose As exposure was associated with increased growth of *E. fetida* (Chapter 5). Such a hormetic (biphasic) *response* may come about through an unknown 'purely hormetic' *mechanism*. Alternatively, it might be caused by a more conventional mechanism such as low-dose As causing a reduction in internal parasites (Chapter 5).

Varied outcomes have been reported for the effects of As on cocoon production, but in general, the pattern is that cocoon production decreases among worms grown in As contaminated soil, and can cease at higher concentrations. Lee and Kim (2013) reported cocoon production is low in As spiked soil, but with considerable variations. Neaman *et al* reported that a mixture of soil organic matter and soil As both influenced reproduction. However when As concentrations increase the cocoon production decreased. The author noted that there is a peak in the cocoon and juvenile production for intermediate As concentrations (around 30 mg/kg) (Neaman et al., 2012).

One of the most important tools to evaluate the biochemical effects of environmental pollutants such as As is the use of molecular biology techniques. Toxicogenomics has become a powerful technological tool in environmental risk assessment (Pennie et al., 2004). In the laboratory, experiments have shown multiple effects at the molecular level following exposure, such as increased oxidative damage to DNA (Li et al., 2001, Liu et al., 2001). As an example, in a study using *Fundulus heteroclitus* (Mummichogs) as model organism, 13 genes were altered in the hatchlings after 10 d As exposure (230 ppb) of parental fish(Gonzalez et al., 2006).

Soil dwelling organisms including earthworms have the ability to accumulation As in their body. According to the literature, at lower concentrations worms have resistance to As through upregulation of MT(Scott-Fordsmand and Weeks, 1998). Langdon *et al* suggested that the most toxic effects will occur after the As-binding

capacity of MT is exceeded (Langdon et al., 2003b). Evidence of this effect was found in this work for longer-term Cd exposures (Chapter 4).

Co-exposure of metals and gene expression

There is a lack of research on co-exposure and gene expression. In the study done on *E. fetida*, Ricketts *et al.* (2004) identified the neuropeptide 'annetocin' as a reproductive biomarker that could be used in earthworm ecotoxicology. These authors measured the annetocine gene expression after exposure of worms to Zn (1750 µg/g) and Pb (650 µg/g) contaminated soil. Their results showed a 20-fold reduction in relative expression of annetocin in worms living in the contaminated environment (Ricketts et al., 2004).

2.5 Research gaps

Despite previous work on Cd and As exposures in earthworms, this review of the literature reveals that number of research uncertainties exist, and the shorter term focus of many experiments leaves a number of research gaps. These include:

- Understanding of the uptake and impact of longer term exposures over more than one generation, under circumstances where offspring are born into the contaminated environment;
- Determining more about differences between uptake and toxicity of chemically different trace elements, when other factors are standardised;
- Determining more about whether thresholds exist beyond which recovery is unlikely, especially for contaminants such as Cd with a long biological half-life;
- Confirming whether there are cases where Cd and/or As may be stimulatory for growth or cocoon production and (if so) determining whether this is beneficial and possible reasons for observed effects;
- Determining impacts of co-exposure to As and Cd in an annelid;
- Understanding effects that might occur on other trace elements; and

- Exploring how any of the above relate to levels of gene expression.

In the last area, little to no research has examined gene expression in worms exposed to metals over consecutive generations, or from a first generation exposure to unexposed worms and/or differences in gene expression that may occur with co-exposures. One aim of the current research was to provide some data to start to address these research gaps in the field of ecotoxicology. This has real-life relevance because most soil contamination persists over long-time periods. Therefore it is important to study more than one generation to gain a better understanding of long-term exposure.

The aims of this research (**Section 1.2**) are designed to explore and address some of these uncertainties and gaps.

Chapter 3: Materials and methods

3.1 Exposure and recovery experiments - common features

3.1.1 Outline of the OECD protocol

OECD guidelines for the testing of chemicals were followed in these experiments (OECD, 2016). This test guideline is designed for assessing the effect of test chemicals in soil on the reproductive output and other sub-lethal endpoints of the earthworm species *Eisenia fetida* and *Eisenia andrei*. The principle of the test is to expose adult worms to a range of concentrations of the chosen test chemical that is mixed into an artificial soil.

3.1.2 Earthworm

E. fetida were obtained from a local retailer of vermi-cultural supplies (Bunnings NZ–Wellington). All earthworms used for the experiments were same aged adults with well-developed clitellum and individual weights between 129 mg and 392 mg.

Worms were sold as and assumed to be *E. fetida*. During the work it became evident that in some cases *E. fetida* may co-occur with *Eisenia andrei*. These two species are almost identical (in morphology), and in earlier work were not distinguished. However, molecular analysis and breeding experiments have led to an acceptance that *E. fetida* and *E. andrei* are two distinct but closely-related species (Plytycz et al., 2018). To establish proportions of each in these experiments, RNA results for 18 individual worms were analysed using the Basic Local Alignment Search Tool (BLAST) database. Results (Appendix 5) showed that 15 of the 18 worms (83.3%) assessed were *E. fetida*. The other two worms were confirmed as *E. andrei*. Results indicate that most (almost 85%) of the worms in the experiments outlined in this thesis were likely to have been *E. fetida*.

3.1.3 Preparation, characterization and optimization of artificial soil

3.1.3.1 Preparation

Artificial soil was prepared according to the OECD guideline (OECD, 2015). Sphagnum peat, kaolin clay and fine sand were ordered from Mitre 10 Petone, Gordon Harris Art Supplies, and PlaceMakers Evans Bay respectively. Artificial soil was prepared using 10% of sieved sphagnum peat, 20% of kaolin clay, 70% of acid-washed quartz sand, and a small amount of calcium carbonate **(Section3.1.3.3)**. The soil was mixed intensely in a Kenwood household mixer until thoroughly mixed.

3.1.3.2 Bulk parameters

Organic matter and moisture content

Moisture content and organic content were measured in both the peat and soil mixture **(Table 3.1)**. To measure moisture content, 6 x 5 g replicates of each were measured into dry pre-weighed 100 mL beakers, which were then placed in a laboratory oven at 120 °C overnight, cooled, and reweighed. For organic content estimation, dry samples were ashed in a muffle furnace at 450 °C overnight. Organic content was estimated as the weight loss on ashing.

Maximum water holding capacity

Maximum water holding capacity was measured in the soil mixture. Three syringes (50 mL) were used to make the setup. Firstly the bottom of the syringes was cut and covered using Whatman 541 filter paper, and weights were measured. The opened cylinders were then filled with dry soil mixture with gentle packing and weighed again. The syringes were gradually submerged in a beaker until the level of water is above the soil level **(Figure 3.1)**. After three hours submerged in the water, the soil samples were allowed to drain for a period of two hours by placing the tubes on a bed of very wet finely ground quartz sand contained within a covered vessel. After drainage of excess water from the unit the samples were weighed, and the water holding capacity (WHC) was calculated using following equation.

WHC (in % of dry mass) = $\frac{S-T-D}{D} \times 100$

Where:

S = Water saturated substrate + mass of tube + mass of filter paper

T= Tare (mass of tube + mass of filter paper)

D= Dry mass

For experiments with worms, the moisture content of the final artificial soil mixture was adjusted to 50% of maximum water holding capacity.

Figure 3.1: The structure used to measure soil water holding capacity

pH

Initial pH of the artificial soil mixture was measured using 5 g soil suspended in 20 mL of 0.01 M AR $CaCl_2$. The mixture was shaken thoroughly using magnetic stirrer for 5 min and the suspension allowed to settle for 3 h. The pH of the liquid phase was measured using a buffer-calibrated pH-meter.

Bulk characteristic results

Results properties of the artificial soil and peat are shown in **Table 3.1**.

Table 3.1: Bulk properties of artificial soil mixture and peat

Soil	Soil type	Mean (n=3)
Moisture content	Soil mixture	0.04±0.002 g
	Peat	0.5±0.005 g
Organic content	Soil mixture	9.6±0.12%
	Peat	84±0.22%
Maximum water holding capacity	Soil mixture	30±81%
pH Value	Soil mixture	3.8±0.13

The moisture content of artificial soil mixture and peat were (0.04±0.002) g and (0.5±0.005) g respectively. The organic content of the sphagnum peat was (84.9±0.2)% and this provided the majority of organic matter in the mixed soil, which had a final organic percentage of (9.6±0.1)%. The maximum water holding capacity of soil the mixture was determined to be (30.8±1.4)%. Before experiments, the dry artificial soil was moistened by adding 50% of the maximum water holding capacity.

Overall, testing of the soil components and the artificial soils confirmed that these were suitable for use in the subsequent experiments.

3.1.3.3 pH optimization and equilibration

Before experiments the pH of the artificial soil needed to be brought up to a suitable range. The average pH of the artificial soil mixture was pH 3.87±0.135 **(Table 3.1)**. According to OECD guidelines the initial pH of the soil mixture should be pH (6±0.5) (OECD, 2015). Adjusting the pH of the soil mix involved the addition of small amounts of $CaCO_3$. Three additions were tested and pH measurements were undertaken in $CaCl_2$ with a 3 h settling time as described above. Based on the

results **(Table 3.2)** 0.5% $CaCO_3$ was selected as suitable for the artificial soil mixture.

Table 3.2: Added $CaCO_3$ amount and average pH in pH optimization process

$CaCO_3$%	pH (average and standard deviation, n=3)
0	3.87±0.14
0.5	5.39±0.08
0.6	5.40±0.03
1	5.84±0.20

However, it was subsequently discovered that when moist soil was left to equilibrate, the pH of the soil continued to increase over time to reach pH 7.1-7.3 over 1-4 weeks to the soil mixture. **Table 3.3** shows the increase in pH with the time after spiking 0.5% of $CaCO_3$ to the soil mixture.

Table 3.3: Changes of pH with the time in artificial soil mixture

$CaCO_3$%	Time (days)	pH
0.5	0	5.76±0.03
0.5	1	6.94±0.16
0.5	7	7.15±0.07
0.5	14	7.32±0.04
0.5	28	7.24±0.10

The increment of pH with the time was observed and increment was rapid during the first 24 h and had reached pseudo-equilibrium after 7 d. The reason for this phenomenon was not determined, but a net reduction in H^+ suggests that after the OECD soils are moistened, a component of the soil mixture becomes capable of net proton adsorption. This effect may have been overlooked as a factor in standardized toxicity testing by the OECD methodology. Acidity itself is likely to arise mainly from low molecular weight acids in the peat component of the artificial soil. A possible reason for the increase in pH over a week (following addition of soil moisture) is adsorption of protons initially released from the peat components to clay minerals. This would most likely involve surface reactions on

the variable-charge kaolin clay mineral phase, which is capable of adsorbing both hydrogen ions and humic substances (Zhu et al., 2015, Huang and Yang, 1995)

Acidity can modify toxicity; for example more acid conditions can promote Cd release (Farrah and Pickering, 1979). As noted above the existence of this change in pH is not explicitly recognised in the OECD protocol, but would be expected to occur in any OECD artificial soil mixture. Under the protocol pH is set at the outset of a trial would rarely be measured during it. This may warrant further investigation for its potential to influence the shorter term toxicity experiments.

3.1.3.4 Background metal concentrations

For metals testing 5 g subsamples of artificial soil mixture, peat, sand and clay (3 replicates each) were measured separately into 100 mL beakers. Samples were ashed overnight at 450 °C and the ash was digested in 20 mL of 4 M AR HNO_3. Digests were made up to 50 mL with distilled deionised water. Metal content was analysed against matrix-matched standards using Flame and Graphite Furnace Atomic Absorption Spectroscopy (AAS) (Analytik Jena Continuum Source ContrAA 700). Results are shown in **Table 3.4.**

Table 3.4: Natural metal concentrations (acid recoverable, mg/kg) of the artificial soil and its main components, based on an analysis of 3 samples

Component	Statistic	Measured element concentration, mg/kg (ppm)						
		Cd	As	Pb	Hg	K	Mg	Zn
Mixture	Mean	0.011	1.31	6.02	0.036	362	182	3.55
	Std dev	0.008	0.02	0.06	0.006	29.7	9.3	0.17
Sand	Mean	0.005	0.07	0.03	0.037	7.40	10	0.2
	Std dev	0.007	0.03	0.18	0.008	0.30	0	0
Clay	Mean	0.003	0.25	4.21	0.101	171	757	1.94
	Std dev	0.003	0.03	0.06	0.005	2.10	51	0.16
Peat	Mean	0.043	0.39	0.13	0.039	141	1357	5.07
	Std dev	0.009	0.01	0.07	0.004	1.20	68.1	0.26
Background soil (Waikato region NZ)	Average	0.110	5.10	11	0.19	490	760	28
Crustal abundance	-	0.150	1.80	14	0.085	20900	23300	70

The metals chosen for measurement included four toxic 'heavy elements' (Cd, As, Pb and Hg), two representative major cations (K and Mg) and one representative essential trace element (Zn), which is commonly elevated when soil shows evidence of anthropogenic influence.

Results are compared with both earth crustal abundances (Haynes, 2014) and natural background values from surface (0-10 cm) soils under native vegetation in the Waikato region of New Zealand (Taylor et al., 2010).

Results show that the individual soil components generally contained lower levels of trace elements than typical background soils, with the exception of peat which had higher Mg. However, the mixed artificial soil consistently had lower levels of every element than reference soils or crustal averages. The study involved spiking soils with Cd and As, and relies on concentrations of these and other toxic elements being low in the artificial soil mixture, before spiking. Concentrations of Cd in the unspiked artificial were 10 times lower than the natural soil in Waikato region, and arsenic values were 3.9 times lower. Pb and Hg concentrations were also low in the artificial soil mixture. This trend continued for the major and essential elements K, Mg and Zn – which are also lower in the artificial soils. However, this was not thought to pose any problem for the worm experiments, because (a) essential elements are also provided from the food source (oats); and (b) the methodology follows a standard OECD protocol.

3.1.4 Preparation of spiked soil

Methods used to determine the effect Cd and As on reproduction and growth were based on OECD protocols (OECD, 2015) for testing of chemicals with experiment-specific modifications in some cases (such as an extension of exposure times beyond 28 d).

3.1.4.1. Selection of optimum exposure concentrations

Artificial soil was spiked with three different concentration Cd solutions prepared from cadmium nitrate ($Cd(NO_3)_2$. Concentrations were prepared in a way that allowed addition of 19% total water to each batch of artificial soil while delivering

the target Cd concentration. Controls were un-spiked artificial soil containing 0.011 mgCd/kg **(Table 3.4**). Final target concentrations in spiked soils were selected based on published toxicological endpoint data for *E. fetida* and related species (Cavanagh and Munir, 2016). The aim was to select a suitable range that would be likely to correspond to low, medium and higher levels of toxicity; at the higher level ideally without causing excess mortality. The lowest concentration (30 mg/kg) of Cd is less than the no observed effect concentration. The medium concentration (90 mg/kg) is three times higher than low value and it is less than lowest observed effect concentration for mortality which is 100 mg/kg (Lock and Janssen, 2001a). The highest concentration (270 mg/kg) is three times higher than medium concentration.

A parallel approach was used for As. Initial As concentrations of 20 mg/kg, 60 mg/kg and 180 mg/kg were selected based on literature values. However, in this case, worm mortality after one week was found to be near total under the 60 mg/kg exposure condition, and complete at 180 mgAs/kg.

Table 3.5: Mortality of *E. fetida* after one week of As exposure

	Mortality after one week		
	20 mgCd/kg	**60 mgCd/kg**	**180 mgCd/kg**
Number	2	38	40
Percentage(%)	5	95	100

Reasons for the difference between these results and toxicological endpoints cited in the literature are unclear but may reflect a lower As adsorption capacity in the OECD soils than natural soils. This is because natural soils form part of the toxicological dataset. Hydrated iron oxides in natural soils are well known to act as an As-binding phase (Parfitt and Smart, 1978). Iron oxides are present in natural soils in ranges from 1-5% and may account for up to 80% of the available surface area. Artificial OECD soils do not contain a discrete iron oxide phase (such as goethite α-FeO(OH) or haematite Fe_2O_3) as part of their formulation but instead rely on clay minerals and humic matter in the peat for most of the binding capacity. This may be a weakness of the OECD methodology when it comes to contaminants

including As which normally show extensive binding to iron or manganese oxides (together called hydrated metal oxides). Bioavailability and toxicity of As may be higher from OECD soils than from ordinary soils.

After failure of the initial experiments two lower As concentrations of 10 mgAs/kg and 20 mgAs/kg were adopted for trials of *E. fetida* in OECD soils in this work, to ensure that exposed worms were challenged, but also survived the first generation. As with Cd clean artificial soil was used as the control condition.

3.1.4.2 Spiking methodology

For Cd, the soil was spiked using Cd nitrate. First, a 10,000 mg/L stock solution was prepared in deionised water from a AR $Cd(NO_3)_2$. Target Cd concentrations (30, 90, and 270 mgCd/kg) in each 2000 g of artificial soil (4 x 500 replicates) were achieved by calculating based on the mass of Cd required in a volume of deionised water that would take the mixture to 60% of the soils' maximum water holding capacity of 30.8% **(Table 3.1)**, followed by thorough mixing of each batch in a Kenwood mixer for 10 min. This approach of using a large water volume ensured an even distribution and good mixing, while simultaneously moistening the spiked soil for experiments. Control samples had the same amount of deionised water and the same treatment, except with no Cd added. After homogenisation, soil mixture was transferred to the beakers and allowed to equilibrate for one week by incubating at (23±1) °C.

It was expected that during the equilibration period, a significant portion of the added Cd^{2+} would progressively bind to adsorptive soil phases to reach a pseudo-equilibrium, and the pH would increase to stabilise **(Section 3.1.3.3)**. Adsorption of Cd to spiked soils reaches pseudo-equilibrium within about 72 h, with the added Cd becoming less available over this time through fixation to soil adsorption sites (Bradl, 2004).

Allowing some time for equilibration was seen as preferable to starting experiments immediately after spiking, at the point when the Cd would be most bioavailable, because that approach would introduce the new variable of lowering Cd availability over time. A lack of allowance for contaminant equilibration times

in the OECD protocol may be seen as another potential blind-spot of the OCED methodology.

Four replicates from each concentration and control were maintained. The initial pH of the medium was adjusted to 5.8 with powdered calcium carbonate. It was again noted that pH of the spiked and moistened soils gradually increased to reach a stable equilibrium value of 7.2 after one week **(Section 3.1.3.3)**.

The same spiking method was used for As exposure experiments. For As, sodium arsenate ($Na_2HAsO_4.7H_2O$) was used to prepare the original stock solution to a concentration of 5000 mgAs/L, and target soil concentrations (after initial experiments) were 10 mg/kg and 20 mg/kg. Spiked soils were prepared in a way that allowed addition of 19.2 % total water to each batch of artificial soil (to achieve 60% of total water holding capacity) while delivering the target of As concentration. Un-spiked artificial soil containing 1.31 mgAs/kg (measured by strong acid extraction) was used as the control. In this case the unspiked soils contained a higher proportion of contaminant relative to the lowest exposure condition (6.5% of 20 mgAs/kg); however, it is expected that the forms of As present in the synthetic OECD soils would be less bioavailable on average than As introduced from the spiking solution. As with Cd, spiked soils were incubated for a week before introducing worms to allow optimal time for equilibration.

3.1.5 Approach to exposing the worms

After the equilibration (pre-incubation) period worms were randomly introduced to beakers as 10 worms per 1L beaker. All worms were mature with well-developed clitella. Individual weights ranged from 129 mg to 392 mg. Worms were then observed for 10 min to confirm that all worms were buried in the soil. The beakers were covered by fabric net and incubated at (23±1) °C in the incubator under dark conditions. During the test period the worms were fed rolled oat (5 g per each beaker with 10 worms) one a week and water lost from the experimental beakers by evaporation was replenished every 2 d, based on a measurement of each beaker's total weight.

3.2 Measurements on worms

3.2.1 Phenotypic variables

At the end of days 28, 56 and 84 in first generation and end of 28 and 56 days in Generation 2 and 3, cocoon production was assessed and any unusual behaviour such as slow movements and morphological changes such as size of the worm were recorded.

At the start and end of each test period the body weights of the earthworms were measured. Before the weight measurements, worms were washed using distilled water and excess water was removed using a paper towel. The growth of each batch of worms during each test phase was calculated based on the changes in weights (as a whole).

3.2.2 Trace element testing

Earthworms were sampled after certain time (28, 56, 84 days) periods. Worms taken for trace element testing were washed two times with tap water and two times with distilled water prior to depuration. For depuration, worms were placed in a 250 mL beaker with a filter paper wetted with deionized water. Beakers were covered by a plastic paper and with air holes to prevent the worm escaping. After a 72 h depuration period, wet weights of worms were measured (g) on a four decimal place balance.

3.2.2.1 Cd and As analysis at Massey University

Cd concentrations in tissue samples (n=3) were determined by acid digestion followed by atomic absorption spectroscopy (Analytik Jena ContrAA 700 tandem flame and graphite furnace instrument). Pre-weighed sampled worms were placed in an oven under 50 °C for 12 hours to evaporate the water from the worm tissues, and then placed in a muffle furnace at 450 °C in order to remove organic matter in the worm tissue. Cadmium was extracted by acid digestion using 4 *M* AR grade HNO_3. Calibration standards were prepared using the same acid matrix. Most Cd concentrations in most samples were sufficiently high to measure in flame atomization mode; where required lower concentrations were determined in

graphite furnace atomic absorption spectroscopy. Arsenic measurements were all undertaken using graphite furnace mode.

3.2.2.2 Selected testing at a commercial lab

A selected set of 34 worm tissue digest samples were sent to an IANZ accredited laboratory (Hill Laboratories, Hamilton) for testing by ICP-MS. Samples were drawn from across all exposure conditions (Cd-only, As-only and co-exposure), as shown in **Tables 3.6-3.8**. All 34 samples were tested for 9 elements: As, Cd, Ca, Co, Fe, Mg, P, Se and Zn.

Table 3.6: Samples selected for external testing from Cd exposure experiments

	Name	Exposure condition	Cd concentration (Massey) (µg/mL)	Weights (g)	Notes
1	Cd/c/1/28 - 1	Control	0.013	0.926	Generation one Cd exposure and recovery test
2	Cd/30/1/28- 3	30 mgCd/kg	0.738	0.545	
3	Cd/90/1/28-1	90 mgCd/kg	0.565	0.279	
4	Cd/270/1/28-1	270 mgCd/kg	0.846	0.284	
5	Cd/1/C/56-1	Control	0.007	0.714	
6	Cd/1/30/56-2	30 mgCd/kg	1.016	0.514	
7	Cd/1/90/56-3	90 mgCd/kg	1.634	0.600	
8	Cd/1/270/56-2	270 mgCd/kg	1.519	0.216	
9	Cd/c/1/84-1	Control	0.018	1.107	
10	Cd/30/c/1/84-2	30-CS	0.907	0.700	
11	Cd/90/c/1/84-1	90-CS	1.192	0.481	
12	Cd/270/c/1/84-1	270-CS	0.858	0.166	
13	Cd/2/C/56-1	Control	0.038	0.768	Cd Generation 2 exposure and recovery
14	Cd/30/2/56-2	30 mgCd/kg	2.063	0.767	
15	Cd/30/c/2/56-2	30-CS	0.583	0.824	
16	Cd/90/2/56-2	90 mgCd/kg	1.522	0.453	
17	Cd/90/C/2/56-1	90-CS	1.453	0.980	
18	Cd/3/C/56-1	Control	0.019	0.553	Cd Generation 3 Cd exposure and recovery
19	Cd/3/30/56-2	30 mgCd/kg	0.756	0.363	
20	Cd/3/30-CS/56-3	30-CS	0.165	0.287	

Table 3.7: Samples selected for external testing from As exposure experiments

Sample no	Name	Exposure condition	As Concentration (Massey) (µg/mL)	Weight (g)	Notes
21	As/1/C/28-2	Control	0.56	0.25	
22	As/1/10/28-2	10 mgAs/kg	3.99	0.30	
23	As/1/20/28-3	20 mgAs/kg	11.7	0.18	
24	As/1/C/56-2	Control	1.70	0.15	Generation 1
25	As/1/10/56-3	10 mgAs/kg	13.4	0.11	As exposure
26	As/1/20/56-3	20 mgAs/kg	38.1	0.29	and recovery
27	As/1/C/84	Control	9.40	0.13	
28	As/1/10-CS/84-2	10-CS	11.9	0.79	
29	As/1/20-CS/84-2	20-CS	14.2	0.45	
30	As/C/2/56-1	Control	1.70	0.27	
31	As/2/10/56-3	10 mgAs/kg	13.4	0.39	Generation 2 As exposure
32	As/2/20/56-2	20 mgAs/kg	0.39	0.29	

Table 3.8: Samples selected for external testing from co-exposure (Cd and As) experiments

Sample No	Sample name	Exposure condition	Cd concentration (µg/mL)	As concentration (µg/mL)	Weight (g)	Notes
33	Co-ex/1/T/56-2	90 mgCd/kg 20 mgAs/kg	0.529	10.0	0.341	Co-exposure
34	Co-ex/1/C/56-1	Control	0.031	0.470	0.228	

3.2.2.3 Assessment of accuracy

Cd and As results for tissue digest samples tested at both locations (Massey University and Hill Laboratories) provide the opportunity to assess the analytical accuracy of the determinations made in this work. **Table 3.9** provides a comparison of results.

Table 3.9: Inter-laboratory comparison of Cd and As concentrations

Concentration range	Cadmium			Arsenic		
	Hill Laboratories, ICP-MS	Massey, AAS	Ratio M/H	Hill Laboratories, ICP-MS	Massey, AAS	Ratio M/H
lower-level determinations (< 1 µg/mL)	0.13	1.75	13.5	0.20	0.56	2.79
	0.2	0.68	3.40	0.48	0.74	1.54
	0.21	0.82	3.90	0.50	0.66	1.30
	0.29	0.50	1.72			
	0.44	6.80	15.5			
	0.58	2.49	4.29			
		Mean	**7.04**		*Mean*	**1.88**
		% RSD	**83.1**		*% RSD*	**42.6**
higher level determinations (> 1 µg/mL)	27.7	28.7	1.04	2.05	1.70	0.83
	38.2	35.4	0.93	4.13	3.99	0.97
	65.7	64.8	0.99	7.77	7.17	0.92
	74.8	77.6	1.04	8.89	10.5	1.18
	75.2	67.7	0.90	11.9	11.7	0.98
	102	74.1	0.73	12.1	11.9	0.99
	106	98.8	0.93	15.3	13.4	0.88
	106	104	0.98	15.4	14.2	0.92
	111	101	0.91	36.3	38.1	1.05
	135	124	0.92	41.2	45.7	1.10
	147	136	0.93	47.8	57.9	1.21
	150	134	0.90			
	166	149	0.90			
	199	168	0.84			
	280	258	0.92			
	379	35	0.93			
		Mean	**0.92**		*Mean*	**1.00**
		% RSD	**7.9**		*% RSD*	**12.1**

Results show generally good agreement for higher-level determinations, defined here as those >1 µg/mL, which comprised the bulk of determinations in this work as they related to the exposed worms. For Cd and As the mean ratio of Massey/Hill Laboratories results were 0.92 and 1.00, respectively, and low relative standard deviations 9% RSDs **(Table 3.8).** These results suggest good accuracy for both Cd and As, but potential for a mean ~8% lower result for Cd in samples tested at Massey.

Results show some problems for the lower level determinations, defined here as those (<1 µg/mL). At these levels Cd testing at Massey showed greater variability, and results for both elements were consistently higher in samples tested at Massey than those tested at Hill Laboratories. This outcome may reflect the proximity of some Massey determinations to instrumental detection limits (and superior detection limits of ICP-MS), and is likely to have introduced some noise to the lower level measurements for samples tested only at Massey. Despite this problem there was no prospect of confusing the high and low level determinations, because results for exposed worms were always substantially above the highest results for worms in control soils. This can be seen in graphs that compare the different exposure settings **(Chapters 4, 5 and 6)**, where mean results for Cd and As worms harvested from control soils are always very low compared with those of worms taken from any other exposure condition.

3.2.3 RNA transcriptome measurements

3.2.3.1 Samples and their preparation

E. fetida RNA was extracted for further analysis in key samples **(Tables 3.10-3.12)** after 28, 56 and 84 d for Generation 1 and after 28 and 56 days in Generations 2 and 3. At the end of each period worms were removed from the soil and depurated prior to sampling for the sequencing. After depuration, the worms were washed with RNAase free water and a QIAGEN mini kit was used to extract RNA. Extraction was carried out in a clean PC2 environment. The extracted RNA was stored in a freezer at -80°C until all samples were collected. RNA concentrations were measured by Denovix (Spectrometer/Fluorimeter). Samples chosen for sequencing are identified in **Tables 3.10 to 3.12**.

Table 3.10: Samples collected for sequencing from Cd exposure experiments

Sample name	Generation	Sampled time (d)	RNA concentration (ng/µL)
Control	Generation 1	28	97.6
30 mgCd/kg	Generation 1	28	64.1
90 mgCd/kg	Generation 1	28	31.8
270 mgCd/kg	Generation 1	28	119
Control	Generation 1	84	106
30-CS	Generation 1	84	176
90-CS	Generation 1	84	162
270-CS	Generation 1	84	133
Control	Generation 2	56	97.6
30	Generation 2	56	44.1
90	Generation 2	56	104
30-CS	Generation 2	56	63.0
90-CS	Generation 2	56	86.9

Table 3.11: Samples collected for sequencing from As exposure experiments

Sample name	Generation	Sample time (d)	RNA concentration(ng/µL)	Total RNA amount
As-control	1	28	156.2	3130
As-10 mg/kg	1	28	200.6	4010
As-20 mg/kg	1	28	193.9	3880
As-control	1	56	143.4	2890
As-10 mg/kg	1	56	119	2380
As-20 mg/kg	1	56	132.8	2660
As-Control	1	84	212.9	4260
As-10-CS	1	84	122.8	2460
As-20-CS	1	84	130.4	2608
As-Control	2	56	118.2	2360
As-10 mg/kg	2	56	187.5	3750
As-20 mg/kg	2	56	199.6	3990

Table 3.12: Samples collected for sequencing from co-exposure experiments

Sample Name	Generation	Sample time (d)	RNA concentration (ng/µL)	Total amount of RNA
Co-ex/1/C/28	1	28	163.9	4590
Co-ex/1/T/28	1	28	65.34	1830
Co-ex/1/C/56	1	28	128.8	3610
Co-ex/1/T/56	1	28	103.1	2890

Samples were tested in two main batches, the first (in Dunedin, New Zealand) for Cd-only samples; and the second (in China) for samples from the As and co-exposure experiments.

As each of the two batches of tests cost in the order of NZD10,000, no replicate testing was able to be undertaken as part of this work. (Funding was made available from both internal Massey University sources and by way of external contract work undertaken by a PhD supervisor, but was only sufficient to allow testing of single samples.)

3.2.3.2 Testing undertaken

RNA sequencing was carried out at Otago University (Dunedin New Zealand) and through Custom Science NZ Ltd (preserved samples tested in China) using an Illumina HiSeq 2500. Which is composed on four basic steps; sample preparation, cluster generation sequencing and data analysis.

3.2.3.3 Approaches to data analysis

A range of software tools were used in interpreting the sample results.

A de novo transcriptome assembly of *E. fetida* was assembled using Trinity software (Nature biotechnology). *Trinity* combines three independent *software* modules: Inchworm, Chrysalis, and Butterfly; which are applied sequentially to process large volumes of RNA-Seq reads (Grabherr et al., 2011). Trimmomatic (Bolger et al., 2014) was used to quality trim reads. This package is designed as a flexible trimmer for Illumina sequence data. Trinotate (Nature biotechnology) was

used to functionally annotate the transcriptome file and TransDecoder (Kim et al., 2017) to generate peptide sequence files. Results from BLASTX, BLASTP and HMMER searches of the transcriptome file were loaded into a Sqlite3 which is a relational database management system database. To identify differentially expressed transcripts; each of the original sequence files were aligned to the de novo assembled transcriptome using Bowtie (Langmead and Salzberg, 2012) and then RSEM (Li and Dewey, 2011) was used to estimate expression values based on the alignments. EdgeR (differential expression analysis of digital gene expression data) (Lai, 2010) was used to calculate differentially expressed genes between the four conditions.

3.3 Pilot run

Before exposure experiments commenced, a pilot run was undertaken to ensure that worms would grow in the OECD artificial soil and identify any problems with smooth operation.

In this run, dry artificial soil was moistened by using deionized water (60% of total water holding capacity). Water and soil were thoroughly mixed using Kenwood household mixture for 10 min. Wetted soil was added to separate beakers and incubated under 23±1 °C for one week under dark conditions.

After this period worms were weighed individually and randomly introduced to test containers (10 worms per container) with uncontaminated artificial soil. The worms were washed with deionized water prior to weighing and excess water was removed by placing them on filter papers. The beakers were covered by fabric net and placed in an incubator at 23±1 °C. Five g of rolled oats were added to each beaker once a week. Beaker weights were measured every 2 d and weight losses were replenished by water. pH of the soil was also measured during the trial run. After 28 d, cocoon production and weights of the worms were assessed to confirm the growth and reproduction of worms in the OECD artificial soil. Results confirmed worms had grown and reproduced proving the artificial soil is a good medium to grow worms for the experiments.

Figure 3.2: Beaker setup in the incubator

3.4 Cadmium toxicity tests

3.4.1 First generation exposure and recovery trials

3.4.1.1 Cd exposure experiments

These experiments were designed to assess the effects of Cd exposure on *E. fetida*. Before the trial began, worms were first conditioned by introducing them to the artificial OECD soil and leaving them to grow for one week. After conditioning they were introduced to control and Cd spiked artificial soils for the Cd exposure experiments. During the experiment the measurements were taken after 28, 56 and 84 days. The design of experiments to assess the impacts of three Cd exposure levels on the first generation of worms is as represented in **Figure 3.3** and **Figure 3.4.**

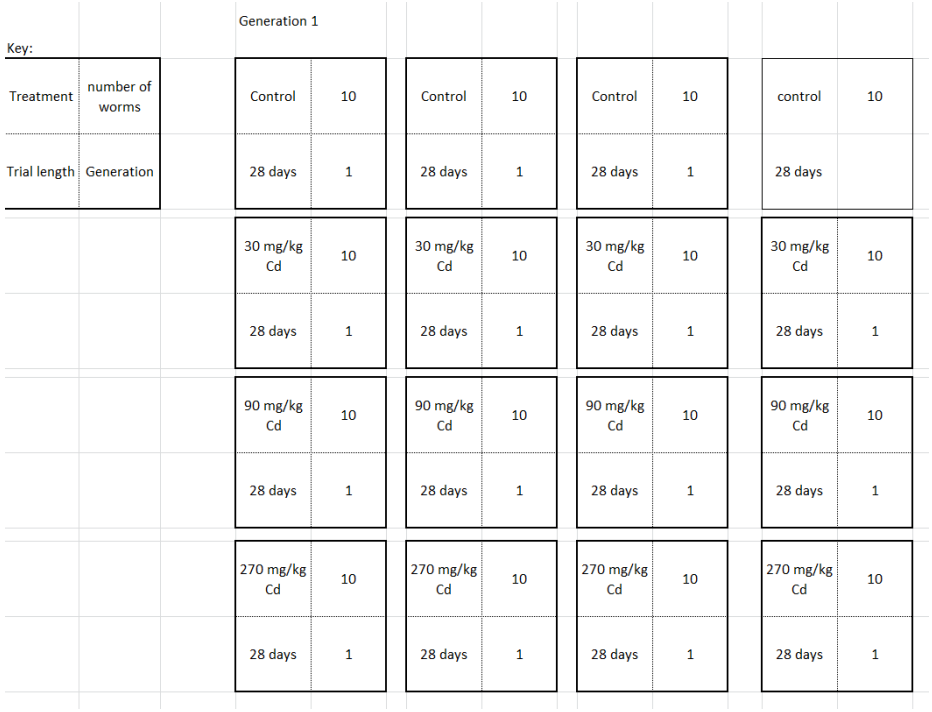

Figure 3.3: Experimental design for Cd exposure experiments on first generation worms-28 d. Each box represents one beaker of worms**.**

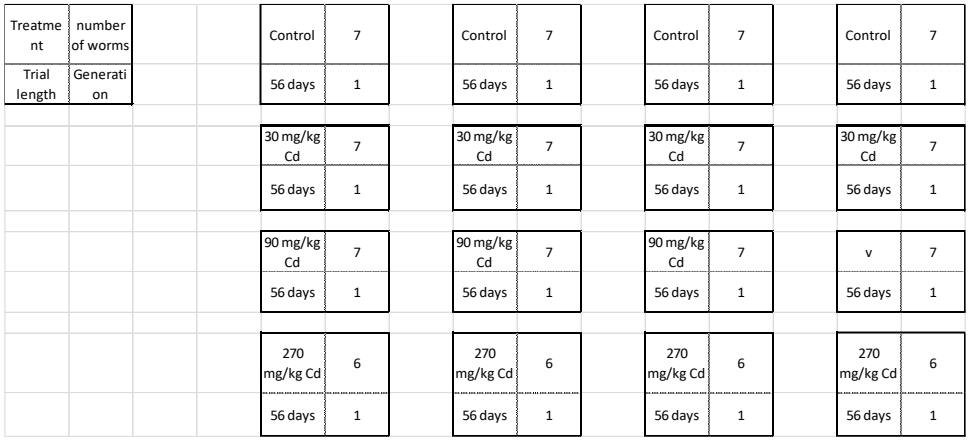

Figure 3.4: Experimental design for Cd exposure experiments on first generation worms-56 d. Each box represents one beaker of worms.

3.4.1.2 Split 28 day recovery and 84 day exposure experiments

After 56 d of Cd exposure, half of the earthworms grown in 30, 90 and 270 mgCd/kg soils were transferred to clean soil for a 28 d recovery period. The other half were transferred to newly prepared Cd spiked soils of the same Cd concentrations as worms had previously experienced, giving these worms a total exposure period of 84 d. After the further 28 d, weights, cocoon production, and Cd in the worm tissue were measured **(Section 3.2)**. The design of these experiments is shown in **Figure 3.5.**

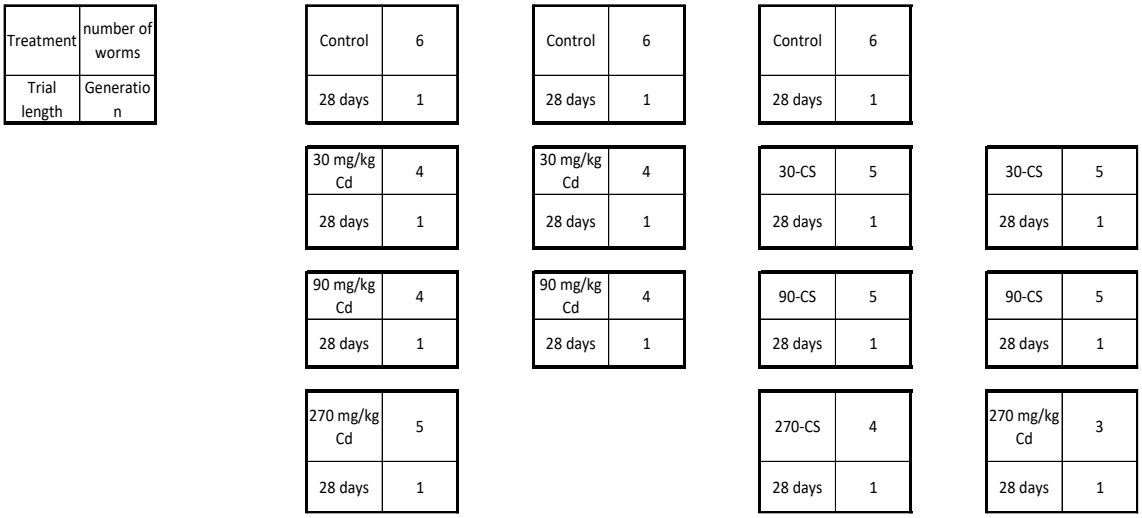

Figure 3.5: Experimental design for generation one experiments after 56 d involving a split between a further 28 d exposure, and 28 d recovery.

3.4.2. Second generation exposure and recovery trials

Hatchlings from the first generation worms that had been exposed for 56 d were taken to second generation experiments. Only the control soil, 30 mgCd/kg and 90mgCd/kg soils contained cocoons and hatchlings after 56 d of Cd exposure; none were present in 270 mgCd/kg soils. After 56 d generation one, adult worms were removed from each beaker, with eggs and hatchlings being retained in order to hatch and grow. 5 g of oats and replacement water were periodically added as outlined above **(Section 3.3)**. When the hatchlings became adults with developed

clitella (after 3 months) worms were ready for second generation experiments. Worms reached adulthood in control and 30 mgCd/kg soils. However juvenile worms grown in the 90 mgCd/kg soils did not fully mature even after 3.5 months. It is likely that the high concentration of Cd worked to delay the growth of the worms, either delaying or preventing their sexual maturation. As evidence suggested that growth may have ceased it was decided to proceed with the experiments and introduce the 90 mgCd/kg worms to the new exposure conditions as they were. New adults in each concentration condition were divided into two groups. One group was introduced to Cd spiked soil which had same concentration of Cd as to the soil they had been living in. The other group was introduced to clean artificial soil which had only trace (0.011 mgCd/kg) levels of natural Cd as a recovery test both has 10 worms per each. Measurements (weights, cocoon counts, and tissue [Cd]) were taken after 28 and 56 d.

The design of experiments to assess impact of Cd exposure levels on second generation worms as represented in **Figure 3.6** and **Figure 3.7**.

Treatment	number of worms			Control	10		Control	10
Trial length	Generation			28 Days	2		28 Days	2
				30 mg/kg Cd	10		30 mg/kg Cd	10
				28 Days	2		28 Days	2
				90 mg/kg Cd	10		90 mg/kg Cd	10
				28 Days	2		28 Days	2
				30-CS	10		30-CS	10
				28 Days	2		28 Days	2
				90-CS	10		90-CS	10
				28 Days	2		28 Days	2

Figure 3.6: Experimental design for Cd exposure and recovery experiments on second generation worms-28 d. Each box represents one beaker of worms. Measurements were undertaken at 28 and 56 d.

Treatment	number of worms			Control	7		Control	6
Trial length	Generation			56 days	2		56 days	2
				30 mg/kg Cd	7		30 mg/kg Cd	6
				56 days	2		56 days	2
				90 mg/kg Cd	7		90 mg/kg Cd	6
				56 days	2		56 days	2
				30-CS	7		30-CS	6
				56 days	2		56 days	2
				90-CS	7		90-CS	6
				56 days	2		56 days	2

Figure 3.7: Experimental design for Cd exposure and recovery experiments on second generation worms-56 d. Each box represents one beaker of worms. Measurements were undertaken at 28 and 56 d.

3.4.3 Third generation exposure trials

Experiments for third generation Cd toxicity tests paralleled those of second generation tests. The main difference was that as generations progressed, fewer worms became available for each test condition. In second generation experiments, worms in both control and 30 mgCd/kg soils produced cocoons during the experimental period. Therefore in third generation experiments were based on only one test condition (30 mgCd/kg) and control. After the growth period the offspring from the second generation were introduced to new soil beakers in order to start Generation 3 experiments. Half of worms from 30 mgCd/kg soils were introduced to clean artificial soil, and other half were introduced to the Cd spiked soil.

After 28 and 56 d, cocoon counts, weights, and Cd concentrations in worm tissue were measured. The design of experiments to assess impact of Cd on Generation 3 of worms is represented in **Figure 3.8** and **Figure 3.9**.

Treatment	number of worms			Control	10		Control	10
Trial length	Generation			28 days	3		28 days	3
				30 mg/kg Cd	10		30 mg/kg Cd	10
				28 days	3		28 days	3
				30-CS	10		30-CS	10
				28 days	3		28 days	3

Figure 3.8: Experimental design for Cd exposure and recovery experiments on third generation worms-28 d. Each box represents one beaker of worms. Measurements were undertaken at 28 and 56 d.

Treatment	number of worms			Control	10		Control	10
Trial length	Generation			56 days	3		56 days	3
				30 mg/kg Cd	10		30 mg/kg Cd	10
				56 days	3		56 days	3
				30-CS	10		30-CS	10
				56 days	3		56 days	3

Figure 3.9: Experimental design for Cd exposure and recovery experiments on third generation worms-56 d. Each box represents one beaker of worms. Measurements were undertaken at 28 and 56 d.

3.5 Arsenic toxicity experiments

3.5.1 Initial experimental setup

In first design of the As experiment three test concentrations were used: the lowest was 20 mgAs/kg, the intermediate was 60 mgAs/kg and the highest was 180 mgAs/kg. After spiking artificial soil with As the soil was pre-incubated for 1 week at (23 ± 1) °C before worms were introduced to the beakers.

Before introducing worms to the soil, they were cleaned (twice with tap water and twice with distilled water). Excess water was removed using a filter paper. After weighing, worms were randomly introduced to the beakers. After 24 h, the majority of the worms were lethargic and some of the worms were dead. After 48 h, all worms in 180 mgAs/kg soils were dead, and only 2 worms were left in the 60 mgAs/kg soils. Worms in control soils were healthy and alive, and out of 40 worms only 4 were dead in 20 mgAs/kg soils. 38 (95%) worms were dead in 60 mgAs/kg and all worms in 180 mgAs/kg soils dead within a seven day period.

As describe above (**Section 3.1.4.1**) the reason for this phenomenon is likely to relate to a higher availability of As in the OECD artificial soil compared with ordinary soils (on which some of the toxicological information was based).

Because of higher mortality observed under medium and higher As concentrations, it was decided to decrease the test concentrations. The new highest spiked concentration was limited to 20mgAs/kg, and lowest one was 10 mgAs/kg **(Section 3.1.4.1)**. The As exposure tests were conducted on up to two generations.

3.5.2 First generation exposure and recovery trials

Four replicate beakers of worms were maintained for each of the three conditions: controls, 10 mgAs/kg, and 20 mgAs/kg. The initial pH of the soil was adjusted to pH 5.8 and after 2 d of pre-incubation period, this had increased up to pH 7.1 (as in the Cd experiments). After one week of pre-incubation, 10 worms were randomly introduced into each beaker and observed for 10 min to confirm that all worms were buried to the soil. The beakers were incubated under (23±1) °C in continuous darkness. Samples were collected and measurements undertaken after 28, 56 and 84 d in first generation trials, and after 28 and 56 d in second generation experiments. As for Cd, measurements included weight, number of cocoons per beaker, and tissue As concentrations, and RNA was extracted from for further testing **(Table 3.10).**

Firstly worms were picked from the soil and checked for phenotypic changes. Before weighing worms were washed (twice with tap water, once with distilled water) and excess water was removed using a clean filter paper. Six worms from across replicates in each condition were sampled: three worms to measure As concentrations, three worms to extract RNA. The other worms were re-introduced to the same soil after 28 d and new soil with the same condition after 56 d.

Recovery experiments for As were design in the same way as Cd recovery experiments **(Section 3.4.1.2)**. After 56 d, the worms in each condition divided in to two groups. One group was introduced to clean artificial soil and other group was introduced to As spiked soils containing the same As concentrations as worms had experienced over the previous 56 d. After a further 28 d worms were weighed,

cocoons per beaker were counted, and selected worms were sacrificed to determine tissue As concentrations or extract RNA for further testing **(Table 3.11)**.

Treatment	Number of worms	Control	10	Control	10	Control	10	Control	10
Trial length	Generation	28days	1	28 days	1	28 days	1	28 days	1
		10 mg/kg As	10	10 mg/kg As	10	10 mg/kg As	10	10 mg/kg As	10
		28 days	1	28 days	1	28 days	1	28 days	1
		20 mg/kg As	10	20 mg/kg As	10	20 mg/kg As	10	20 mg/kg As	10
		28days	1	28 days	1	28 days	1	28days	1

Figure 3.10 : Experimental design for As exposure experiments on first generation worms-28 d. Each box represents one beaker of worms.

Treatment	Number of worms	Control	7	Control	7	Control	7	Control	7
Trial length	Generation	56 days	1	56 days	1	56 days	1	56 days	1
		10 mg/kg As	7	10 mg/kg As	7	10 mg/kg As	7	10 mg/kg As	7
		56 days	1	56 days	1	56 days	1	56 days	1
		20 mg/kg As	7	20 mg/kg As	7	20 mg/kg As	7	20 mg/kg As	7
		56 days	1	56 days	1	56 days	1	56 days	1

Figure 3.11 : Experimental design for As exposure experiments on first generation worms-56 d. Each box represents one beaker of worms.

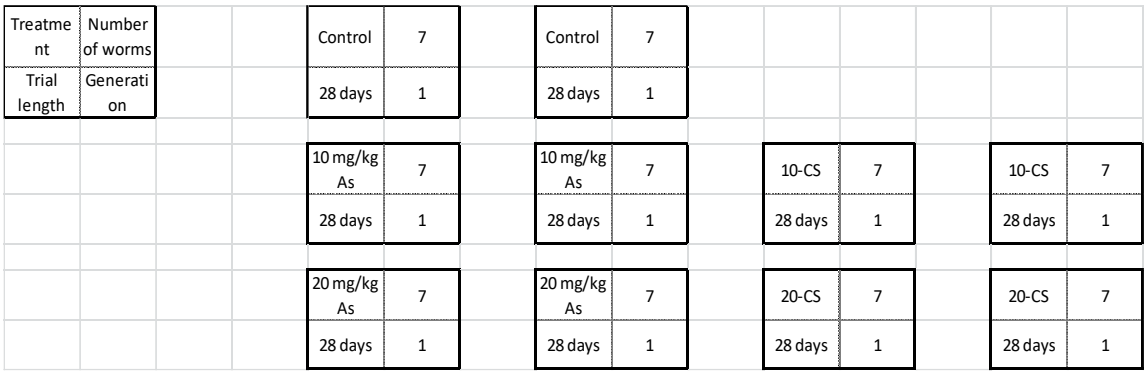

Figure 3.12 : Experimental design for split exposure and recovery experiments on first generation worms.

3.5.3 Second generation exposure trials

Hatchlings from the first generation were taken to second generation trials. After 56 d of As exposure adult worms were removed from the beakers and cocoons were left behind to hatch under the same conditions. Hatched worms were left in the same beaker to sexually mature. Other aspects of the As second generation experiments were the same as those outlined in the Cd toxicity experiments **(Section 3.4.2)**. The design of experiments to assess impact of As exposure on second generation worms is provided in **Figure 3.13** and **Figure 3.14**.

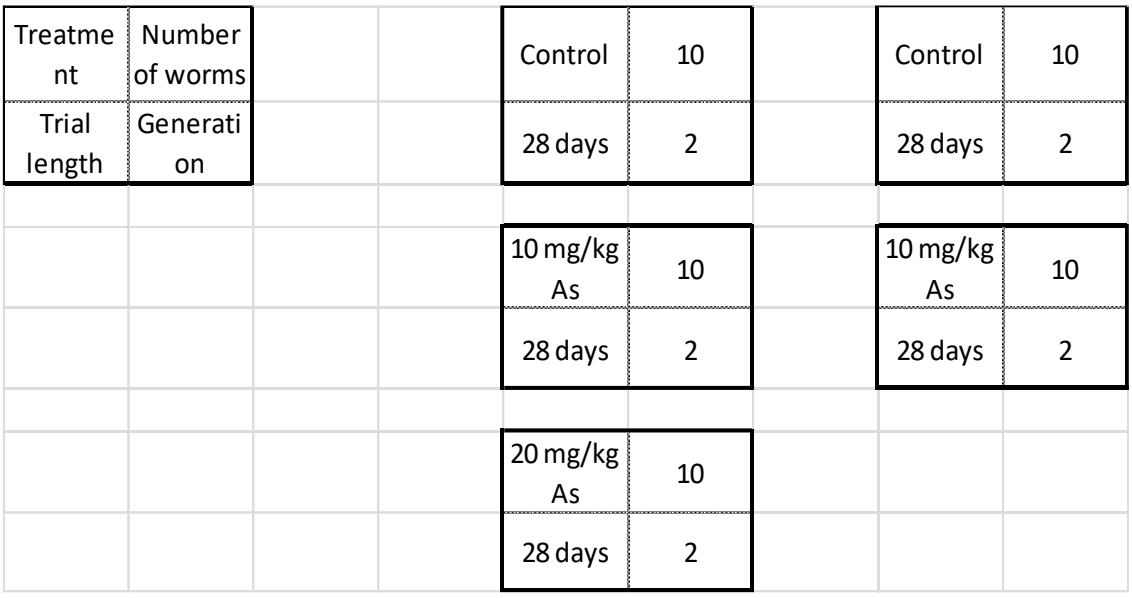

Figure 3.13 : Experimental design for As exposure experiments on second generation worms-28 d. Each box represents one beaker of worms.

Treatment	Number of worms			Control	6		Control	7
Trial length	Generation			56 days	2		56 days	2
				10 mg/kg As	6		10 mg/kg As	7
				56 days	2		56 days	2
				20 mg/kg As	3			
				56 days	2			

Figure 3.14 : Experimental design for As exposure experiments on second generation worms-56 d. Each box represents one beaker of worms.

For As, the number of juvenile worms in a cocoon was analysed by dissecting cocoons. Five cocoons from each condition were dissected under the dissection microscope and number of juvenile worms in the cocoons were counted.

3.6 Co-exposure experiments

The co-exposure of As and Cd was also studied. For this experiment, one concentration was chosen for each element to allow matching with individual As and Cd experiments. 90 mgCd/kg was selected as the Cd concentration because this concentration gave interesting results in the Cd experiments without causing significant mortality. 20 mgAs/kg was selected as As concentration for the co-exposure experiment for similar reasons and as the highest concentration used.

OECD artificial soil was spiked with 90 mgCd/kg and 20 mgAs/kg. As with the individual experiments, dilutions were prepared in a way that allowed addition of a set amount of water to each batch of artificial soil (to reach 60% water holding capacity) while delivering the target As and Cd concentrations. After spiking soil with Cd and As the soil medium was incubated at (23±1) °C in the dark for 7 d to allow equilibration.

After this period the cleaned and weighed worms were randomly introduced to soil beakers which were set up as one test condition and control, all in triplicate. 5 g of oats was given to each beaker as food. After confirming that all worms had buried themselves the beakers were covered with fabric net and incubated at (23±1) °C in dark conditions. Weight loss caused by evaporation was replenished by addition of water in every 2 d.

After 28 d and 56 d exposure cocoon production was assessed. Weights of re-cleaned worms were measured and selected worms were sacrificed for RNA extraction and tissue metal analysis. RNA and metal extractions were as outlined in **Sections 3.2.3** and **3.2.2**, respectively. The design of experiments to assess the impact of co-exposure of Cd and As on generation one of *E. fetida* is presented in **Figures 3.15 and 3.16**.

Treatment	Number of worms	Control	10	Control	10	Control	10
Trial length	Generation	28 days	1	28 days	1	28 days	1
		90 mgCd/kg 20 mgAs/kg	10	90 mgCd/kg 20 mgAs/kg	10	90 mgCd/kg 20 mgAs/kg	10
		28 days	1	28 days	1	28 days	1

Figure 3.15: Experimental design for Cd and As co-exposure experiments-28 d. Each box represents one beaker of worms.

Treatment	Number of worms	Control	7	Control	7	Control	7
Trial length	Generation	56 days	1	56 days	1	56 days	1
		90 mgCd/kg 20 mgAs/kg	7	90 mgCd/kg 20 mgAs/kg	7	90 mgCd/kg 20 mgAs/kg	7
		56 days	1	56 days	1	56 days	1

Figure 3.16: Experimental design for Cd and As co-exposure experiments-56 d. Each box represents one beaker of worms.

Chapter 4: Uptake and effect of Cd exposure to *E. fetida* over three generations

4.1 Introduction

Earthworms are one of the first organisms affected by heavy metal contamination in soils. Toxicity and bioaccumulation of soil toxicants have been observed in different species of earthworms such as *Eisenia fetida* (Neuhauser et al., 1985, Spurgeon and Hopkin, 1999), *Eisenia Andrei* (Van Gestel et al., 1993a, Peijnenburg et al., 1999) and *Lumbricus terrestris* (Šrut et al., 2017, Kennette et al., 2002). In this study, the earthworm *E. fetida* was used as the test organism. A particular advantage of *E. fetida* is rapid maturation; in fact the worms were reproductively mature within 3 to 4 months in clean artificial soil. Prior to the investigations reported here there was limited literature available on the effects of Cd across different generations of *E. fetida*.

The purpose of this part of the study was to determine how different Cd concentrations affect growth, reproduction and gene expression of *E. fetida* in three consecutive generations, and also characterise recovery when exposure stops. These experiments involved measurements of the Cd concentrations in worm tissues, weight, cocoon production and differential gene expression. The same parameters were measured during the recovery period. Principle objectives of the experiments outlined in this section of the thesis were to determine:

- how different Cd concentrations in soil and time related to Cd uptake, growth, reproduction and gene expression of *E. fetida*;
- the extent to which worms have the ability to recover from effects of Cd toxicity after exposure; and
- whether and how the effects of Cd exposure impact on the next generations, including whether offspring of exposed parents show any ability to limit their Cd uptake.

4.2 Phenotypic effects

Phenotypic variables that were assessed included weight, cocoon count, mortality, size, and activity. Results for measured phenotypic variables in the Cd exposure experiments for Generations 1, 2 and 3 are provided in **Appendix 2**

In general, weights show very good agreement between replicate experiments set up in different beakers, with most identical exposure cases being statistically indistinguishable from each other based on Student's t-test 95% confidence intervals about mean values. This result itself demonstrates good reproducibility of the effects on growth recorded under each exposure condition, giving confidence that observed impacts are likely to be caused by the toxic exposures involved.

The good reproducibility between beakers also makes it possible to combine the results for worms under each exposure condition across beakers, providing better statistical power. Combined weight and cocoon count data (for the three generations) and full results for the chemical analysis of worm tissue are provided in **Appendix 2**.

4.2.1 Generation One

4.2.1.1 Overview

Summary statistics showing mean weights, cocoon counts and Cd content of worms in Generation 1 over time are provided in **Table 4.1**. Results cover both accumulation of Cd, and losses after worms that had been exposed for56 d were returned to clean soil for 28 d.

Table 4.1: Summary statistics relating to Cd effects on *E. fetida* – Generation 1. Results are reported as means with standard deviation in bracket

Soil cadmium condition and [Cd]	Measured variable	Time (days) 0	28	56	84	84 – CS [f]
Unspiked (control) soil: 0.011 mg/kg	Mean weight (g)	0.27 (0.06)	0.30(0.06)	0.39 (0.09)	0.38	
	Tissue [Cd] (mg/kg)	1.17 (0.01)	1.25 (0.01)	0.870(0.50)	1.52 (0.94)	
	Mass Cd per worm (µg)	0.33	0.38	0.34	0.59	
	Cocoons/10 worms (summed)	0	8.30 (2.80)	44.5 (4.10)§	6.11 (3.87)	
	Percent fatalities	0	0	3.6	0	
Lowest spike: 30 mg/kg	Mean weight (g)	0.24 (0.07)	0.29 (0.05) [a]	0.33 (0.07) [b]	0.29 [b]	0.31(0.11) [b]
	Relative weight	1	0.97	0.85	2.03§	0.89
	Tissue [Cd] (mg/kg)	1.17(0.01)	72.2 (4.6)[d*]	100 (10) [d]	138 (7.19) [d]	81.3(8.1)[d]
	Mass Cd per worm (µg)	0.28	21.5 [d *]	33.5 [d]	41.2 [d]	25.2 [d]
	Cocoons/10 worms (summed)	0	8.30 (3.30)	27.9 (6.8) [a]	5 (1.4)	10 (7.1)
	Percent fatalities	0	0	3.6	0	0
Medium spike: 90 mg/kg	Mean weight (g)	0.293 (0.06)	0.224(0.06)[d]	0.324(0.10)[b]	0.198 [b]	0.290(0.1[b]
	Relative weight	1	0.73	0.82	1.34	2.79
	Tissue [Cd] (mg/kg)	1.17(0.01)	108(14) [d *]	135 (2) [d]	240 (4) [d]	123 (0.4) [d]
	Mass Cd per worm (µg)	0.34	24.4 [d *]	43.9 [d]	47.6 [d]	35.9 [d]
	Cocoons/10 worms (summed)	0	2.00 (4.00)	11.3 (6.2) [b]	1.25 (1.41)	5.00(2.12)
	Percent fatalities	0	0	7.1	0	0
Highest spike: 270 mg/kg	Mean weight (g)	0.280 (0.050)	0.150 (0.040) [d]	0.110 (0.020) [d]	0.04	0.12 (0.05) [d]
	Relative weight	1	0.50	0.28	0.11	0.92
	Tissue [Cd] (mg/kg)	1.17(0.01)	149(22)[d*]	368(24.02)[d§]	-	260 (3.13) [d]
	Mass Cd per worm (µg)	0.33	22.6 [d*]	38.9 [d]	-	30.3 [d]
	Cocoons/10 worms (summed)	0	0	0	0	0
	Percent fatalities	0	10.0	21.4	80.0	14.3

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001
Difference between means compared with previous 28 d time period; § <0.05, ¶ <0.01, * <0.001, # <0.0001
[f] The worms from 56 days were separated to clean soil (84-CS) and As contaminated soil (84).

4.2.1.2 Cd uptake by *E. fetida*

Average Cd concentrations in Generation 1 *E. fetida* after 28, 56 and 84 d are shown in **Figure 4.1**. Estimated bioconcentration factors are provided in **Table 4.2**.

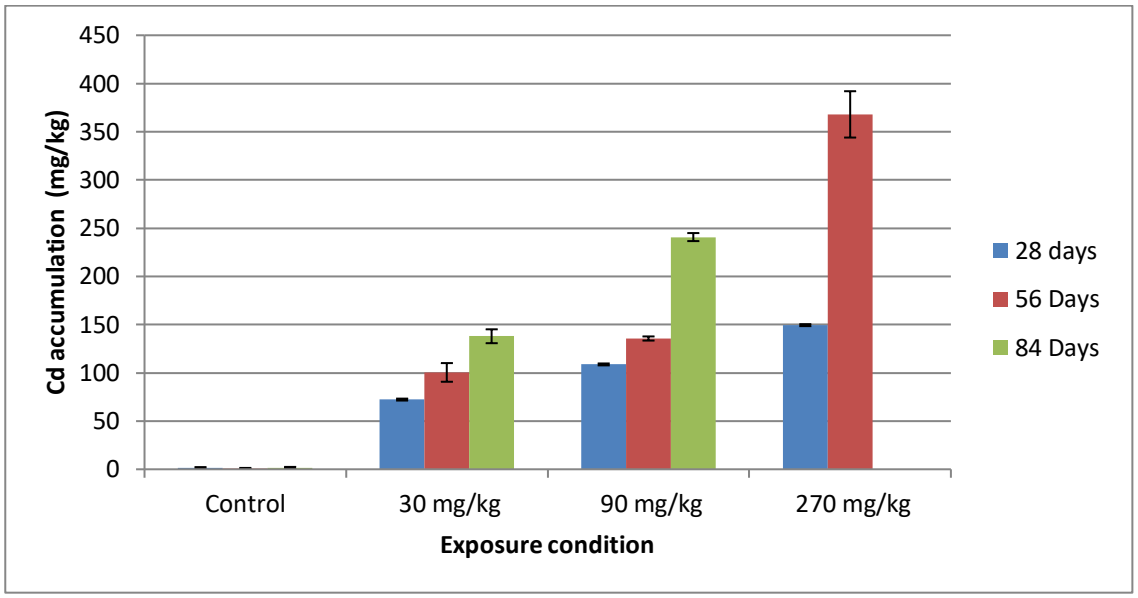

Figure 4.1: Average Cd concentrations in *E. fetida* of Generation 1after being exposed to Cd spiked soil for 28, 56 and 84* d.* no data collected in 270 mgCd/kg due to mortality. Error bars represent standard deviation from the mean. Refer to Table 4.1 for statistical significances of differences between means.

Table 4.2: Cd concentrations and bioconcentration factors(BCFs, bold) for uptake of Cd in *E. fetida* from OECD artificial soil spiked with three different Cd concentrations.

	Cd in soil (mg/kg)			
Variable	0.011	30	90	270
Cd in worm tissue after 28 days (mg/kg)	1.24	72.2 ^{d*}	108 ^{d*}	149 ^{d*}
BCFs	**113**	**2.40**	**1.20**	**0.55**
Cd in worm tissue after 56 days (mg/kg)	0.87	100 ^{d*}	135 ^{d}	368 ^{d§}
BCFs	**79.6**	**3.35**	**1.50**	**1.36**
Cd in worm tissue after 84 days (mg/kg)	1.52	137 ^{d}	241 ^{d}	-
BCFs	**138**	**4.59**	**2.67**	-

Difference between means compared with control condition; ^{a} <0.05, ^{b} <0.01, ^{c} <0.001, ^{d} <0.0001
Difference between means compared with previous 28 d time period; § <0.05, ¶ <0.01, * <0.001, $^{\#}$ <0.0001

Cd accumulation by E. *fetida* was positively correlated with the soil Cd concentrations, and with exposure time (**Figure 4.1**).

As two examples:

- At 28 d, the relationship with soil Cd concentrations (excluding the unspiked soils) is: **Tissue Cd = 81.01 x log(soil [Cd] in mg/kg) - 48.17** (R=0.999, p<0.001).
- For the 90 mg/kg results, the relationship with time is: **Tissue Cd = 2.66 x (number of days) + 9.70** (R=0.978, p<0.01).

In control samples, worms absorbed negligible amounts of Cd.

After 28 d, worms in soils with 30mgCd/kg, 90 mgCd/kg and 270mgCd/kg absorbed (72.3±4.6) mg/kg, (108±14) mg/kg and (149±22) mg/kg of Cd, respectively. At 30 mgCd/kg in soil the Cd concentration in *E. Fetida* was more than 2 times higher the level of Cd in the soil. Worms in soil with 90 mgCd/kg also absorbed Cd to higher tissue concentrations than Cd in the soil. However, for the

worms in 270 mg/kg Cd concentrations in worm tissue were significantly ($p=0.001$) lower than those in soil. This indicates a diminishing efficiency of uptake as soil Cd level increases, and is also reflected in bioconcentration factor (**Table 4.2**).

After 56 d worms in 30 mgCd/kg soil had absorbed (100±10) mg/kg of Cd. This concentration is three times higher than the Cd concentration in the soil. The average levels of Cd in worms in 90 mg/kg and 270 mg/kg soils were 135±2 mg/kg and 367±24 mg/kg, respectively.

Accumulation of Cd continued steadily through the third period of 28 d (84 d total). After this time worms in soils with 30 mg/kg and 90 mg/kg Cd contained (137±7) mg/kg and (240±4) mg/kg Cd respectively. Cadmium data not available for the 84 d 270 mgCd/kg test condition, because there was only one worm remaining alive. This was used for RNA extraction rather than metal analysis.

Cd accumulation was a relatively fast and continuous process during the experiment, and an equilibrium plateau was not reached for Cd uptake after even 84 d. Some other researchers have reported the same effect, but for shorter time scales. Lock and Janssen (2001) reported that internal Cd concentrations in *E. fetida* did not reach equilibrium during an uptake period of 25 d (Lock and Janssen, 2001b). Li *et al* (2010) reported that accumulation of heavy metals in earthworms depends more on the metal bioavailability than the total metal content in the soil (Li et al., 2010). Therefore uptake is commonly found to depend on both soil type and total metal amount in the soil. In OECD artificial soil mixtures the bioavailability of the metal is usually taken to be somewhat higher than in typical soils (Spurgeon and Hopkin, 1996), although key adsorptive components represented by clay minerals and peat are present in similar proportions.

Another reason to expect a higher bioavailability of Cd in the artificial soil is that the Cd was spiked from a soluble form of Cd which was cadmium nitrate ($Cd(NO_3)_2$; although during spiking Cd, 7 d was allowed to give time for adsorption and equilibration. The average strength of bonding increases over that equilibration period and would have reduced the average bioavailability to levels closer to those in normal soils exposed to anthropogenic Cd. However, as total Cd

concentrations increase, the bioavailable proportion of Cd also increases, because the strongest binding sites in the soil are occupied first. At higher Cd loadings a larger proportion of the Cd is bound to weaker sites.

In terms of relative bioconcentration factors (BCF values) (**Table 4.2**), the results show that although overall Cd uptake was positively linked to Cd concentrations in soil, the efficiency of uptake **decreases** as total soil Cd concentration increase. Proportionately, Cd accumulation was most efficient on clean artificial soil which has 0.011 mg/kg of Cd.

Previous researchers have reported that the bioconcentration factor for Cd decreases with increasing Cd concentrations in the soil (Neuhauser et al., 1995, Lock and Janssen, 2001b) and others have reported the same phenomenon for other metals (Wright and Stringer, 1980, Sample et al., 1999, Van Gestel et al., 1993a).

This effect is interesting because is over-rides expectations based on bioavailability, i.e. the decreasing BCF works in the opposite direction to the likely change in exchangeable Cd. As noted above, because the high energy soil adsorption sites are occupied first, it is likely that the proportion of exchangeable Cd increases with increasing Cd in the soils. By itself, that factor should increase Cd uptake in the worms. This contradiction is likely to point to the existence of decreasing efficiency or saturation of uptake pathways as Cd concentrations in soil increase. An attempt to reduce uptake of Cd may come about as a toxicity defence mechanism, or may be a by-product of the natural limitations of uptake pathways. A feature of the Cd results is a very high BCF (138 at 84 d) for Cd uptake from the natural soil. A possible reason for this is the co-extraction and uptake of $Cd^{2+}$with similar but essential elements that are required by the worm.

Two possible candidates are calcium (Ca) and (Zn):

- $Ca^{2+}$and Cd^{2+} are ionic mimics of each other, because they both form divalent ions which have very similar ionic radii (Cd^{2+}=95 pm and Ca^{2+}=100 pm). This means that Cd^{2+} is taken up with Ca^{2+}, and conversely that high dietary Ca may reduce Cd uptake. This relationship between Cd and Ca is a well-known aspect of the human Cd toxicity: for example, the primary

osteotoxic effects of *itai-itai* disease were caused by Cd substituting for Ca in bone tissue (**Chapter 2**).

- Zn is positioned above Cd in Group 12 (IIB) of the Periodic Table and the two elements share some similar chemical behaviours. For example, Zn^{2+} is known to compete with Cd^{2+} for soil adsorption sites (Zhang et al., 2008), and highly dietary Zn is known to reduce Cd absorption through the human gastrointestinal tract (Brzóska and Moniuszko-Jakoniuk, 2001).

The observed behaviour of very high relative Cd uptake at very low concentrations may be caused by worms having evolved very efficient systems extract Ca and/or Zn from soils at their normal soil concentration ranges.

It is possible that uptake systems for these essential elements may either become less efficient, or maybe actively down-regulated, when concentrations in soil increase to excessive levels. This is because at higher concentrations, essential elements such as Zn^{2+} become toxic in their own right. If so the effect of decreasing BCF with increasing soil Cd concentration may also reflect Cd^{2+} 'piggy-backing' on the same uptake pathways that normally regulate the uptake of similar essential metals.

For reference, concentrations of these two essential elements in worm tissues in this study were found to average 551 mg/kg for Ca and 30.4 mg/kg for Zn (**Table 7.1**). These means were similar magnitudes to values in control soils (e.g. Cd Generation 1: 616 mg/kg for Ca and 25.4 mg/kg for Zn) indicating the likelihood that their uptake and order-of-magnitude ranges were being maintained by homeostatic processes. The natural level of Cd in worm tissues was ~0.2 mg/kg, and the highest concentration recorded in the Cd exposure experiments was 379 mg/kg (**Table 7.1**).

4.2.1.3 Mortality and growth

Summary information relating to worm mortality is provided in **Table 4.3** Growth can be expressed in both absolute terms and relative to control soils. Results are provided in graphical form in **Figure 4.2** and **Figure 4.3**, which are derived from the data in **Table 4.1**.

Table 4.3: Percent mortality among generation one worms exposed to Cd.

Condition	Exposure time (days)				
	0	**28**	**56**	**84**	**84 – CS[a]**
Control	0	0	3.6	0	0
30 mg/kg	0	0	2.5	0	0
90 mg/kg	0	0	7.1	0	0
270 mg/kg	0	10	21	80	0

[a] Worms assessed at day 84 that had been returned to control soils after day 56.

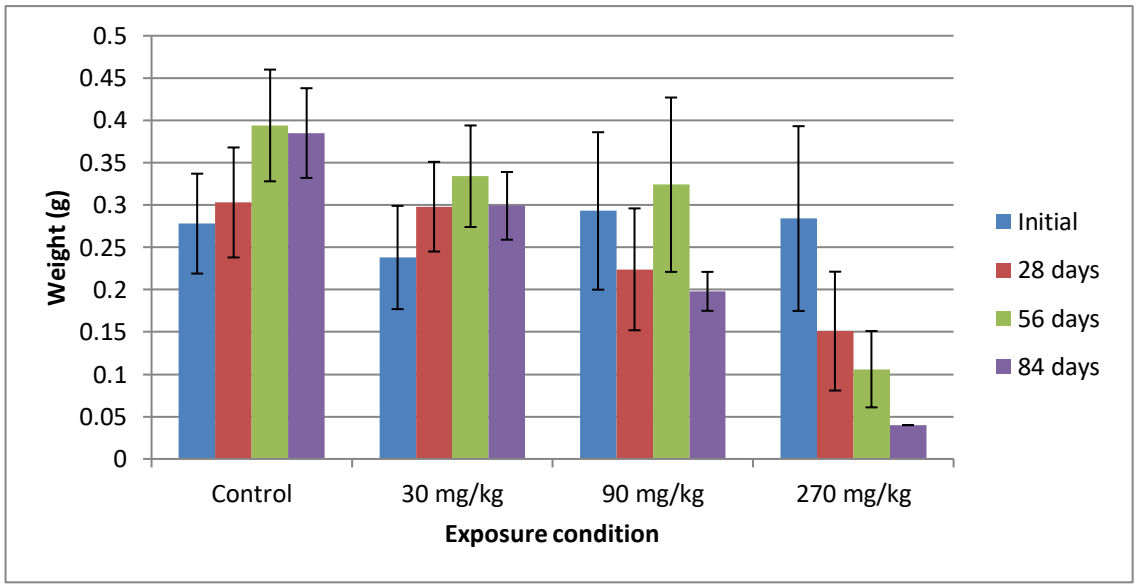

Figure 4.2: Changes in the fresh mean weight of *E. Fetida* in OECD artificial soil spiked with different Cd concentrations (Generation 1). Error bars represent standard deviations from the mean. The results are based on 40 worms**.** Refer to Table 4.1 for statistical significances of differences between means.

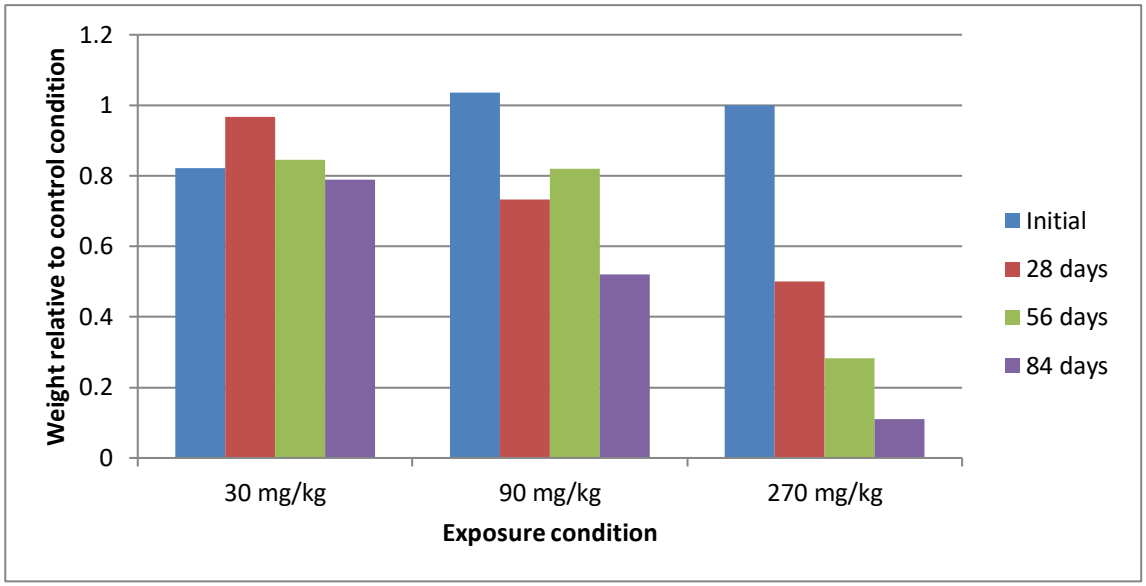

Figure 4.3: Mean relative weights of Generation 1 Cd-exposed worms over time, compared with those in control soils. Refer to Table 4.1 for statistical significances of differences between means.

Different levels of Cd in soil were associated with some obvious phenotypic changes in the worms. These changes were more prominent with increasing Cd concentration.

Mortality of worms increased with increasing Cd concentration and exposure time (**Table 4.3**). In the first 28 d, no mortality occurred in soils with 30, or 90 mgCd/kg and only 10% (4 worms) of worms died in the 270 mgCd/kg condition. In the second 28 d, only 3.6% (1 worm) of worms died in control and 30mgCd/kg soils (respectively) and 7.1% (2 worms) in the 90 mgCd/kg soils. A significant ($p=0.006$) proportion of worms died when exposed to 270 mgCd/kg, adding to 21% (6 worms) after 56 d. By 84 days 80% of worms under this condition had died. This also corresponded to the extremely low weights of the surviving worms relative to those in control soils (see above). Interestingly no further worms died in the 30 mg/kg or 90 mg/kg conditions. Mortality and the weight loss results for the two higher Cd conditions soils were ideal in relation to the aim to examine second and third generation impacts.

Growth of *E. fetida* under lower Cd concentrations was rapid (**Figure 4.2**). When Cd concentrations increased the growth rate of *E fetida* decreased. Under higher

(270mg/kg) and intermediate (90mg/kg) Cd concentrations the worms tended to lose weight during the first 28 d period. However, the worms living under lower Cd concentrations gained weight. The highest growth percentage was showed by worms in 30 mgCd/kg soils (27.9%).

During the second 28 d worms in 90 mgCd/kg soils showed positive growth and the highest growth percentage (28.7%). Worms in 270 mgCd/kg soils showed weight loss throughout the experiment but the weight loss percentage was reduced in the second 28 d. Growth in 30 mgCd/kg soils was not significantly different ($p>0.05$) from that in control soils. Worms in 90 mgCd/kg soil showed significantly ($p=0.0072$) lower weights to controls during first 28 d. However, the same worms showed recovery in growth, exceeding weights of controls after 84 d.

Viewed in terms of relative growth (**Figure 4.3**): after the first 28 d, worms exposed to 30 mg/kg Cd were indistinguishable from those grown in control soils; while those at higher Cd conditions (90 and 270 mg/kg) showed relative weight losses. For worms exposed to the highest Cd levels of 270 mg/kg, this weight loss continued to 56 d and then 84 d. Relative weight losses in this group were on average proportional to total Cd concentrations. Under this condition in 270 mgCd/kg soils, weights relative to those for worms in controls soils approximately halved every 28 d, from 105% (day 1) to 50% (day 28) to 27% (day 56) to only 10% (day 84). Worms under the 90 mg/kg condition (which also initially lost weight) appeared to show a slightly recovery between 28 and 56 days, but the growth was still well below that seen in control soils.

Overall, the growth of worms was clearly affected by Cd, especially during first 28 d. However, with the exception of worms in 270 mgCd/kg soils, all other worms did show positive growth during the second 28 d period.

Significant negative effects of Cd on growth have been reported in the literature (Žaltauskaitė and Sodienė, 2014, Burgos et al., 2005, Spurgeon et al., 2004b). However, this is not always consistent, and depends on the total amount of Cd. Under some conditions, growth is not affected by Cd (Van Gestel et al., 1993a, Spurgeon et al., 1994b, Žaltauskaitė and Sodienė, 2010a). At low Cd concentrations worms did not show significant differences in weight with those grown in control

soils. However, under higher Cd concentrations worms showed weight loss (Burgos et al., 2005, Žaltauskaitė and Sodienė, 2014). Žaltauskaitė and Sodienė (2014) introduced worms to 1,2,5,10,40,100,250, and 500 mg/kg of Cd contaminated soil. These authors reported a significant effect to juvenile growth by Cd ($p<0.001$). Worms at lower concentrations of Cd (1-2 mg/kg) showed very similar growth to the control group. Earthworms treated with 1-40 mg/kg Cd gained weight during 14 weeks which is same as worms were in 30 mgCd/kg soils, while those in 250 and 500 mg/kg Cd soils showed weight loss (Žaltauskaitė and Sodienė, 2014). This is consistent with results for worms in the highest Cd concentration group in this study (270 mgCd/kg).

4.2.1.4 Implications of Cd uptake behaviour for the lethal dose

In these experiments, the lethal dose of Cd in soils could not be viewed as being a fixed number, because Cd by its nature continued to accumulate in worm tissues. What can be said is that lethality was reached over the shortest time period at the highest Cd concentrations. Over 56 d, 270 mg/kg Cd was found to be lethal to 20% of *E. fetida* (**Table 4.3**), and the tissue Cd concentration was ~370 mg/kg (**Table 4.1**). Over 84 d the same concentration resulted in 80% mortality (**Table 4.3**) with no suitable samples being available for tissue analysis. However, at all exposure levels, Cd steadily accumulated. Tissue concentrations, toxicity, and lethality are therefore a function of both soil Cd (including all edaphic factors), and time. These results imply that the lethal dose of Cd cannot be derived from a 28 d test, but has to be considered in relation to:

(a) the rate of Cd accumulation **relative to the worm's optimal life-span**. This is significant, because under favourable conditions, *E. fetida* may have a life-span of more than two years (Venter and Reinecke, 1988), which is equivalent to 26 x 28 d periods.

(b) The fact that the efficiency of Cd uptake (BCF) appears to **increase** with decreasing Cd concentration. This is significant because it implies that extrapolation of results from higher to lower exposures Cd may be invalid. Cadmium may be more toxic to earthworms at lower soil concentrations

than might otherwise be expected, because at the lower concentrations proportionately more Cd is taken up.

Taking the lowest exposure setting in this study (30 mg/kg), a graph can be developed to estimate the time over which Cd may reach 20% lethality. The main uncertainty is about how accumulation continues to occur after the 84 d. For a linear relationship the equation from the 30 mg/kg soils is Cd_{tissue}= 1.5796 (day number) + 11.177; (R = 0.977). These and the other two soil concentration results are shown as extrapolated relationships in **Figure 4.4**.

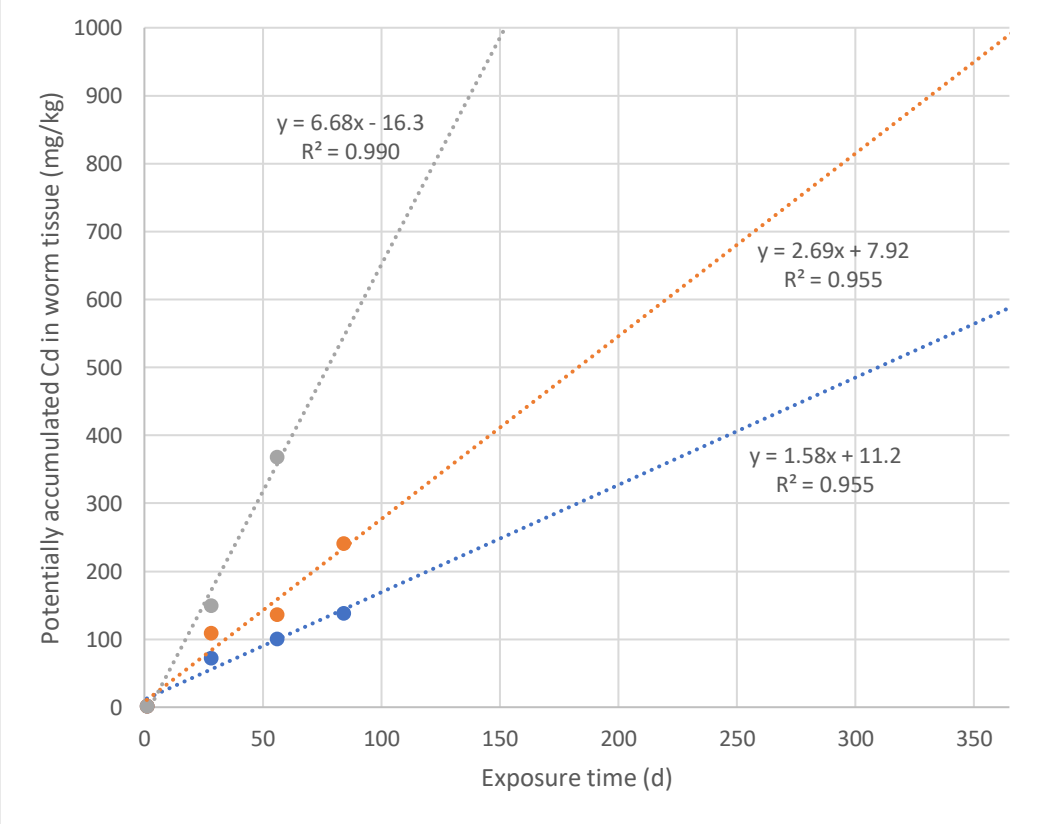

Figure 4.4: Extrapolation of Cd accumulation in worm tissue at soil values of 30 mg/kg, 90 mg/kg and 270 mg/kg (Linear regression). (Equations edited for clarity. Note that some worm tissue results would not be reached due to lethality; 20% lethality occurred once in worm tissue reached 370 mg/kg Cd.)

From this graph it can be suggested that at soil Cd concentrations of 30 mg/kg and 90 mg/kg, tissue concentrations corresponding to 20% lethality might be reached after 227 d and 134 d, respectively.

More widely instead of a single Lethal Concentration (LC) value, there should exist a Cd lethality **envelope** which is defined by soil [Cd], relative BCF, and exposure time. This result for Cd suggests that there is an urgent need to re-think toxicological testing for contaminants like Cd that accumulate over the organism's lifespan.

4.2.1.5 Sexual maturation and cocoon production

Results for cocoon production are shown in **Table 4.4** and **Figure 4.4**.

Table 4.4: Cocoon production by *E. fetida* (per 10 worms) exposed to Cd spiked soil: expressed as both the cumulative total and increase over each 28 d period.

Statistic	**exposure period**	**Control**	**30 mgCd/kg**	**90 mgCd/kg**	**270 mgCd/kg**
Cumulative	0	0	0	0	0
total	28	8.30	8.30	2	0
production	56	44.5 §	27.9 [a]	11.3 [b]	0
(per 10	84	50.6	32.9 [a]	12.6 [b]	0
worms)	84 (recovery)	N/A	37.9	16.3	0
New production during each 28 day exposure period (per 10 worms)	**exposure period**	**Control**	**30 mgCd/kg**	**90 mgCd/kg**	**270 mgCd/kg**
	0	0	0	0	0
	28	8.30	8.30	2.00	0
	56	36.2§	19.6 [a]	9.30 [b]	0
	84	6.11	5.00 [b]	1.25 [b]	0
	84 (recovery)	N/A	10.00	5.00	0

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001
Difference between means compared with previous 28 d time period; § <0.05, ¶ <0.01, * <0.001, # <0.0001

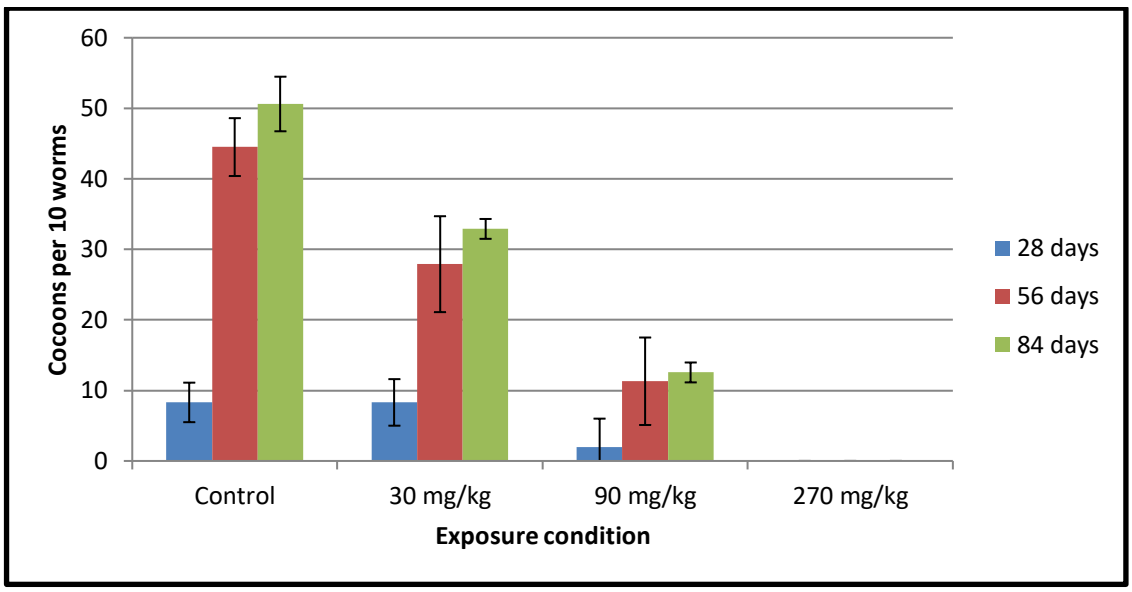

Figure 4.5: Cumulative cocoon production by *E. Fetida* after exposed to Cd spiked artificial soil.). Error bars represent standard deviations from the mean. Refer to Table 4.1 for statistical significances of differences between means.

The concentration of Cd in soil was negatively correlated (R=0.587) ($p<0.05$) with cocoon production (**Figure 4.4**). In the Generation 1 worms, average cocoon production after 28 d was 8.3, 8.3 and 2 per 10 worms exposed to clean soil, 30 mgCd/kg and 90 mgCd/kg respectively. At 28 d there was no significant difference in production between worms under the 90 mgCd/kg and control conditions. However, worms in 30 mgCd/kg and 90 mgCd/kg soils showed significant reductions ($p<0.05$) in cocoon production after 56 d with average number of cocoons per beaker being 44.5±4.1, 27.9±6.8 and 11.3±6.2 per 10 worms in control, 30 mgCd/kg and 90 mgCd/kg soils respectively. These trends continued to 84 days (**Figure 4.4**). There was no cocoon production by worms in 270 mgCd/kg soils. The worms in 90 and 270 mgCd/kg soils decreased their weight during the first 28 d and because of the weight loss the clitella had disappeared from worms. Thereafter *E. fetida* in the highest Cd concentration soil did not reach sexual maturity during the experiment, and the number of worms that reached sexual maturity in 90 mgCd/kg soils was low.

Cadmium severely affected sexual maturity and reproductive success of *E. fetida.* The time needed to reach a sexually mature state increased for increasing Cd concentrations in the soil. These results are consistent with literature reports.

Žaltauskaitė and Sodienė (2014) reported that *E. fetida* incubated at 10 mgCd/kg could reach maturity size after 11.3 weeks, whereas those incubated at 40 mgCd/kg took 12.4 weeks. The number of worms that reached sexual maturity decreased with increasing Cd concentration in soils (Žaltauskaitė and Sodienė, 2014).

Reduction in cocoon production due to Cd exposure was also reported in several studies (Žaltauskaitė and Sodienė, 2010a, Žaltauskaitė and Sodienė, 2014, Van Gestel et al., 1993a, Spurgeon et al., 1994b). Several researchers have reported cocoon production rate is decreased with increasing Cd concentrations in soil (Žaltauskaitė and Sodienė, 2014, Reinecke et al., 1999a, Van Gestel et al., 1993a). In general these results are as might be expected for contaminant exposure. In this sense the results for Cd provide a useful benchmark for interpreting the As exposure results, where unusual impacts on cocoon production occurred (**Chapter 5**).

4.2.1.6 Recovery tests

At the end of the 56 d exposure period the worms under each exposure condition were divided into two groups. One set was transferred to untreated artificial soil, and the other to artificial soil with same Cd concentration as in the previous 56 d, for exposure to continue (**Section 3.4**). At the end of the 28 d recovery period, the concentration of Cd in worm tissue and weights of worms were measured. The amount of cocoons per beaker and number of dead worms was counted and phenotypic changes were observed.

Losses of accumulated Cd

Concentrations of Cd in worm tissue measured for each condition at 84 d are shown in **Figure 4.6** and **Table 4.1**. Results for worms returned to control soils expressed as mass of Cd per worm are provided in **Table 4.5**.

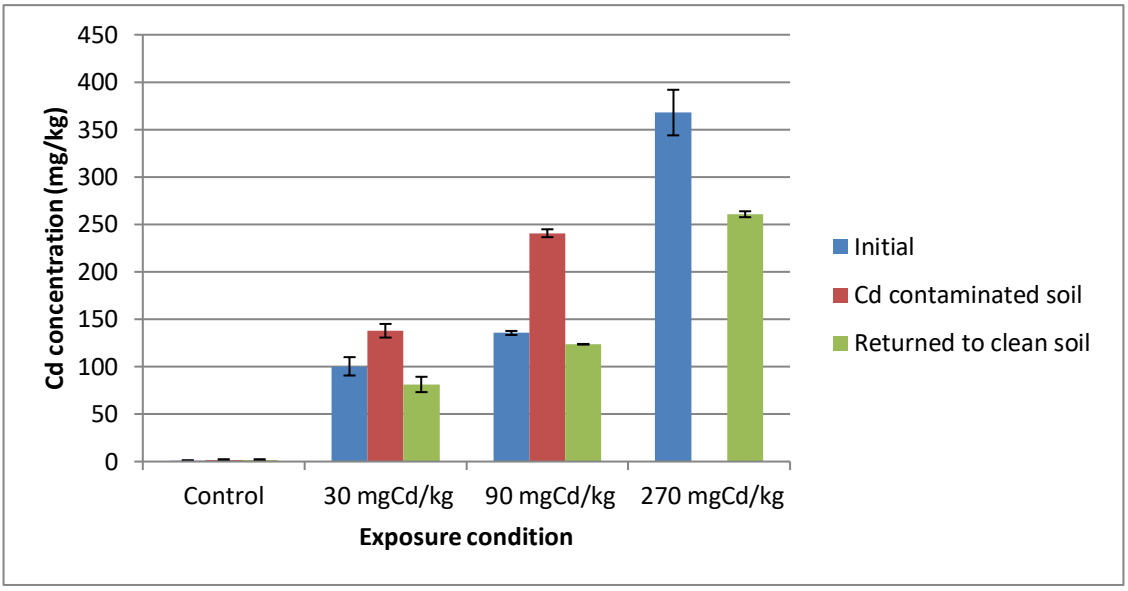

Figure 4.6: Cd concentrations in *E. Fetida* after 84 d of Cd exposure. 'Clean soil' refers to worms that were returned to spend 28 d in clean soil after 56 d of Cd exposure. Error bars represent standard deviations from the mean. Refer to Table 4.1 for statistical significances of differences between means.

Table 4.5: Mass of Cd per worm before and after the 28 d recovery test.

Cd exposure condition to 56 d	Mass Cd per worm (µg)	
	After 56 d of Cd exposure	After 28 d recovery in clean soils
Control soils	0.340	0.590
30 mgCd/kg	33.5	25.2
90 mgCd/kg	43.9	35.9
270 mgCd/kg	38.9	30.3

As previously discussed, worms that continued to be exposed to Cd spiked soil from 56 to 84 d showed an increase in Cd concentrations in their tissues over that final period. Those transferred from contaminated soil to clean artificial soil showed a decrement in Cd concentrations. However, Cd loss was slow. Even after this 28 d recovery period Cd concentrations in worm tissues remained significantly elevated (**Figure 4.6**, **Table 4.5**).

After 28 d worms transferred from 30 mgCd/kg, 90 mgCd/kg and 270 mgCd/kg conditions to clean soil showed Cd concentrations in tissues of 81.3±8 mg/kg,

123±0.3 mg/kg, and 260±3 mg/kg, respectively. By contrast the worms transferred to 30 mg/kg and 90 mg/kg soils continued to accumulate Cd up to 138±7 mg/kg and 240±4 mg/kg, respectively. Only one worm survived in 270 mgCd/kg soil to 84 d and this was used to extract RNA.

Compared with accumulation, elimination was a slow process. The worms transferred from 30 mgCd/kg to clean soil decreased their Cd concentrations by 19.1 mg/kg on average, while in the other group Cd increased by 36.6 mg/kg. Similarly, tissue Cd concentrations in worms transferred from 90 mgCd/kg soils decreased by 12 mgCd/kg when transferred to clean soils, compared with an increase of 105 mgCd/kg for those that continued in spiked soils.

Comparatively, Cd accumulation was a faster process than elimination as mentioned above. This might be because Cd strongly binds with the tissues of *E. fetida.* There were very few reports about recovery, but Gestel *et al* (1993) reported that elimination during a recovery period was a slow process. These authors found that significant Cd residues in the worm tissues remained at the end of their experimental trial period of 3 weeks. An experiment done using *Eisenia andrei* showed elimination of Cd during a 21 d recovery phase which had followed 21 days of Cd exposure. However the variability of Cd elimination was high (Smith et al., 2010).

When considered in terms of the mass of Cd per worm, the worms also showed a reduction in the amount of Cd during the recovery test.

By comparing concentrations and masses it can be shown that almost all (94%) of the Cd concentration reduction in worms that had been exposed to 30 mg/kg soils was actual Cd loss, rather than dilution caused by worm growth and greater tissue mass. In worms that had been exposed to 90 mg/kg soils, half (50%) of the Cd loss was 'genuine', and the other half was caused by the dilution effect of a recovery in worm growth.

An elimination half-life for Cd of ~6 months can be estimated from the mass data (**Table 4.5**) by assuming first-order elimination kinetics, as has been reported for *E. andrei* by Smith et al. (2010). Results are shown in **Table 4.6**.

Table 4.6: Estimated half-lives for Cd loss based on first-order kinetics and total Cd masses.

Condition	Calculated half-life (d)
30 mg/kg	157
90 mg/kg	222
270 mg/kg	179
Average	**186**
Std dev	33

Changes in mortality and growth

During the recovery period mortality only occurred in worms that had been transferred from 270 mgCd/kg to clean soil, and was only 14.3% (1 worm). This compared with 80 % mortality (4 worms) for worms that continued to be exposed to 270 mgCd/kg soils. After 28 d, worms that had remained in control soils showed no significant difference in weight reflecting their maturity, so absolute weight differences between paired conditions also reflect changes relative to control soils. Absolute weight changes are shown in **Figure 4.7**.

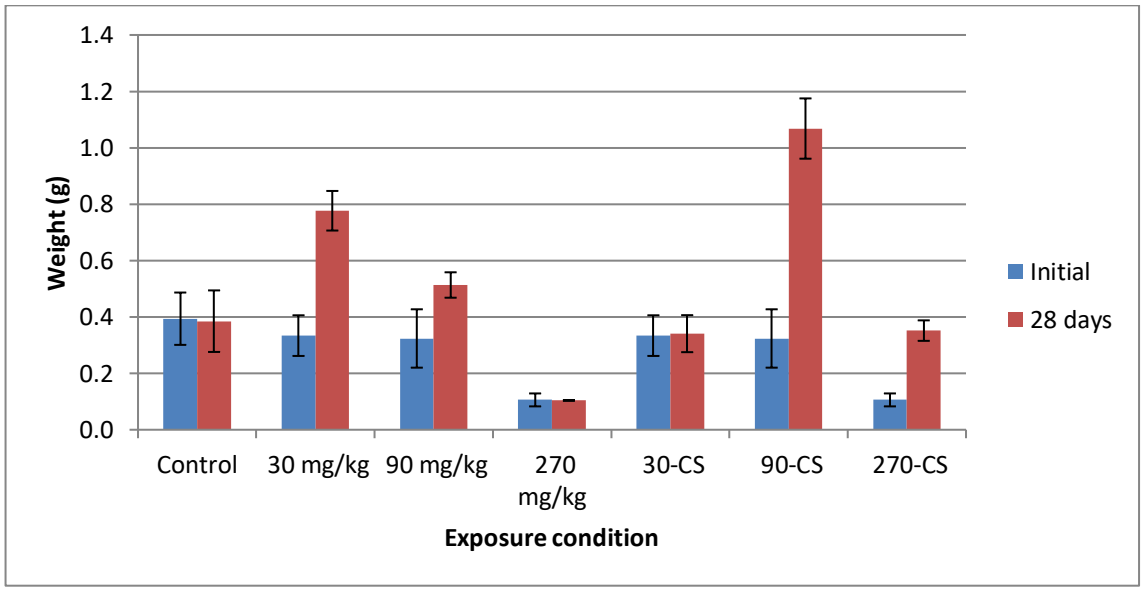

Figure 4.7: Average weights of *E. fetida* groups at beginning (56 d, 'Initial weight') and end (84 d, 'Final weight') of the third 28 d period, for both continued exposure and worms returned to control soils (30-CS, 90-CS and 270-CS). Error bars represent standard deviations from the mean. Refer to Table 4.1 for statistical significances of differences between means.

After 28 d, the average weights of worms in 30 mgCd/kg and 90 mgCd/kg soils were higher than those of the controls and the average weight of *E. fetida* in 270 mgCd/kg soils was 0.28 mg lower than that of controls (Figure 4.7). Worms transferred from 30 mgCd/kg soils showed no significant change in growth. However, those transferred from the two higher Cd-contaminated soils (90 mg/kg and 270 mg/kg) showed significant ($p<0.05$) positive growth. Interestingly, although worms in 30 mgCd/kg and 90 mgCd/kg soils started with equivalent weights, only the second group showed a major increase in growth rate when Cd was removed, to over twice the mean weight of controls. Worms in 270 mg/kg soils started with lower weights, but also saw an almost proportionate rebound in growth when Cd was removed, achieving the same weights as worms in the 30 mgCd/kg, 30-CS and control soils within 28 d of recovery.

These results give an idea that resources that were being used to counter toxicity were able to be redirected towards growth when the Cd stressor was removed. It is possible that worms exposed to the lower (30 mgCd/kg) condition can overcome the stress and grow normally; whereas those with higher exposures devote increasingly more resources to repair. Some biochemical systems that have been upregulated to deal with Cd toxicity may then persist after the Cd is removed to create a rebound growth effect. Evidence for this is strongest in worms that had been exposed to 90 mgCd/kg soils, where weights after 28 d in clean soils far outstrip those of worms in control soils. One literature report shows similar results (Van Gestel et al., 1993a) where removing the stressor resulted in worms showing greater growth than controls.

Changes in cocoon production

Changes in cocoon production for worms experiencing continued exposure compared with those returned to control soils are shown in **Figure 4.8**.

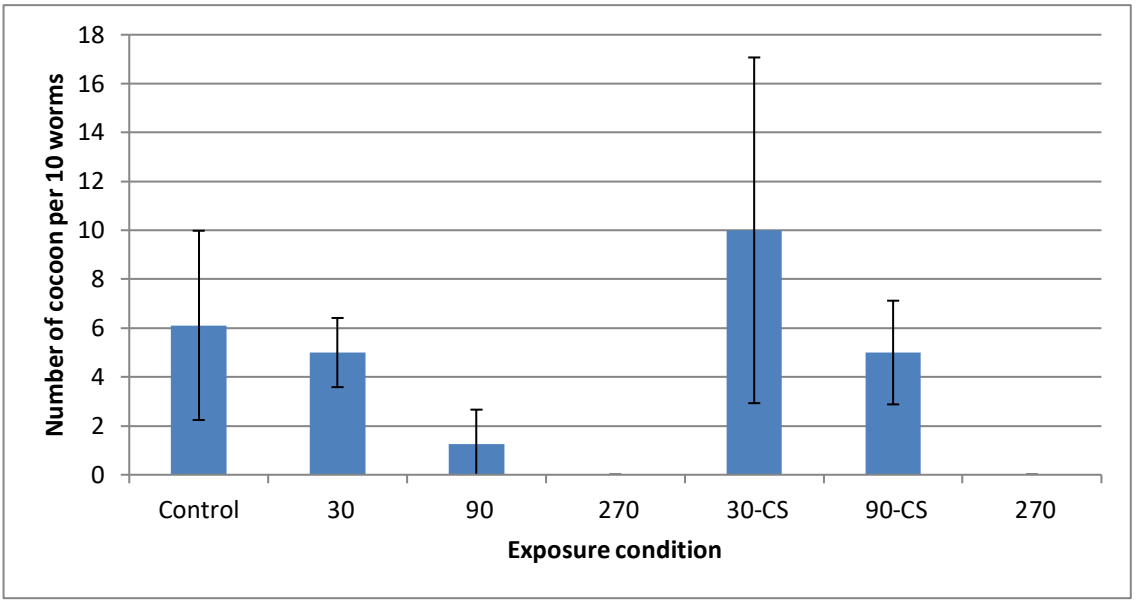

Figure 4.8: Average numbers of cocoons produced after 28 d by *E. fetida* under continuation of the original exposure condition and return to control soils from 56-84 d. Error bars represent standard deviations from the mean. Refer to Table 4.1 for statistical significances of differences between means.

As can be seen (**Figure 4.8**) in worms that had been exposed to 30 mgCd/kg and 90 mgCd/kg soils, cocoon production increased after transferral to clean soil. Mean cocoon production increased more than 100% in worms moved from 30 mgCd/kg soils to clean soils (though the difference between the two means is not statistically significant ($p<0.1$)). In worms moved from 90 mgCd/kg soils, cocoon production increased by 82% ($p<0.05$).

Recovery of reproduction for worms that experienced the two lower exposure conditions are consistent with literature reports. Van Gestel *et al* (1993) reported results of research where after 3 weeks of Cd exposure (0, 10, 18, 32, 56, 100 mgCd/kg), worms were transferred to clean soil and monitored for another three weeks. After this recovery period worms from all concentrations showed complete recovery of reproduction (Van Gestel et al., 1993a).

In worms either remaining in or moved from 270 mgCd/kg soils, no cocoons were produced. The worms were underweight that other conditions and would have still had high levels of Cd binding to their tissues. Under this situation, they are likely to need more time to reproductively mature, and the change in weights to match

those of controls was only evident after 28 d (**Figure 4.7**). With longer-term recovery it would be expected that reproduction would eventually resume.

4.2.2 Generation two

4.2.2.1 Overview

One objective of this research was to assess effects across generations. In chronic reproductive toxicity assays with annelids, adult worms are exposed to Cd for several weeks and the numbers of cocoons and juveniles produced are counted. However, this procedure may easily underestimate the effects of Cd, because in the natural environment worms are exposed to the contaminant during their whole lifetime, and consecutive generations as well. This research goes beyond testing for acute effects and acclimatisation of worms to the situation to focus on longer-term effects on the population.

Hatchlings from Generation 1 exposed worms were used for the Generation 2 experiments. In the first generation experiments, the worms in control, 30mgCd/kg and 90 mgCd/kg soils produced cocoons; whereas those in 270 mgCd/kg soils did not. Therefore the second generation experiments were limited to the lower two experimental exposure concentrations (30 mgCd/kg and 90 mgCd/kg), and control soil.

After 56 d of the first-generation exposure, adult worms were removed from the beakers leaving cocoons behind in the same beaker to incubate. During the growth phase, the worms in control and 30 mgCd/kg soils showed fast growth. In contrast, hatchlings in 90 mgCd/kg soils were lethargic and showed slow growth which is consistent with results reported by Žaltauskaitė and Sodienė (2014). After the worms in control and 30 mgCd/kg soils became adults with well-developed clitella (3 months) they were introduced to new beakers with new soil. The juvenile worms in 90 mgCd/kg soils were underdeveloped and had no visible clitella. However, they were introduced to the new soils in order to also continue the second generation experiments at this concentration.

Some adult worms were transferred to the same exposure condition as they had experienced during their growth phase, and some that had been exposed to

cadmium (30 mgCd/kg, and 90 mgCd/kg) were transferred to clean control soils to monitor their recovery. Measurements on the second generation adult worms

4.2.2.2 Summary statistics

Summary statistics showing mean weights, cocoon counts and Cd content of worms in generation 2 over time are provided in **Table 4.8**, (which is based on the raw data provided in **Appendix 2**.)

Table 4.7: Summary statistics of Cd effects on *E. fetida* - Generation 2. Results are reported as means with standard deviations in brackets.

Soil cadmium condition and concentration	Measured variable	Time (days) 1	28	56
Unspiked (control) soil: (0.011 mgCd/kg)	Mean weight (g)	0.36 (0.01)	0.37 (0.01)	0.43 (0.02)
	Tissue [Cd] (mg/kg)	4.44 (1.22)	2.31 (0.42)	3.11 (0.88)
	Mass Cd per worm (µg)	1.59	0.84	1.32
	Cocoons/10 worms (summed)	0	5.00 (1.41)	27.5 (0.71)
	Percent mortalities (%)	0	0	0
30 mgCd/kg	Mean weight (g)	0.30 (0.08)	0.32 (0.07)	0.43 (0.003)
	Tissue [Cd] (mg/kg)	74.2 (11.2)	84.2 (17.1)	129 (7)
	Mass Cd per worm (µg)	22.5	26.6	55.6
	Cocoons/10 worms (summed)	0	1.50 (0.71)	5.00 (1.41)
	Percent mortalities (%)	0	0	5
90 mgCd/kg	Mean weight (g)	0.23 (0)	0.18 (0)	0.21 (0)
	Tissue [Cd] (mg/kg)	113 (0)	127 (8)	173 (8)
	Mass Cd per worm (µg)	26.3	23.4	36.8
	Cocoons/10 worms (summed)	0	0	0
	Percent mortalities (%)	0	5	5
30-CS (30 mg/kg during growth phase, but returned to control soils)	Mean weight (g)	0.34 (0.04)	0.36 (0.03)	0.45 (0.06)
	Tissue [Cd] (mg/kg)	74.2 (11.2)	69.9 (2.5)	39.7 (6.2)
	Mass Cd per worm (µg)	25.2	25.2	17.7
	Cocoons/10 worms (summed)	0	9 (0)	56.0 (1.41)
	Percent mortalities (%)	0	0	0
90-CS (90 mg/kg during growth phase, but returned to control soils)	Mean weight (g)	0.23 (0.12)	0.24 (0.07)	0.32 (0.10)
	Tissue [Cd] (mg/kg)	113	78.0	51.2
	Mass Cd per worm (µg)	25.9	18.4	16.4
	Cocoons/10 worms (summed)	0	3	5
	Percent mortalities (%)	0	0	0

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001
Difference between means compared with previous 28 d time period; [§] <0.05, [¶] <0.01, [*] <0.001, [#] <0.0001

4.2.2.3 Cd uptake and loss by *E. fetida*

Average Cd concentrations in Generation 2 *E. fetida* initially, and after 28 and 56 d of continued exposure to Cd (compared with controls) are shown in **Figure 4.9**. Losses of Cd in worms returned from elevated Cd exposures to control soils are shown in **Figure 4.10**.

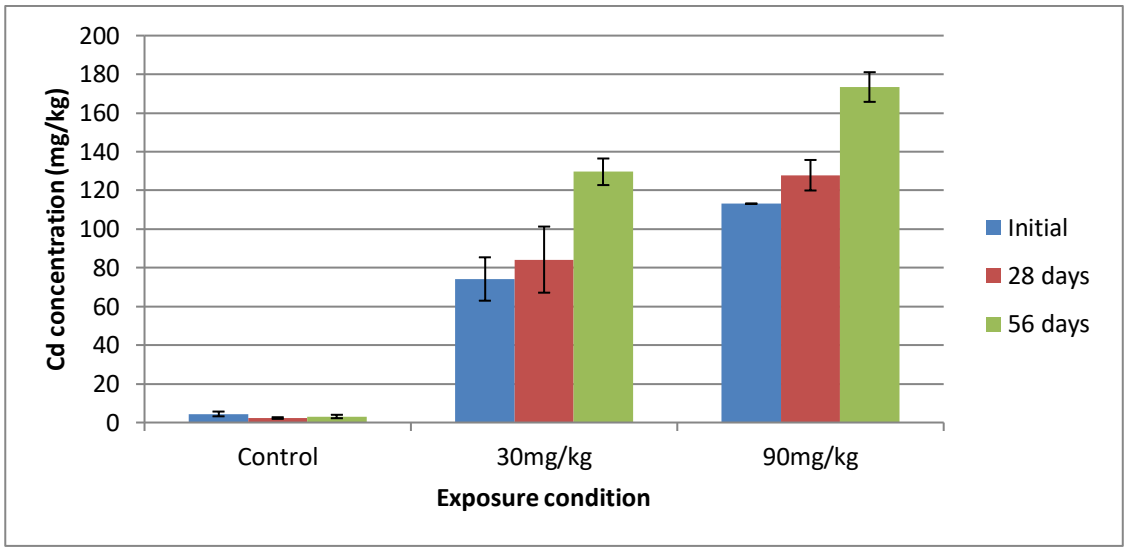

Figure 4.9: Average Cd concentrations in *E. fetida* of Generation 2 at 1, 28 and 56 d. Error bars represent standard deviations from the mean. Refer to Table 4.7 for statistical significances of differences between means.

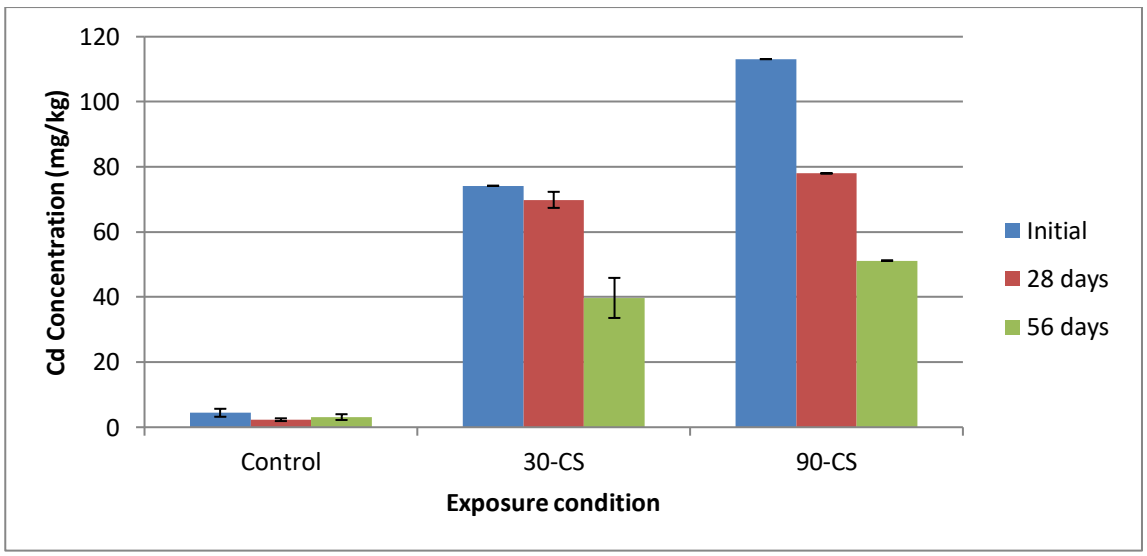

Figure 4.10: Average Cd concentrations in the *E. fetida* of generation two introduced to control soils at 1, 28 and 56 d. Error bars represent standard deviations from the mean. Refer to Table 4.7 for statistical significances of differences between means.

Cd accumulation under ongoing exposure

Cd-exposed worms were born and grew into adults in Cd contaminated soil. Therefore, compared with control worms, they carried a significant body burden of Cd at the beginning of the Generation 2 experiments (**Figure 4.9** and **Figure 4.10**). From that point, the results were as for Generation 1, in that Cd accumulation continued, and was positively correlated with the levels of Cd in the soil ($R=0.864$, $p< 0.05$) and exposure time ($R=0.919$, $p<0.0001$).

This bioaccumulation of Cd shows up both as an increase in concentrations in worm tissue (mg/kg), and as an increase in the total mass of Cd per worm (**Table 4.7**)

Worms in 30 mgCd/kg soils also showed a greater increment in tissue Cd during the second 28 d (+45.4 mg/kg) than the first 28 d (+10.0 mgCd/kg). Worms in 90 mg/kg also showed the same pattern (**Figure 4.9**). Reasons for this are unclear but may reflect a longer initial period of acclimation following transfer to the new beakers compared with first generation worms. That may have come about due to the second generation Cd exposed worms being less robust. It is probable that in worms already containing 70-110 mg/kg Cd, some normal biochemical pathways and functions are already being compromised.

Cd loss on recovery

In the recovery experiment Generation 2 worms showed a reduction in Cd concentrations and masses after they were transferred from Cd contaminated soils (30 mgCd/kg and 90 mgCd/kg, respectively) to clean soil (**Figure 4.10**).

As expected levels in control worms remained low. For the other worms, tissue Cd concentrations decreased with time. Initially worms in the experimental beakers contained substantially more Cd in their bodies than controls because they were born and grew to maturation in the Cd contaminated soil.

Tissue Cd concentrations in worms transferred to clean soil from 30 mgCd/kg soil decreased only slightly (by 4.32 mg/kg) during the first 28 d, but by a further 30.1 mgCd/kg during the second 28 d. The decrement was significantly higher in the

second 28 d ($p<0.05$). When the results are expressed as mass of Cd per worm (**Table 4.7**) it is clear that no Cd was lost overall in 30-CS worms over the first 28 d (25.2 µg per worm in both cases), indicating that the 4.32 mg/kg concentration decrement shown in **Figure 4.10** is due to worm growth causing dilution of Cd concentrations with clean worm tissue. By contrast, the reduction in tissue concentrations of over 30 mg/kg Cd in the second period is likely to be caused genuine Cd loss (as the total mass drops by 30% from 25.2 µg per worm to 17.7 µg per worm) as well as dilution through the growth of new tissue.

Worms transferred from 90 mgCd/kg soils showed faster loss of Cd from the first 28 d. In mass terms these worms lost 29% of their total Cd (25.9 to 18.4 µg per worm) in the first 28 d, and proportionately less (another -11%) in the second 28 d. Reductions in tissue Cd concentrations for the worms previously grown in 90 mgCd/kg soils were mainly due to actual Cd loss in the first 28 d (as mean weights did not change), and tissue dilution (+33%) accompanied by Cd loss (-11%) over the second 28 days (**Table 4.7**)

An interesting feature of the results is that in mass terms, actual losses from 30-CS worms from 28-56 days (25.2 to 17.7 µg per worm) were almost identical to those shown in 90-CS worms over the first 28 d period (25.9 to 18.4 µg per worm). As with the Generation 1 worms, Cd elimination was a slower process than Cd accumulation.

Half-life estimates were calculated based total and first-order elimination over the 56 d period. These are 239 d for worms previously exposed to 30 mgCd/kg, and 193 d for worms previously exposed to 90 mgCd/kg soils. These compare with estimates ranging from 157-222 d for the Generation 1 worms (**Table 4.6**)

Aggregating both datasets a refined half-life loss over n=5 trials and two generations estimate is **198 d** (standard deviation 33 d), or **6.5 months**.

An interesting feature of the results is that loss rates appear to be faster if the estimates are based on tissue Cd concentrations, due to the effect of tissue dilution.

4.2.2.4 Mortality and growth

Minimal mortality occurred during the experimental period among the Generation 2 worms. In the first 28 d, 0% and 5% (1 worm) mortality occurred in worms in 30 mgCd/kg and 90 mgCd/kg soils, respectively. During the second 28 d, 5% (1 worm) mortality was recorded for worms living under both conditions.

These mortality percentages are similar to a certain degree with Generation 1 mortality percentages which showed no mortality during first 28 days and 2.5% and 7.5% in 30 mgCd/kg soils and 90 mgCd/kg soils respectively.

Based on mortality the worms in Generation 1 and Generation 2 are similar. Changes in growth are shown in **Figure 4.11** (absolute growth) and **Figure 4.12** (growth relative to controls).

At the outset, Generation 2 worms raised in 30 mgCd/kg soils showed slightly lower mean weights than control worms. Both those returned to clean soils, and those that remained exposed to 30 mgCd/kg showed modest positive growth (**Figure 4.11**), reaching the same mean weight as controls by 28 days and 56 days, respectively (**Figure 4.12**). Worms returned to clean soils matched control weights by 28 d.

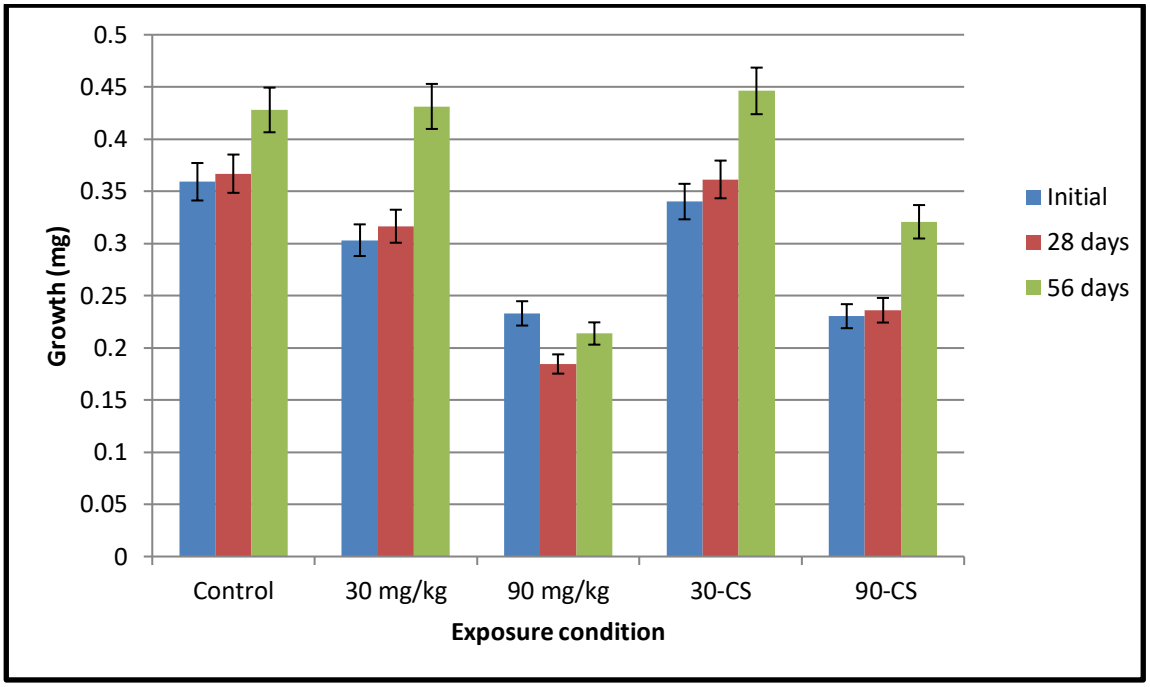

Figure 4.11: Changes in mean weights of *E. Fetida* exposed to different Cd concentrations and clean soil after 28 and 56 d. Error bars represent standard deviations from the mean. Refer to Table 4.7 for statistical significances of differences between means.

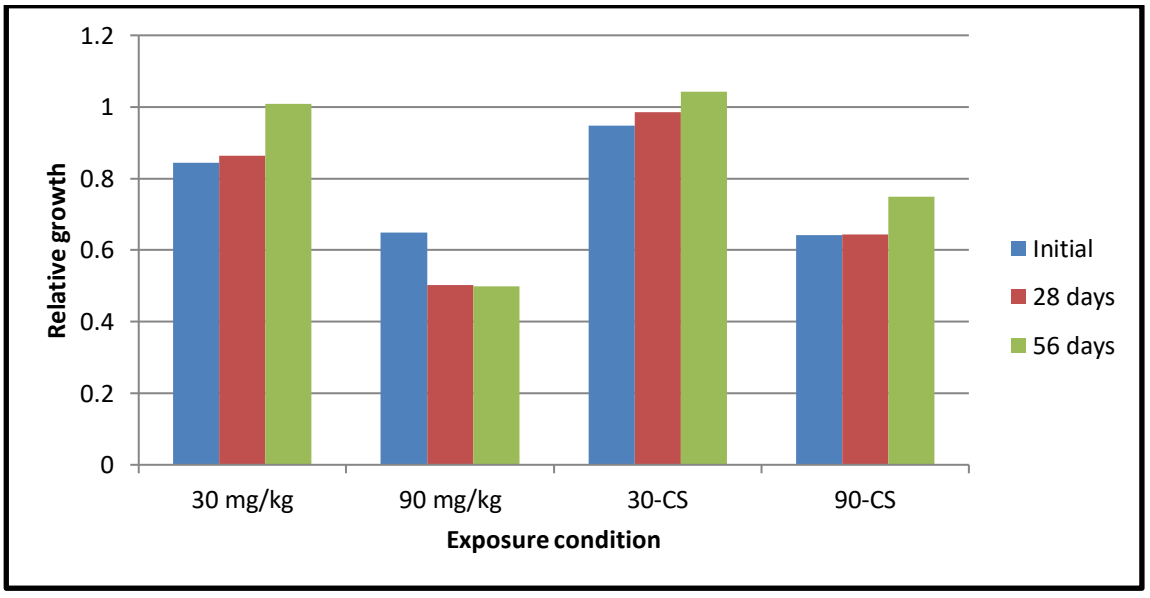

Figure 4.12: Changes in relative growth of *E. fetida* in Generation 2 after 28 and 56 d. Error bars represent standard deviations from the mean. Refer to Table 4.7 for statistical significances of differences between means.

The worms that were raised and continued in 90 mgCd/kg soils showed a modest decrease in mean weights to day 28 which then stabilized relative to controls. In contrast, those previously exposed to 90 mgCd/kg that were returned to control soils showed a relative weight gain of 10% (from 64% to 74% of the mean weight of controls).

4.2.2.5 Sexual maturation and cocoon production

Cocoon production results for Generation 2 worms are provided in **Table 4.8** and **Figure 4.13**.

Table 4.8: Cocoon production by generation two *E. fetida* (per 10 worms) exposed to Cd spiked soil, expressed as both the cumulative total and increase over each 28 d period.

Statistic	exposure period	Control	30 mgCd/kg	90 mgCd/kg	30-CS	90-CS
Cumulative total production (per 10 worms)	0	0	0	0	0	0
	28	5	1.5	0 [a]	9	3
	56	27.5 [§]	5 [b]	0 [d]	56 [c §]	5 [d]
New production during each 28 d exposure period (per 10 worms)	**exposure period**	**Control**	**30 mgCd/kg**	**90 mgCd/kg**	**30-CS**	**90-CS**
	0	0	0	0	0	0
	28	5	1.5	0 [a]	9	3
	56	22.5 [§]	3.5 [b]	0 [d]	47 [c §]	2 [d]

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001
Difference between means compared with previous 28 d time period; [§] <0.05, [¶] <0.01, [*] <0.001, [#] <0.0001

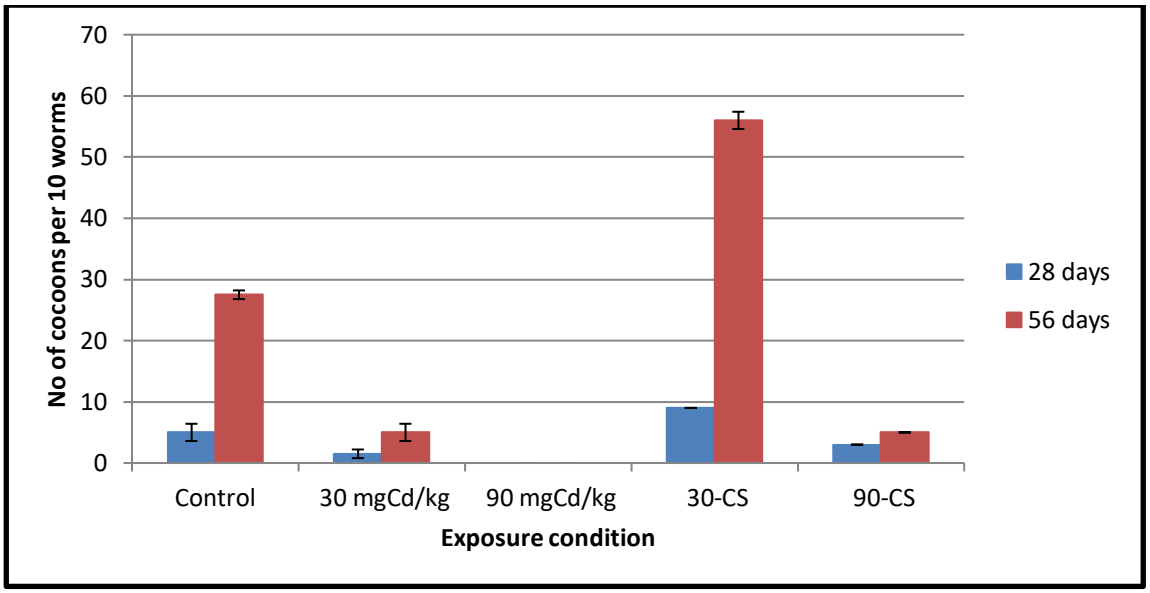

Figure 4.13: Cumulative cocoon production by second generation *E. Fetida* after exposure to Cd spiked and clean artificial soil. Error bars represent standard deviations from the mean. Refer to Table 4.7 for statistical significances of differences between means.

Cocoon production in the Generation 2 worms (**Figure 4.13**) was negatively correlated (R=-0.632, p<0.0001) with soil Cd concentrations. Of the two continuing exposure conditions, worms in 30mgCd/kg soils some showed rates of cocoon production at 1.5 and 5 cocoons per 10 worms in first and second 28 d periods, respectively. In contrast, no cocoons were produced by worms in 90 mgCd/kg soils during the experimental period because the worms were not sexually mature.

Cocoon production of Generation 2 worms in contaminated soil was lower than for Generation 1 worms under the same exposure conditions. In Generation 1, worms in 30 mgCd/kg soils produced (on average) 8.3±3.3 cocoons per 10 worms in 28 d and 27.9±6.8 cocoons per 10 worms in 56 d (**Table 4.1**; **Figure 4.5**). In Generation 1, worms in 90 mgCd/kg soils produced 2 and 11.3 cocoons per 10 worms during first and second 28 d periods (compared with none in the second generation). The continuous Cd exposure had the effect of reducing the maturation and

performance of the exposed generation, and inhibiting onset of reproductive maturity in the second generation.

On average the rate of cocoon production was highest during the second 28 d period. The reason could be the worms were more mature and had adapted to the new soil.

Notably, in the recovery experiment, second generation worms showed more than 100% increase in cocoon production compared with those that remained in Cd spiked soil (**Figure 4.13**). Of the groups, worms from 30 mgCd/kg soils that were returned to control soils showed highest cocoon production overall. They produced 9 and 56 cocoons per 10 worms during first and second 28 d periods, respectively. These figures were noticeably higher than corresponding figures for control soils especially after 56 d. The results also repeat the pattern seen for first generation worms, where a strong increase in cocoon production occurred in worms that had been exposed to 30 mgCd/kg soils and then returned to control soils for 28 days (**Figure 4.8**).

A possible reason is that energy devoted to reproductive effort may increase in response to a recent environmental challenge, provided it is not too severe. However, although an increase in numbers above those of controls was observed for both generations (when exposed worms were returned to control soils), the viability of cocoons was not determined. It is therefore, possible that the increase in cocoon numbers may be a downstream toxic effect of Cd exposure and that this is also accompanied by a higher proportion of non-viable cocoons. Such impact was clear for As exposure (**Chapter 5**), where cocoon production increased but survival of hatchlings born into the same environment was very low.

No other research looking at the downstream impact of a recent Cd exposure was found in the literature. However Aira *et al* (2007)reported that stressed earthworms (*E. fetida*) laid 25% fewer cocoons than control worms and the cocoons were 30% lighter than cocoons laid by control earthworms (Aira et al., 2007). The difference in results might be because of the worms were under relatively high stress compared with worms from 30 mgCd/kg soils, and therefore required more time to recover.

4.2.3 Generation three

4.2.3.1 Summary statistics

Summary statistics showing mean weights, cocoon counts and cadmium content of worms in generation three over time are provided in **Table 4.9**, based on the raw data provided in **Appendix 2**.

Table 4.9: Summary statistics of Cd effect on *E. fetida* - Generation 3. Results are reported as means with standard deviation in brackets.

Soil cadmium condition and concentration	Measured variable	Time (days) 1	28	56
Unspiked (control) soil: (0.011 mgCd/g)	Mean weight (g)	0.25 (0.04)	0.27 (0.04)	0.51 (0.16)
	Tissue [Cd] (mg/kg)	1.26 (0.05)	3.01(0.57)	2.02(0.24)
	Mass Cd per worm (µg)	0.32	0.84	1.02
	Cocoons/10 worms (summed)	0 (0)	5.50 (0.70)	21.1 (0.17)
	Percent fatalities (%)	0	0	0
30mgCd/kg	Mean weight (g)	0.25 (0.001)	0.27 (0.01)	0.39 (0.04)
	Tissue [Cd] (mg/kg)	61.6 (0.76) [d]	76.1 (6.66) [d]	103(5.96) [d]
	Mass Cd per worm (µg)	15.5 [d]	20.2 [d]	41.0 [d]
	Cocoons/10 worms (summed)	0 (0)	2.5 (0.07) [a]	10.7 (0.98) [a]
	Percent fatalities (%)	0	10	0
30-CS (30 mg/kg during growth phase, but returned to control soils)	Mean weight (g)	0.25 (0.03)	0.29 (0.03)	0.52 (0.03)
	Tissue [Cd] (mg/kg)	61.6 (0.75) [d]	47.8 (5.10) [d]	29.7 (3.18) [d]
	Mass Cd per worm (µg)	15.6 [d]	13.7 [d]	15.4 [d]
	Cocoons/10 worms (summed)	0 (0)	6.50 (2.12)	52.5 (2.12) [a §]
	Percent fatalities (%)	0	0	0

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001
Difference between means compared with previous 28 d time period; [§] <0.05, [¶] <0.01, [*] <0.001, [#] <0.0001

4.2.3.2 Cd uptake and loss by *E. fetida*

Mean uptake and losses of Cd in generation three *E. fetida* are shown in **Figure 4.14**.

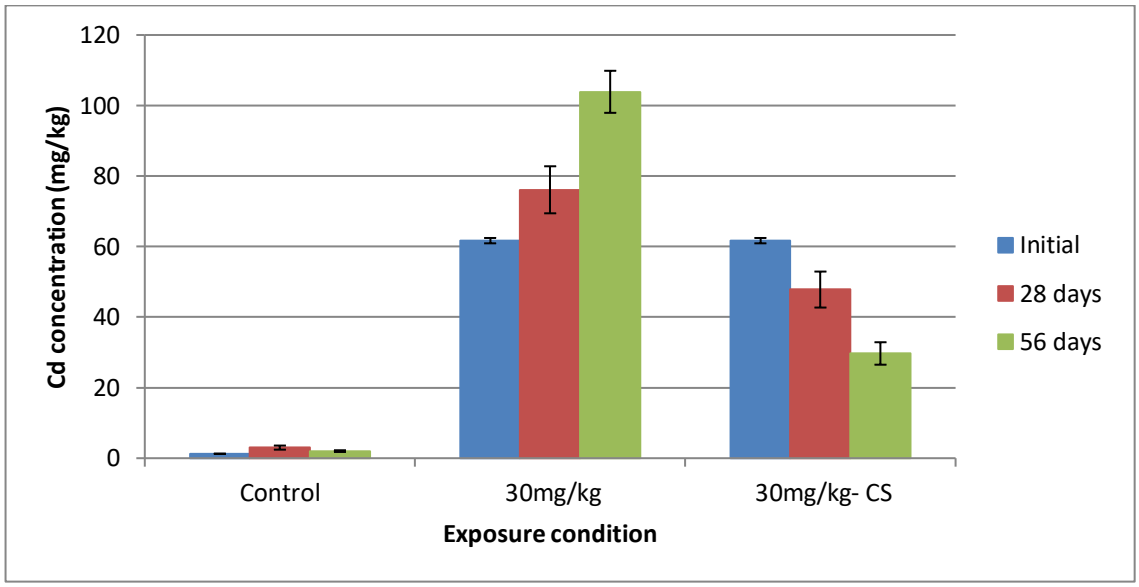

Figure 4.14: Average Cd concentrations in *E. fetida* exposed to 30 mgCd/kg soils and worms transferred to clean soil. Error bars represent standard deviations from the mean. Refer to Table 4.9 for statistical significances of differences between means.

Worms of Generation 3, which were from cocoon production of Generation 2, involved only the 30 mgCd/kg exposure condition, clean soils and controls. Compared to controls, worms in both test conditions started with and accumulated significant amounts of Cd. Worms transferred from and to new 30 mgCd/kg soils continued to accumulate Cd. Worms transferred to clean soil showed a decrease in the mean Cd concentration (**Figure 4.14**), but this was all accounted for by increased tissue mass. However, in third generation there was no apparent decrease in total Cd over 56 d (**Table 4.9**).

This result may be because initial Cd levels in third generation worms were lower to begin with, and the lowest levels seen in second and third generation worms may represent some form of plateaux where actual losses become stalled. Total Cd losses in first, second and third generation worms exposed to 30 mgCd/kg and returned to clean soils (30-CS worms) can be compared:

- First generation: 33.5 µg accumulated (56 days) reduced to 25.2 µg (84 days)
- Second generation: 25.2 µg (initially), 25.2µg (28 days), 17.7 µg (56 days)
- Third generation: 15.6 µg (initially), 13.7 µg (28 days), 15.4 µg (56 days)

(The slight variation in generation three means is attributable to experimental and sampling error.) Results indicate that third generation worms were growing, but were not losing Cd; and that their final mean Cd loading was essentially equivalent (within errors) to that reached by 30-CS worms from Generation 2.

4.2.3.3 Mortality and growth

The worms in all three conditions (including control soils) were well developed and healthy. The worms transferred from 30 mgCd/kg soils to clean soil were much bigger than worms in other conditions. There was no significant mortality during the experiment. Only 10% (2 worms) of worms were dead in 30 mgCd/kg soils during the first 28 d period.

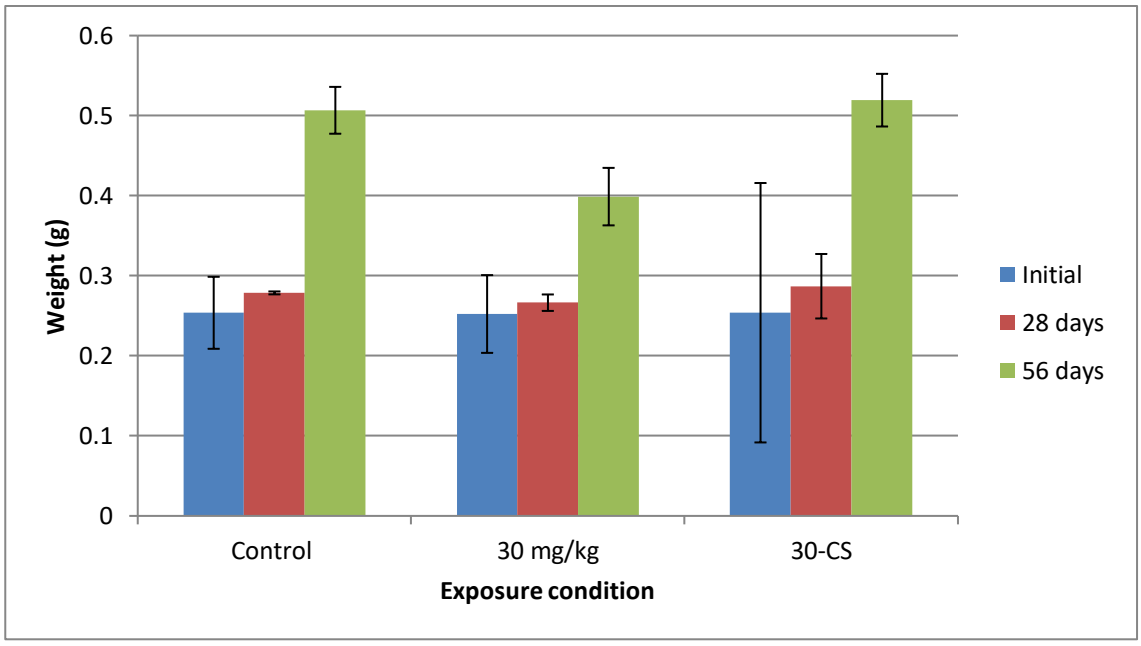

Figure 4.15: Changes in mean weights of Generation 3 *E. fetida* exposed to 30 mgCd/kg and clean soil after 28 and 56 d. Error bars represent standard deviations from the mean. Refer to Table 4.9 for statistical significances of differences between means.

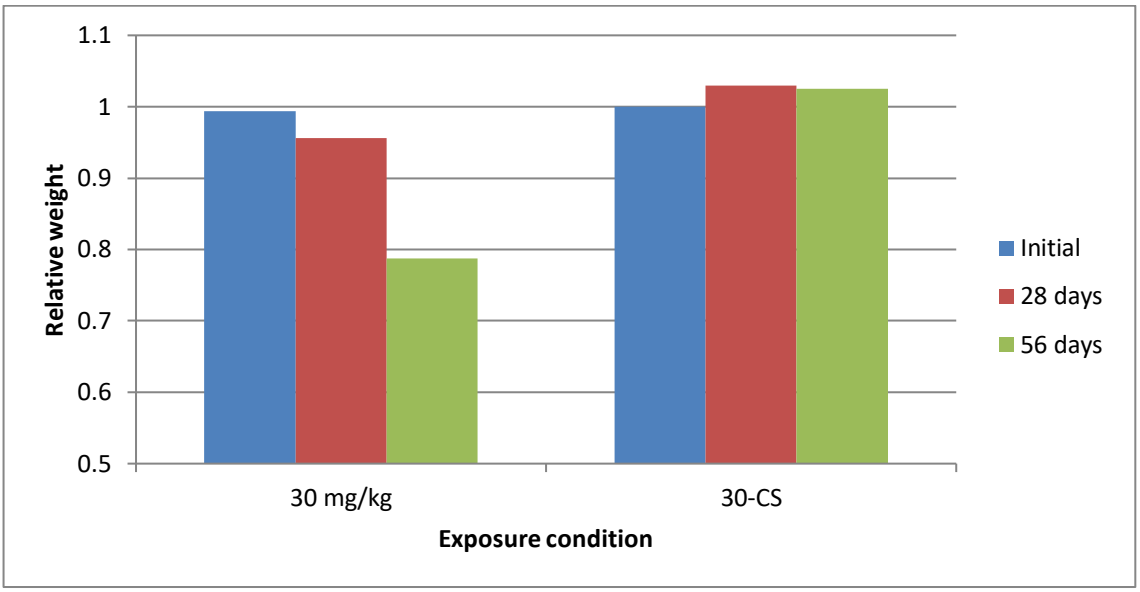

Figure 4.16: Changes in relative growth of Generation 3 *E. fetida* after 28 and 56 d.
Refer to Table 4.9 for statistical significances of differences between means.

All third generation worms showed low growth rates during the first 28 d and then considerably increased their growth during second 28 d (**Figure 4.15** and **Figure 4.16**).

Worms in 30 mgCd/kg soils showed slower growth compared to controls. The difference is much higher in second 28 d where the average weight of the Cd-exposed worms was 0.108 g lower than for controls. As means, the highest growth was by worms transferred to clean soil from 30 mgCd/kg soils. However, the growth was not significantly higher than those of control worms during either the first ($p=0.564$) and second ($p=0.692$) 28 d periods.

4.3.3.4 Sexual maturation and cocoon production

Table 4.10: Cocoon production by Generation 3 *E. fetida* (per 10 worms) exposed to Cd spiked soil, expressed as both the cumulative total and increase over each 28 d period.

Statistic	exposure period	Control	30 mgCd/kg	30-CS
Cumulative total production (per 10 worms)	0	0	0	0
	28	5.5	2.5	6.5
	56	21.1 §	10.7 a	52.5 a §
New production during each 28 day exposure period (per 10 worms)	**exposure period**	**Control**	**30 mgCd/kg**	**30-CS**
	0	0	0	0
	28	5.5	2.5	6.5
	56	15.6	8.2 a	46 a §

Difference between means compared with control condition; a <0.05, b <0.01, c <0.001, d <0.0001
Difference between means compared with previous 28 d time period; § <0.05, ¶ <0.01, * <0.001, # <0.0001

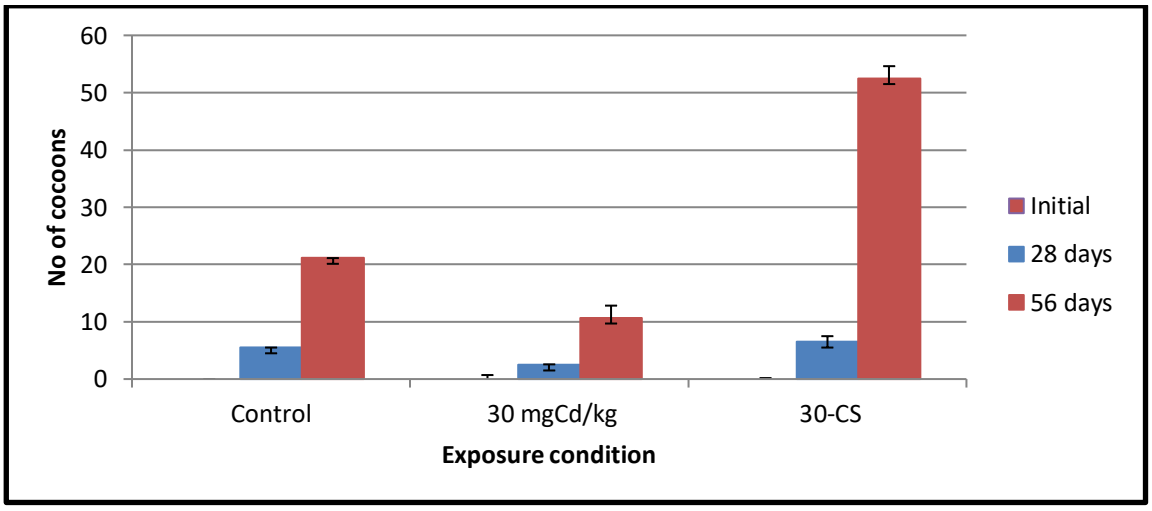

Figure 4.17: Cumulative cocoon production by Generation 3 *E. fetida* after exposure to Cd spiked and clean artificial soil. Error bars represent standard deviations from the mean. Refer to Table 4.9 for statistical significances of differences between means.

The third generation hatchlings in both 30 mgCd/kg and control soils were healthy and grew fast. The worms became sexually mature within three months with well-developed clitella.

Worms in 30 mgCd/kg soils showed lower cocoon production than those in controls. The number of cocoons produced in Cd-exposed worms was significantly lower than in control soils at both 28 d (p=0.05) and 56 d (p=0.037).

Repeating the pattern seen for first and second generation recovery experiments, exposed worms that were returned to clean soils showed a strong increase in cocoon production; within first 28 d producing on average 6.5 cocoons; and this was not significantly (p=0.201) higher than controls. However, during the second 28 d the production significantly increased to above that seen in controls (p=0.029) with on average 31 more cocoons (per 10 worms) than controls.

4.2.3.3 Comparison of Cd uptake across the three generations

For the 30 mg/kg exposure condition, there are three generations of comparative data. For the 90 mg/kg exposure condition, there are two.

To 84 d:

- Generation 1 accumulation from 30 mg/kg soils can be described by a polynomial equation with the formula: µg/worm = $0.8761x - 0.0046x^2 - 0.2887$ ($R^2 = 0.998$), where x is the number of days elapsed.
- Generation 1 accumulation from 90 mg/kg soils can be described by a polynomial with the formula: µg/worm = $1.1653x - 0.0069x^2 - 1.3492$ ($R^2 = 0.996$), where x is the number of days elapsed.

The following comparisons can be made to total Cd uptake in Generation 2 and 3 worms.

- Generation 2 worms exposed to 30 mgCd/kg started with 22.5 µg Cd/worm, the equivalent of 29 d of Generation 1 Cd accumulation. After 56 d they accumulated a total of 55.6 µgCd/worm. On a Generation 1 graph, (29 + 56) d would make 85 d in total, and this would translate to a predicted uptake of 40.9 µg Cd/worm, giving an observed/predicted ratio = **1.36**.
- Generation 2 worms exposed to 90 mgCd/kg started with 26.3 µg Cd/worm, the equivalent of 29 d of Generation 1 Cd accumulation. After 56 d they

accumulated a total of 36.8 µgCd/worm. On a Generation 1 graph, (29 + 56) d would make 85 d in total, and this would translate to a predicted uptake of 47.8 µg Cd/worm, giving an observed/predicted ratio = **1.29**.

- Generation 3 worms exposed to 30 mgCd/kg started with 15.5 µg Cd/worm. This is the equivalent of 16 days of Generation 1 Cd accumulation. After 56 d they accumulated a total of 38.9 µgCd/worm. On a Generation 1 graph, (16 + 56) d would make 72 d in total, and this would translate to a predicted uptake of 41.0 µg Cd/worm, giving an observed/predicted ratio = **0.95**.

On this basis it appears that:

Cd uptake in Generation 2 worms may exceed uptake in Generation 1 by a factor of about one-third (mean of the two observations = **1.33**)

Cd uptake in Generation 3 worms was back to the same level as seen for Generation 1 worms. The result for Generation 3 worms might be 'as expected'; and is consistent with results for As uptake (Chapter 5), where there is no evidence for any differences in uptake between generations.

It is uncertain whether this effect seen in Generation 2 is real, and more experiments would be needed to be certain that Cd uptake may increase in the second generation when expressed on a mass basis. If it is really a possible reason may be the early priming of genetic pathways for metallothionein production, meaning that more Cd would be sequestered and retained earlier in the worm's life-span.

Table 4.11: Overview of cocoon production across the three generations exposed to 30 mgCd/kg in soil.

	Controls (per 10 worms)		Exposed worms (per 10 worms)		Control/exposed (Ratios)		Apparent relative Cd uptake (56 d)
	28 d	56 d	28 d	56 d	28 d	56 d	
Gen 1	8.33	44.5	8.33	27.9	1.00	0.63	1.00
Gen 2	5	27.5	1.50	5	0.30	0.18	1.36
Gen 3	5.50	21.1	2.50	10.7	0.45	0.51	0.95

From this data it can be seen that the apparent 36% increase in Cd uptake in Generation 2 corresponds to a decrease in relative cocoon production; and the apparent return to normal Cd uptake in Generation 3 corresponds with a return to similar cocoon production (after 56 d) as in Generation 1 exposed worms.

4.4 Changes in RNA expression

4.4.1 Generation 1

In Generation 1 worms, the effect of Cd on gene expression was explored in *E . fetida* after 28 d of exposure and after the 28 d recovery period (**Section 3.4.1**). **Table 4.12** provides total numbers of transcripts that were differentially expressed between each of the four exposure conditions (at a significance level of p=0.001) and showed minimum 5-, 10- and 15-fold differences.

Table 4.12: Numbers of differentially expressed transcripts (p=0.001) in Generation 1 *E. fetida*genome between each Cd exposure condition and controls after 28 d.

5-fold differences

	Control	30 mgCd/kg	90 mgCd/kg	270 mgCd/kg
Control	0			
30 mgCd/kg	8272	0		
90 mgCd/kg	7462	11272	0	
270 mgCd/kg	10649	13633	7893	0

10-fold differences

	Control	30 mgCd/kg	90 mgCd/kg	270 mgCd/kg
Control	0			
30 mgCd/kg	1212	0		
90 mgCd/kg	1119	1597	0	
270 mgCd/kg	1953	2516	1333	0

15-fold differences

	Control	30 mgCd/kg	90 mgCd/kg	270 mgCd/kg
Control	0			
30 mgCd/kg	7	0		
90 mgCd/kg	7	9	0	
270 mgCd/kg	28	12	13	0

Large numbers (7-10k) of genes under the exposure conditions showed differential expression of ≥5-fold compared with controls. For 14-18% of these the difference was ≥10-fold, and for 0.6-1.4% the difference was ≥15-fold. As an example: comparing gene expression in the highest 270 mgCd/kg exposures to controls: 10629 transcripts showed a 5-fold difference, almost 1953 showed a 10-fold difference, and 28 showed a 15-fold difference (**Table 4.12**).

A second notable feature of these results is that as many, or more, genes were differentially expressed between exposure conditions, as between each exposure

condition and the controls. For example, there were 1597 genes that showed ≥10-fold differential expression between the 30 mg/kg and 90 mg/kg exposure conditions; and this is higher than the numbers between either condition and controls (**Table 4.12**). This pattern (of significant difference between exposure conditions) was also seen for As (**Chapter 5**). The results may reflect the increasing Cd causing progressive (a) inhibition and (b) activation of different biochemical processes, as its levels in tissues increase.

For practicality and relevance, the following discussion focuses on transcripts differentially expressed at a minimum of 15-fold difference between the conditions. **Table 4.13** provides identities, names and relative changes in transcription copy numbers at increasing exposures for the genes that showing ≥15-fold expression. **Table 4.14** provides the putative functions of the genes that showed a notable pattern.

(It may be noted that **Table 4.13** contains only 24 genes out of 76 differentially expressed genes. The reason for the lower number of genes is that among these 76 genes some genes were repeated (e.g. *CHIT1*) and some gene codes do not have gene names.)

Table 4.13: Genes showing ≥15-fold differential gene expression in Generation 1 worms after 28 d of Cd exposure, sorted according to the consistency of the pattern shown with increasing Cd exposures.

Regulation direction	Gene ID	Gene name	Level of expression (relative number of copies)			
			Control	30 mgCd/kg	90 mgCd/kg	270 mgCd/kg
Apparently up	*FST*	Follistatin	1.82	0	77.2	599
	RL18A	Ribosomal protein 18a 60S large ribosomal subunit	0	0	930	621
Apparently down	*PF07740.11*	PF07740	451	115	0.517	0
	LBP	Lipopolysaccharide binding protein	214	122	95.4	0
	MYSP	Paramyosin	109	59.5	0	0.035
Variably present in controls and lower exposures but absent at highest exposure (270 mgCd/kg)	*FRIS*	Ferritin-2 heavy chain	845	216	388	0
	LYS	Lysozyme	498	75.2	125	0
	CYB	Mitochondrially Encoded Cytochrome B	391	398	533	0
	NEUV	Vasotocin-neurophysin VT	331	130	223	0
	TYR3	Putative tyrosinase-like protein	173	28.4	175	0
	CPG2	Chondroitin proteoglycan-2	123	313	105	0
	CETP	Cholesteryl Ester Transfer Protein	113	29.3	48.4	0
	CHIT1	Chitinase 1	107	7.67	134	0
	109	CD109 antigen	54.9	8.93	24.7	0
Equivocal results: no consistent pattern	*GLB2*	Extracellular globin-2	732	0	1290	449
	AGRIN	Agrin	232	1.58	577	0.043
	NCS2	Neuronal Calcium Sensor 2	228	0	110	132
	SAP	Histone deacetylase complex subunit	112	75.6	0	74.1
	NCS2	Neuronal calcium sensor 2	62.3	0	16.5	272
	CADN	Neural-cadherin	2.06	0	488	0.121
	CO4A1	Collagen alpha-1(IV) chain	0	23.6	0	0
	SAP3	Ganglioside GM2 activator	0	6.45	232	11.5
	KLOM	Lombricine kinase	0	104	9.89	0
	PCKG	Phosphoenolpyruvate carboxykinase	0	125	0	138

Table 4.14: Putative functions of genes showing ≥15-fold differential gene expression in Generation 1 worms after 28 d of Cd exposure.

Gene name	Putative function	Apparent change
Follistatin	Binds directly to activin and functions as an activin antagonist. Specific inhibitor of the biosynthesis and secretion of pituitary follicle stimulating hormone (FSH).	**Increase**
Ribosomal protein 18a 60S large ribosomal subunit	Translation, RNA binding, and translation initiation	
PF07740	Spider toxin, which is a neurotoxin	**Decrease**
Lipopolysaccharide binding protein	Lipid binding	
Paramyosin	Paramyosin is a major structural component of many thick filaments isolated from invertebrate muscles	
Ferritin-2 heavy chain	Iron homeostasis, Ferric ion binding, Iron ion transport	**Present at lower exposures but disappeared at the highest Cd exposure levels (270 mgCd/kg)**
Lysozyme	Has bacteriolytic activity (by similarity). May play a role in digestion and in the host defence mechanisms against invading microbes.	
Mitochondrially Encoded Cytochrome B	Part of the mitochondrial respiratory chain	
Vasotocin-neurophysin VT	Neurohypophyseal hormone activity	
Putative tyrosinase-like protein	Copper ion binding	
Chondroitin proteoglycan-2	Embryo development, chitin binding, chitin metabolic process	
Cholesteryl Ester Transfer Protein	Cholesterol homeostasis	
Chitinase 1	Chitinase activity Degrades chitin, chitotriose and chitobiose. May participate in the defense against nematodes and other pathogens. Isoform 3 has no enzymatic activity.	
CD109 antigen	Modulates negatively TGFB1 signalling in keratinocytes.	
Extracellular globin-2	Heme binding, Iron ion binding	***Equivocal results: no consistent pattern***
Agrin	plays a central role in the formation and the maintenance of the neuromuscular jur	
Neuronal Calcium Sensor 2	Regulator of G protein-coupled receptor phosphorylation in a calcium dependent m	
Histone deacetylase complex subunit	Metal ion binding, DNA binding	
Neuronal calcium sensor 2	calsium ion binding	
Neural-cadherin	Beta-catenin binding, calsium ion binding	
Collagen alpha-1(IV) chain	Embryo development	
Ganglioside GM2 activator	Ganglioside catabolic activity,	
Lombricine kinase	ATP binding, Lombricine kinase activity	
Phosphoenolpyruvate carboxykinase	In parasitic nematodes PEPCK carboxylates phosphoenolpyruvate to oxaloacetate t	

Under Cd stress, some genes were up-regulated compared to the control and some genes were down-regulated. Some genes identified have a linear relationship with Cd concentration whereas others did not.

Of the 24 ≥15 fold differentially expressed genes shown in **Table 4.13**: 5 genes are related to neuronal function, 5 genes are related to ion exchange, binding, transport and ion homeostasis, 4 to the reproduction, and 2 to metabolism. Of particular interest to reproduction are the 4 genes *CPG2, FST, CO4A1* and *MYSP*.

Of these, results for *CO4A1* are equivocal because RNA corresponding to this gene was not detected in worms from the control or two highest exposure conditions, but was assayed as being present at the lowest exposure condition (**Table 4.13**). The protein expressed by *CO4A1* (Collagen type IV) is responsible for restricting presynaptic growth at the neuromuscular junctions in the late larval stage in adult motor neurones (Qin et al., 2014). Hisamoto *et al* reported it may play a role in axon regeneration in embryos following injury in D-type motor neurons (Hisamoto et al., 2016). Its significance in these results is unclear because there was no relationship with gene expression and number of cocoons produced by worms.

Of the other 3 genes involved with reproduction, with increasing Cd concentrations: expression of *FST* expression was apparently upregulated; that of *MYSP* was apparently down-regulated, and that of *CPG2* dropped from detectable to absent at the highest (270 mgCd/kg) exposure condition(**Table 4.13**).

Follistatin (*FST*) is a specific inhibitor of the biosynthesis and secretion of pituitary follicle stimulating hormone (FSH). This gene is highly expressed under high Cd concentrations and it least expressed in clean soil. However, the gene was not expressed in 30 mgCd/kg soils. FSH is a gonadotropin hormone which regulates growth and development, reproductive maturation and reproductive processes in the body. When *FST* inhibits the production of FSH it negatively affects growth and reproduction. When considering growth, the worms in control and the lowest Cd concentration had no expression of *FST* and increased weight during the first 28 d. The worms in 90 mgCd/kg and 270 mgCd/kg soil lost weight and this was positively related with *FST* expression levels. Cocoon production also negatively

correlated with *FST* expression. As an association, when *FST* gene up-regulated, the cocoon production decreased in worms.

The protein expressed by the gene *MYSP* is paramyos in which is a structural component of the muscle thick filament (Qadota et al., 2016, Kagawa et al., 1989). However, a novel research project on *C. elegans* found that this gene is involved in ovulation (Ono et al., 2007). According to Ono *et al* paramyosin was specifically expressed in myoepithelial sheaths. Myoepithelial cells of the proximal ovary are smooth muscle-like cells that provide contractile forces to push mature oocytes in to the spermatheca for fertilization. Therefore *MYSP* is an essential gene for positive regulation of ovulation. Consistent with this, in the current results, expression of *MYSP* shows a negative correlation with the Cd concentration in the soil. This gene is highly expressed in control soils, and this reduces in 30 mgCd/kg soils. When Cd exposures increases above 90 mgCd/kg, the *MYSP* expression ceases. This pattern corresponds with the reduction in cocoon production by *E. fetida*. Both *MYSP* gene expression and cocoon production decrease with increasing Cd concentrations.

In terms of muscle regulation, paramyosin is an invertebrate specific coiled-coil dimer protein that is homologous to the rod portion of myosin heavy chains (Cervera et al., 2006) and this protein is an important factor for muscle development. Decreased expression of *MYSP* with increased Cd corresponded with weight reduction of the worms. Parts of the observed weight reductions could be related to under-expression of this gene.

Chondroitin proteoglycan-2 (*CPG2*) controls polar body extrusion during cytokinesis in embryo development and affects cortical granule size. It also has a role in meiotic chromosome segregation (Olson et al., 2006, Lee and Schedl, 2001, Johnston et al., 2006). This developmental protein could affect embryo development and cocoon production of *E. fetida*. According to the results, high Cd concentrations had a significant impact on the level of expression of this gene. The gene was expressed highly in worms in low Cd concentration soil, less in worms exposed to medium Cd concentrations, and not at all in those experiencing the highest Cd exposures. There is a relationship with cocoon production and expression of *CPG2*. Even under Cd exposure, the worms in 30 mgCd/kg soil

produced on average the same number of cocoons (8.3) as worms in control conditions. Worms in 90 mgCd/kg showed decreased *CPG2* expression and much lower cocoon production after the first 28 d (average 2 per 10 worms). Worms in 270 mgCd/kg soils that did not express the gene did not produce cocoons. Expression reduction of one CPG type has no effect on embryonic viability, but concurrent depletion of *CPG-1* and *CPG-2* can cause lethality (Olson et al., 2006).

Among the significantly differentially expressed genes there are also some metal regulating genes; *TYR3* regulates copper ion binding, *NCS2* regulates calcium ion binding, *FRIS* regulates ferric ion binding and iron ion transport, *GLB2* regulates haem and iron ion binding, and *SAP* regulates DNA binding and metal ion binding. The elevated Cd in soil would affect the regulation of other metals in *E. fetida*, especially those involved with homeostasis of divalent cations. Previous research has reported elevated Cd^{2+} levels have a detrimental effect on Fe^{2+} metal regulation, an essential metal for cell function (Dharmadasa et al., 2017). However, in this experiment the expression of these genes (whilst significantly differentially expressed) was variable with respect to Cd concentration and not positively or negatively related with increasing Cd concentration in the soil or tissues (**Table 4.13**). In **Chapter 7** it is shown that total Cd and Fe concentrations in worm tissues were highly correlated. This correlation may reflect Cd^{2+} from the spike displacing some exchangeable Fe from soils, increasing Fe uptake; or other mechanisms may be in play that increase Fe retention. The significance of *FRIS* in this context is unclear, because its levels reduced as Cd levels increased (**Table 4.13**). This seems to imply that Cd has an impact on Fe homoeostasis, and that in the Generation 1 worms, production of Fe-storage proteins reduced with increasing Cd exposure.

Five of the identified genes encode proteins related to the nervous system; these are: *PF07740, SAP3, NEUV, AGRIN*, and *NCS2*. The extracellular matrix protein 'agrin' plays a central role in the formation and maintenance of the neuromuscular junctions (Gingras et al., 2005). In fact, the neuromuscular junction in the skeletal muscle is regulated by agrin (Burden, 1998, Sanes and Lichtman, 1999), and this protein is essential for movement and respiration (Gingras et al., 2005). Results for this protein were equivocal, with high expression under control and 90 mgCd/kg exposure conditions, and low expression under the other two conditions (**Table**

4.13). If the results for 90 mgCd/kg soils were an outlier, it could be speculated that low expression at 270 mgCd/kg exposures may relate to worms in these soils being notably slow and lethargic.

The gene *CYB* encodes the protein cytochrome B which is an essential part of the mitochondrial respiration chain (Riccio et al., 1977). This protein contributes to the generation of a proton gradient across the mitochondrial membrane to use for ATP synthesis. The expression of this gene is increased with increasing Cd concentration in the soil and silenced in the 270 mgCd/kg soils. This may be because of the worms need to produce more energy (ATP) to fight with ambient toxic environment. However, under very high Cd concentrations worms are less capable of fighting and less likely to survive. This explanation for the *CYB* results is consistent with two other aspects of the As and Cd results. These were the way that more As was taken up over longer As exposure times (thought to relate to more phosphate uptake and turnover); and that natural As was taken up in proportion to Cd exposures in Cd exposed worms (thought to be a by-product of the same mechanism). Both effects would come about because arsenate (AsO_4^{3-}) is isomorphous with phosphate (PO_4^{3-}) (this idea is discussed further in **Section 7.2.2**).

Other than the above mentioned genes there were ≥15 fold differentially expressed genes encoding for proteins related to metabolism, host defence, chitin production (discussed under the Generation 2 results), transfer protein, lipid binding protein and keratin (**Table 4.13**).

Further analysis in 10-fold differentially expressed genes

Further analysis was undertaken of ≥10-fold differentially ($p<0.001$) expressed genes among the four conditions after 28 d. Of the 5203 trinity gene codes, 509 genes (9.8%) had gene names. A list of genes related to growth and reproduction was taken from National Centre for Biotechnology Information (NCBI), and a further analysis was done to find which of the 509 named genes were on that list. Of these, 5 genes (9.8%) were related to reproduction, and 146 (28.7%) were related to growth. **Table 4.15** shows the names of these.

Table 4.15: Identified and named ≥10-fold differentially expressed genes in Generation 1 *E. fetida* related to growth and reproduction after 28 d Cd exposure.

Reproduction	**Growth**												
ACE	*ABCB6*	*ANXA7*	*CD109*	*DACH1*	*ERAP1*	*FZD4*	*IL16*	*LRP5*	*NF1*	*PTPRD*	*SFRP5*	*THRB*	*XYLB*
MMP12	*ABI2*	*AQP4*	*CDC73*	*DAG1*	*ERCC1*	*GFPT2*	*KANK1*	*MAGI2*	*NRCAM*	*PTPRR*	*SIR2*	*TLL1*	*ZNRF3*
PKD1	*ACD11*	*ARF4*	*CEP83*	*DCLK1*	*FADS2*	*GNAS*	*KDM5A*	*MEIS2*	*NUDT6*	*PTPRT*	*SKI*	*TPM2*	
VDR	*ACE*	*ARNT*	*CETP*	*DDX46*	*FAS*	*GRIA1*	*KEAP1*	*MELK*	*NUP98*	*PURA*	*SMC2*	*TRAK2*	
VRK1	*ACLY*	*ATF2*	*CGAS*	*DDX5*	*FAT1*	*GRN*	*KLF2*	*MET*	*PAR1*	*RAB14*	*SNED1*	*TRIM3*	
	ACSL4	*AXIN1*	*CHIT*	*DDX58*	*FAT4*	*GSTP1*	*KLF5*	*MGMT*	*PCDH9*	*RAC1*	*SOCS3*	*TRIO*	
	ACT1	*BANP*	*CHIT1*	*DLG1*	*FBLN1*	*GULP1*	*LAMP1*	*MMP12*	*PDCD4*	*RHOU*	*SPAST*	*TRPM3*	
	ACTB	*BIRC3*	*CLIP1*	*DMD*	*FBLN2*	*HGF*	*LATS1*	*MVP*	*PEX5*	*ROBO1*	*SRSF2*	*UBE3A*	
	ADT1	*BMI1*	*CPNE3*	*DOK5*	*FBN2*	*HIF1A*	*LATS2*	*MYD88*	*PKD1*	*ROCK2*	*SRSF3*	*VDR*	
	ADT2	*BRD2*	*CPT1A*	*ECE1*	*FKBP4*	*HIP1*	*LBP*	*MYH10*	*PLAC8*	*ROS1*	*STRAP*	*VRK1*	
	AMFR	*BTG1*	*CSK*	*EHF*	*FLNA*	*HPSE*	*LEO1*	*MYLK*	*PPIB*	*RSF1*	*SVIL*	*WWP1*	
	ANXA4	*CBS*	*CTCF*	*EI24*	*FST*	*IFT88*	*LRP4*	*MYO6*	*PRDX6*	*SACS*	*TAB3*	*XIAP*	

4.4.1.2 Recovery experiments

Table 4.16 provides total numbers of transcripts that were differentially expressed between each of the four exposure conditions (at a significance level of p=0.001) and showed minimum 5-, 10-fold differences.

Table 4.16: Numbers of differentially expressed transcripts (p=0.001) in generation one after *E. fetida* allowed to recover in clean soils for 28 after 56 days of exposure.

5-fold differences

	Control	30 mgCd/kg-CS	90 -CS	270 -CS
Control	0			
30 mgCd/kg-CS	93	0		
90 mgCd/kg-CS	242	269	0	
270 mgCd/kg-CS	257	291	143	0

10-fold differences

	Control	30 mgCd/kg-CS	90 mgCd/kg-CS	270mgCd/kg-CS
Control	0			
30 mgCd/kg-CS	0	0		
90 mgCd/kg-CS	1	0	0	
270 mgCd/kg-CS	9	0	0	0

After the 28 d recovery period the number of deferentially expressed genes between controls and previous exposure conditions drastically decreased. There were no genes differentially expressed to the 15 fold cut-off range and only 9 genes were in the 10 fold cut-off range. The number of differentially expressed genes in the 5 fold cut-off range was larger but still significantly lower than when the worms were being actively exposed to Cd contaminated soils (compared **Table 4.12** and **Table 4.16**. For example, for the 270 mg/kg condition compared with controls, the number of ≥5-fold differentially expressed genes drop from 10649 during exposure to only 257 (2.4% of the original) at 28 d post-exposure. This is a good sign of recovery.

The response result is also quite different to that seen for As, where after 28 d of recovery, large numbers of genes were still being differentially expressed in previously exposed worms, compared with controls (see **Section 5.3.1.3**). In this sense Cd and As are showing distinctly different post-exposure behaviour.

A probable reason for the turnaround in the case of Cd is that Cd-binding by metallothionein (MT) and similar proteins is very effective, meaning that until MT is saturated, the main response to Cd to the active exposure situation. In this work and in *E. fetida*, tissue levels of ~370 mg/kg Cd were found to correspond with 20% mortality (**Section 4.2.1.4**; **Table 4.1**).

Together these results suggest that for *E. fetida*, ~370 mg/kg Cd is a tissue level at which all Cd-binding mechanisms have already become saturated.

This has a parallel in humans, for which the common effects of chronic Cd poisoning are kidney and liver damage (**Chapter 2**). In humans, Cd acts as a highly specific inducer of MT, which is synthesized in the liver and kidneys. The resultant complex serves the purpose of locking up Cd and thus blocking its toxic activity. Once the amount of MT available is insufficient to complex all the Cd, the toxicity of the metal is felt (Hutzinger, 1980, Mason, 1984). In other words, a threshold level must be exceeded before serious renal or hepatic damage begins. In human kidney tissue this threshold has been estimated to be about 200 mg/kg (Piotrowski and Coleman, 1980).

The genes *CYB* and *AGRIN* were not silenced (as they were in the 270 mgCd/kg soils) and correspondingly the worms recovering from these exposures showed positive phenotypic changes including higher activity levels, faster movements and increased weight.

Though many fewer genes are differentially expressed, in the 5-fold cut off range there is still an increase in line with the prior Cd exposure level, from 30 mg/kg to the two higher exposures. This residual effect on these genes probably reflects the differences in the amount of Cd that accumulated before exposure ceased.

Table 4.17 shows ≥10-fold significantly differentially expressed genes between the four conditions after the 28-d recovery period.

Table 4.17: Genes showing ≥10-fold differential expression compared with controls after 28 days of recovery from Cd exposure, and their putative functions.

Gene symbol	Protein name	Putative function	Level of expression			
			Control	30-CS	90-CS	270-CS
CHIT1	Chitinase 1	Chitinase activity	120760	2320	65.5	26.8
FRIS	Soma ferritin	Iron homeostasis, ferric ion binding, iron ion transport	98233	846	44.4	30.5
SODC	Superoxide dismutase	Destroys intracellularly produced radicals which are toxic to biological systems	26853	2170	30.5	11.2
CADN	Neural-cadherin	Regulator of G protein-coupled receptor phosphorylation in a calcium dependent manner	70956	2967	17.0	14.2
LYS	Lysozyme	Has bacteriolytic activity (by similarity). May play a role in digestion and in the host defence mechanisms against invading microbes	36425	324	9.15	7.94
LBP	Lipopolysaccharide binding protein	Lipid binding	22160	803	4.50	8.85

As a general pattern, all of these identified genes were down-regulated with respect to increasing Cd concentration in the worm tissues. *SODC* was identified as significantly differentially expressed at 10-fold in the recovery test but not in the Cd exposure experiments. The function of superoxide dismutase is to destroy

radicals produced inside of cells to prevent toxicity to the organism. The increased expression of this in the recovery test could be a marker of relative recovery.

4.4.2 Generation 2

4.4.2.1 Cd exposure

Table 4.18: Number of differentially expressed (p≤0.001) gene after the 56 days exposure experiment in generation 2

5-fold difference			
	Control	30-CS	90-CS
Control	0		
30-CS	142136	0	
90-CS	16125	9524	0
10-fold difference			
	Control	30-CS	90-CS
Control	0		
30-CS	2511	0	
90-CS	2425	587	0
15-fold difference			
	Control	30-CS	90-CS
Control	0		
30-CS	15	0	
90-CS	23	7	0

After 56 days of generation two experiment, a significant number of genes were differentially expressed. The same as occurred for Generation 1 more genes were 5-fold significantly differentially expressed and less number of genes were 10-fold significantly differentially expressed. There were only 31 genes which are 15 fold differentially expressed among three conditions including control.

In Generation 2 worms, RNA testing was carried out after 56 d of exposure. **Table 4.19** shows the differentially expressed genes with gene name, protein, gene ontology and level of expression under different concentrations of Cd.

In total 31 genes were identified as ≥15-fold differentially expressed to $p<0.05$. Only 7 of these genes were able to have names allocated to them. The other 24 genes were made available to the annotation library but no gene names for them were found.

Table 4.19: Name identified genes showing ≥15-fold differential gene expression compared with controls in Generation 2 worms after 56 d Cd exposure.

Gene symbol	Gene name	Gene ontology	Control	30 mgCd/kg	90 mgCd/kg
FIBG	Fibrinogen gamma chain	Metal binding , Has a major function in hemostasis as one of the primary components of blood clots	0	188	396
FRIS	Ferritin	Stores iron in a soluble, non-toxic, readily available form. Important for iron homeostasis. Iron is taken up in the ferrous form and deposited as ferric hydroxides after oxidation.	0	707	522
CHIT1	Chitotriosidase-1	Degrades chitin	0	1620	1260
FAT1	Protocadherin Fat 1	Plays an essential role for cellular polarization, directed cell migration. The molecular function is Ca ion binding	0	365	154
CHIT1	Chitotriosidase-1	Degrades chitin	0	4235	2495
VKT3	Kunitz-type serine protease inhibitor textilinin-3	Serine protease inhibitor (By similarity). Does not inhibit plasmin, and does not reduce blood loss in the mouse tail vein blood loss model	0	335	152
ICIC	Subtilisin-chymotrypsin inhibitor CI-1C	Inhibits both subtilisin and chymotrypsin.	0	289	61.7

Results can be compared with those from Generation 1 (**Table 4.13** and **Table 4.14**). Of the 7 genes in **Table 4.19**, 3 (two *CHIT1 genes & FRIS*) were differentially expressed in both Generation 1 (to ≥15-fold) Cd exposure and the Generation 1 recovery experiment (to ≥10 fold) (**Table 4.19**). The other four genes (FIBG, FAT1, VKT3 and ICIC) were only identified as being differentially expressed in Generation 2.

Table 4.20: *CHIT1* and *FRIS*≥15-fold differential expression levels compared.

CHIT1	**Control**	**30 mgCd/kg**	**90 mgCd/kg**	**270 mgCd/kg**
Gen 1	107	7.7	134	0
Gen 2	0	1620	1260	-
FRIS	**Control**	**30 mgCd/kg**	**90 mgCd/kg**	**270 mgCd/kg**
Gen 1	845	216	388	0
Gen 2	0	707	522	-

CHIT1 controls chitinase activity. In arthropods, chitin is a primary component in the exoskeleton, which worms do not possess. In earthworms, the gizzard epithelium secretes large amounts of chitin-containing material; this does not arrange in layers but is sloughed off at the lumen side and mingles with gut contents to form a type of gastric shield (Peters and Walldorf, 1986). Results for this gene are a little equivocal. In Generation 2 worms the *CHIT1* gene was not expressed in the control worms, but was under the 30 mgCd/kg and 90 mgCd/kg conditions (**Table 4.20**). This result suggests upregulation of a gastric shield in response to Cd, and makes sense. However, in Generation 1 (after 28 d), the gene was expressed at moderate amounts in the control and 90 mg/kg conditions, but only at low levels at 30 mg/kg and not at all under the 270 mg/kg condition (**Table 4.20**). Inconsistencies here may reflect uncertainties in the results of the gene testing which are difficult to assess, due to having only one sample extract to represent each condition.

As mentioned in the Generation 1 discussion, *FRIS* is responsible for making the protein soma ferritin which stores iron in a soluble non-toxic and readily available form. *FRIS* is differentially expressed in Generation 1 worms in both the Cd

exposure experiment (15-fold) and recovery experiments (10-fold). The pattern of expression was not entirely consistent. In Generation 1 worms, FRIS decreased with increasing Cd. In Generation 2, it was absent in controls and at similar levels of differential expression in the two exposure conditions.

In the Generation 2 worms (at 56 d) as Cd concentrations in the soil increased, the expression of *FIBG* also increased (**Table 4.19**). This gene did not show differential expression in Generation 1 worms after 28 d (**Table 4.13**). Possible reasons it showed up significantly in Generation 2 measurements are the longer exposure period (56 d compared with 28 d), actual differences in biochemistry between first and second generation worms, and/or variability in the RNA transcriptome results related to the sequencing depth.

Gene *FIBG* is responsible for expression of the fibrinogen gamma chain which polymerises with fibrinogen alpha (*FGA*) and fibrinogen beta (*FGB*) to form an insoluble matrix. This matrix is one of the primary components of blood clots (Farrell, 2004) and has a major function in homeostasis. In addition this matrix plays a role in the early stages of wound repair (Farrell, 2004) and in guiding cell migration during the re-epithelialization (Uniprot, 2019d).

Histological changes to *E. fetida* caused by Cd exposure include lesions and destruction of the body wall, destruction of cuticle, rupture of the alimentary canal with large lesions and internal bleeding, and bleeding from the skin (Rodriguez et al., 2013). These Cd-induced wounding effects are consistent with increased expression of *FIBG* to prevent bleeding through clotting. For the clotting process need more insoluble matrix which makes *FIBG* with *FGA* and *FGB*. Similarly, re-epithelialization is an essential component in the wound healing process and fibrinogen gamma chain is an essential part of the re-epithelialization.

There were three other named genes (*FAT1, VKT3* and *ICIC*) that were differentially expressed in the Generation 2 Cd exposed worms (**Table 4.19**). *FAT1* is plays an essential role in cellular polarization. The protein protocadherin has ability to direct cell migration and modulating cell-cell contact (Dunne et al., 1995). *VKT3* is express protein Kunitz-type serine protease inhibitor textilinin-3, which is act as seine protease inhibitor by similarity. The function of serine protease is

cleave peptide bonds in proteins (UniProt, 2019c). Subtilisin-chymotrypsin inhibitor CI-1C isinhibits subtilisin and chymotrypsin. Both subtilisin and chymotrypsin are protein digestive enzymes.

Further analysis of ≥10-fold differential gene expression in Generation 2

Of the 10-fold differential-expressed genes, only 6% had gene names. Of named genes, none related to reproduction, and 10 relate to growth. These were PL*AC8, ERAP1, ADK2, DLL1, CHIT, FAT1, CHIT1, FBP1, FCN2*, and *HGF*.

4.4.2.2 Generation 2 recovery

Table 4.21: Numbers of differentially expressed (p≤0.001) genes after the 28 d recovery experiment in Generation 2.

5-fold difference			
	Control	30-CS	90-CS
Control	0		
30-CS	13133	0	
90-CS	12981	6244	0
10-fold difference			
	Control	30-CS	90-CS
Control	0		
30-CS	2315	0	
90-CS	1846	488	0
15-fold difference			
	Control	30-CS	90-CS
Control	0		
30-CS	40	0	
90-CS	14	1	0

After 28 d of recovery the Generation 2 the worms did not show a large amount of recovery in differential gene expression. There were 48 genes which are 15 fold differentially expressed among three conditions including control. This is an

immense number when compared with number of differentially expressed genes after generation one recovery period which is no genes under 15 fold and only 6 genes under 10 fold differentially expressed (**Table 4.16**). However, compared to the generation two differential gene expressions (**Table 4.18**) recovery showed decrement in number of differentially expressed genes in 15- fold and 5 fold differences.

Table 4.22: Total numbers of differentially expressed genes in Generation 2 worms after exposure to Cd and following a 56 days recovery.

Expression level	Cd exposure	Recovery
5-fold	16876	19659
10-fold	2104	3103
15-fold	31	48

With the exception of controls, the worms used for these experiments were born in Cd spiked-soils and therefore had elevated Cd in their tissues at the beginning. New Cd accumulation then built on top of this pre-existing level. Whereas differential gene expression levels in Generation 1 worms dropped substantially after the 28 d recovery, levels in Generation 2 worms remained high.

These results suggest that after 3 months and 56 d of ongoing exposure, tissue Cd levels in the Generation 2 worms had passed a toxic threshold that had not been reached in the Generation 1 worms. For worms born in Cd-contaminated soils, recovery was harder.

Analysis of the small subset of genes within the 15-fold and 10-fold differential expression sets which are able to have names assigned to them was carried out, in the same way as outlined above. To maintain clarity of this Chapter, these results are provided **Appendix 2**

4.4.3 Differential gene expression in Generation 3 (Cd exposure and recovery)

In Generation 3 worms, the effect of Cd on gene expression was explored after 56 d of 30 mgCd/kg exposure and after 56 d recovery. As for Generation 2, worms under the exposure condition were born in Cd-spiked soils.

Table 4.23: Number of differentially expressed (p≤0.001) transcripts in Generation 3 *E. fetida* exposed to 30 mg/kg Cd for 56 d and allowed to recover for 56 d.

5-fold difference			
	Control	30 mgCd/kg	30-CS
Control	0		
30 mgCd/kg	10642	0	
30-Cs	7623	8521	0
10-fold difference			
	Control	30 mgCd/kg	30-CS
Control	0		
30 mgCd/kg	2673	0	
30-CS	1464	1961	0
15-fold difference			
	Control	30 mgCd/kg	30-CS
Control	0		
30 mgCd/kg	42	0	
30-CS	10	14	0

Analysis of the small subset of genes within the 15-fold and 10-fold differential expression sets which are able to have names assigned to them was carried out. Results are provided in **Appendix 2**

Considerable numbers of genes were differentially expressed under the stressed condition (**Table 4.23**).

Table 4.24: Number of differentially expressed genes in the Generation 3 Cd exposure and recovery experiments.

	Number of differentially expressed genes		
Fold of differential expression	**30 mgCd/kg**	**30-CS**	**Recovery percentage (%)**
5	10642	7623	28.4
10	2673	1465	45.2
15	42	10	76.2

Numbers of differentially expressed genes decreased after recovery (**Table 4.23**). The greatest percentage decrease was in genes that had been 15-fold differentially expressed, and the least in those that were 5-fold differentially expressed (**Table 4.23**). Unlike Generation 2 worms (for comparable data see **Table 4.22**), results for Generation 3 worms transferred to clean soil suggested a certain amount of recovery occurred. After a recovery period in clean soils, Cd-exposed Generation 2 worms showed equivalent (apparently ~13% higher) differential gene expression as they had before it. In contrast, Generation 3 worms showed a 28% reduction.

4.5 Findings and Implications

The most notable findings of this part of the work are as follows.

[1] Confirming previous work, the relative efficiency of Cd uptake (as quantified in BCF values) decreased with increasing soil Cd concentrations, in opposition to likely bioavailability. It is speculated that uptake of Cd^{2+} may be via pathways that regulate the uptake of the similarly sized essential ions Ca^{2+} and/or Zn^{2+}.

[2] Results for Cd uptake over longer time periods shows that accumulation continues until lethal levels in worm tissue are reached. This and Finding **[1]** work together to undermine the idea that there may be a fixed soil concentration or (other factors equal) corresponding to any given lethal endpoint (LC value). Instead, the lethal dose would be better viewed as an envelope defined by **soil**

[Cd], **BCF**, and **exposure time**. Tissue levels of ~370 mg/kg Cd in *E. fetida* were found to correspond with 20% mortality.

[3] Accumulation of Cd when worms are exposed is a faster process than the loss of Cd when exposure ceases. Based on first-order elimination kinetics and results of five experiments, the half-life for Cd mass loss from worm tissues is estimated as being 198 days (6.5 months). This long retention time implies that worms exposed to significant soil Cd would be unlikely to lose it again (if transposed to clean soils) over their life-spans.

[4] Although the lowest spike (30 mg/kg) had little impact on worm growth, it markedly reduced cocoon production. In general, Cd severely affected sexual maturity and reproductive success of *E. fetida*. The time needed to reach a sexually mature state increased for increasing Cd concentrations in the soil. This effect compounded from Generation 1 to Generation 2: cocoon production of Generation 2 worms in contaminated soil was lower than for Generation 1 worms under the same exposure conditions. By Generation 3 and for the 30 mg/kg exposure condition, cocoon production had returned to the same level as for exposed Generation 1 worms (see also Findings **[6]** and **[8]**).

[5] Under some conditions when worms were returned to controls soils, large rebounds to well above performance of controls occurred in growth rate (e.g. Gen 1, 90-CS; **Figure 4.7**) and/or cocoon production (e.g. Gen 2 and 3, 30-CS; **Figure 4.13** and **Figure 4.17**); suggesting that resources being used to counter toxicity were redirected when the Cd stressor was removed. For higher exposures or longer exposure times, these effects disappeared, and worms performed more poorly overall. This distinction suggests there is a tissue Cd threshold beyond which recovery from Cd exposure becomes very difficult.

[6] Comparison of Cd uptake at the lowest exposure condition across the three generations suggested that there may have been a mean 33% increase in Cd uptake in Generation 2 worms, with a return to normal levels by Generation 3. This finding remains uncertain but corresponded with the pattern for cocoon production (**Finding 4**), where Generation 2 worms dipped further relative to

Generations 1 and 3. Results for numbers of differentially expressed genes are also consistent (See **Finding [9]**)

[7] Gene expression results suggest that after 28 d recovery, the impacts of prior Cd exposure in Gerneration 1 *E. fetida* were being well-controlled. This result is quite different to that seen for As, where after 28 d of recovery, large numbers of genes were still being differentially expressed. For Cd the likely reason is that Cd-binding by metallothionein (MT) is very effective, meaning that until MT is saturated, the main response to Cd to the active exposure situation. Together with **Finding [2]** these results suggest that for *E. fetida*, ~370 mg/kg Cd is a tissue level at which all Cd-binding mechanisms have already become saturated. In magnitude terms this compares well with the 200 mg/kg estimated threshold for MT-saturation and onset of Cd toxicity in the human kidney.

[8] Gene expression results for Generation 2 and 3 worms show distinct differences to those of Generation 1 worms (see **Finding [7]**). In Generations 2 and 3, levels of differential gene expression after exposure remained very high. This distinction thought to reflect the higher relative body burdens of Cd in the second and third generation worms, which were born into Cd-spiked soils. Results suggest that Generation 2 and 3 worms were finding it hard to recover.

[9] Despite Finding **[8]**, there was a notable difference between Generation 2 and 3 worms in their recovery responses. After a recovery period in clean soils, Cd-exposed Generation 2 worms showed equivalent (apparently ~13% higher) differential gene expression as they had before it. In contrast, Generation 3 worms showed a 28% reduction. This difference may relate to Finding **[6]**, where it appeared that Generation 2 worms showed increased uptake in Cd and reduction in cocoon production compared with worms of Generation 3.

[10] Overall findings [6] to [9] suggest that genetic responses to Cd are sensitive to Cd uptake, accumulated Cd tissue levels, and whether a MT-saturation threshold has been passed.

Implications

Two implications of **Findings [1]** to **[3]** are that Cd may be toxic to soil invertebrates at much lower concentrations than has previously been appreciated; and that for bioaccumulative contaminants including Cd, toxicity should be assessed over the organism's full unencumbered life-span. There is an urgent need to reconsider the reliability of 28 d toxicological testing for bioaccumulative contaminants to allow for these two effects and a move towards life-span linked moderate-to-low dose experiments. Building on this, Finding **[4]** and the gene expression results (Findings **[6]** to **[10]**) imply that for populations of worms (and presumably other soil invertebrates) living on Cd contaminated land, impacts are likely to compound across generations. This is likely to be because each new generation is born into an already contaminated environment and starts with a significant tissue Cd burden. To make things worse still, Findings **[7]** and **[8]** imply that when the tissue burden of Cd crosses a certain threshold, damage may become irreversible. This has a parallel in human biochemistry in cases where Cd levels in liver or kidney tissue saturate available MT capacity, usually resulting in fatality.

Future research on Cd should be directed towards revising acceptable limits for Cd in soil for protection of ecological receptors. For soil invertebrates these should focus on (a) characterising uptake and impacts of lower-level exposures (where BCFs are likely to be highest) that (b) designing exposures that span an appreciable part of the invertebrate's lifespan (allowing Cd accumulation to occur), (c) understanding the nature of the apparent MT-saturation threshold, and (d) investigating the impacts of soil type and co-exposure (**Chapter 6**). Combining these approaches it may be possible to develop a more robust basis for risk-based limits for soil Cd that would be protective of ecological receptors.

Chapter 5: Uptake and effects of As exposure to *E. fetida* over two generations

5.1 Introduction

In this work *E. fetida* was used as a model organism to assess impacts of Cd and As exposure (**Section 1.1**). This chapter provides results and discussion relating to phenotypic and gene expression impacts of the worms' exposure to As.

As discussed in **Chapter 2**, As and its compounds have been widely used by humans, including for medicinal purposes, as pesticides, wood preservatives, and cattle and sheep dips, in glass manufacture, and in a range of electronic devices. Natural As can also be released through other human activity linked to the increasing global population, such as wide-scale use of groundwater resources. The combination of these factors has caused an increase in cases of human and environmental exposures to elevated levels of As.

Methodological approaches to the As exposure and recovery experiments are outlined in **Sections 3.5**; and details of genomic tests are provided in **Section 3.2.3**. For As, the experiments were carried out over two worm generations. Aims and objectives of this work are as outlined in **Section 1.2**. The principal objectives of the experiments outlined in this section of the thesis were to determine:

- patterns of As uptake over longer time-scales;
- how different As concentrations in soil affect growth, reproduction, and gene expression of *E. fetida*
- the extent to which worms have the ability to recover from effects of As toxicity after exposure; and
- whether and how the effect of As exposure impacts on the next generation.

5.2 Phenotypic effects

5.2.1 Generation one

5.2.1.1 Overview

As detailed in **Section 3.5**, these experiments involved worms being exposed to control soils and two elevated As concentrations (10 mg/kg and 20 mg/kg). These As concentrations had been selected after initial experiments at higher soil As concentrations (60 mg/kg and 90 mg/kg) resulted in excessive mortality (**Section 3.5.1**), making them unsuitable for the aims of this work. Measurements were taken after 28, 56 and 84 days.

Phenotypic results for first generation As exposure experiments are provided in **Appendix 3**, and summary statistics for the same data are given in **Table 5.1**. Mean As concentrations in worm tissue under each exposure condition are shown in **Figure 5.1**.

Table 5.1: Summary statistics relating to effects of As exposure on *E. fetida* – Generation 1. Results are reported as means with standard deviations in brackets.

Soil As condition and concentration	Measured variable	Time (days)			
		1	28	56	84
Unspiked (control) soil: 1.31 mg/kg	Mean weight(g)	0.33 (0.01)	0.36 (0.03)	0.38 (0.02)	0.41
	Tissue [As] (mg/kg)	0.95 (0.32)	0.82 (0.23)	1.14 (0.49)	0.97
	Mass As per worm(µg)	0.31	0.29	0.43	0.40
	Cocoon count (per 10 worms)	0	7.25 (2.22)	17.2 (3)	22.9
	percent fatalities	0	0	0	0
Spike 1: 10 mg/kg	Mean weight(g)	0.31 (0.04)	0.34 (0.04)	0.46 (0.05) [a]	0.59 [b]
	Tissue [As] (mg/kg)	0.95 (0.32)	4.46(1.44) [c §]	12.3 (1.7) [d]	36.2 [d]
	Mass As per worm(µg)	0.29	1.51 [c §]	5.53 [d]	21.4 [d]
	Cocoon count (per 10 worms)	0	2 (2.28) [a]	38.2(13.9) [§ a]	138 [c §]
	percent fatalities	0	5	2.5	0
Spike 2: 20 mg/kg	Mean weight(g)	0.29 (0.01)	0.38 (0.20)	0.47 (0.04) [a]	0.51 [a]
	Tissue [As] (mg/kg)	0.95 (0.32)	12.2 (7.1) [d §]	35.3 (3.9) [d]	50.9 [d]
	Mass As per worm(µg)	0.27	4.63 [d §]	16.6 [d]	26.0 [d]
	Cocoon count (per 10 worms)	0	0 [c]	58.7(7.4) [d §]	137 [c]
	percent fatalities	0	0	0	0

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001
Difference between means compared with previous 28 d time period; [§] <0.05, [¶] <0.01, [*] <0.001, [#] <0.0001

Table 5.2: Changes in weights, As uptake and cocoon production in As exposed worms relative to those in control soils.

Soil As condition and concentration	Measured variable	Time (days)			
		1	28	56	84
Spike 1: 10 mg/kg	Relative weight	0.94	0.94	1.24	1.44
	Relative tissue [As]	1.0	5.4	10.8	37.3
	Relative mass As per worm	0.9	5.2	12.9	53.7
	Extra cocoons (per 10 worms)	0	-5	21	115
Spike 2: 20 mg/kg	Relative Weight	0.88	1.06	1.24	1.24
	Relative Tissue [As]	1.0	14.9	31.0	52.5
	Relative mass As per worm	0.9	15.7	38.3	65.3
	Extra cocoons (per 10 worms)	0	-7	42	114

5.2.1.2 As uptake by *E. fetida*

Average As concentrations in Generation 1 *E. fetida* after 28, 56 and 84 d are shown in **Figure 5.1**. Estimated bioconcentration factors are provided in **Table 5.3**.

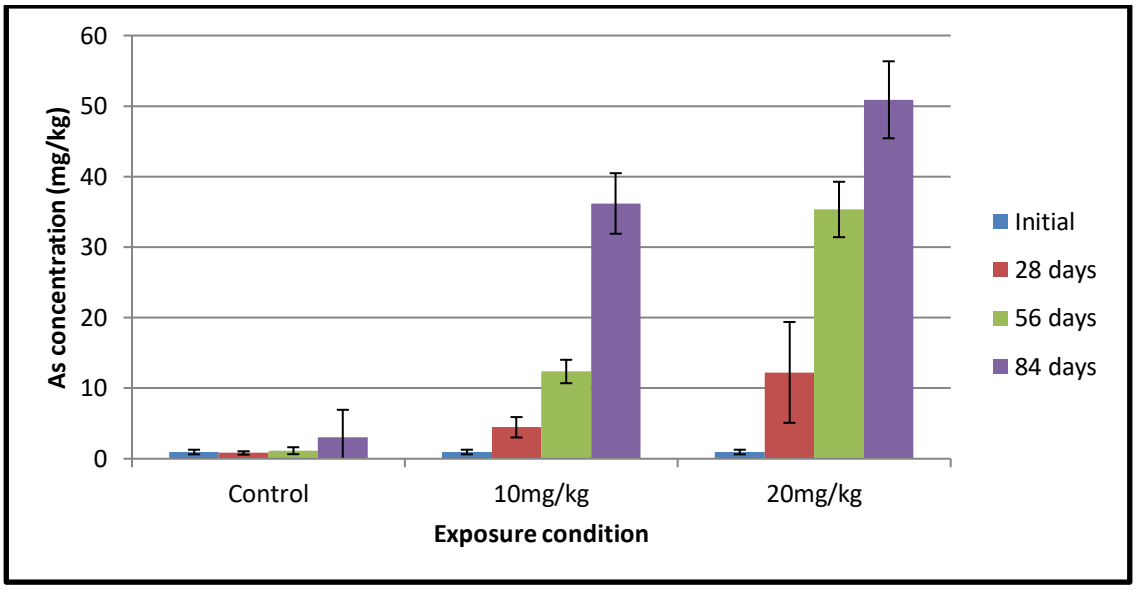

Figure 5.1: Average As concentrations in E. *fetida* of Generation 1 after being exposed to As spiked soil for 28, 56 and 84 d. Error bars represent standard deviations from the mean. Refer to Table 5.1 for statistical significances of differences between means.

Table 5.3: As bioconcentration factors

Variable	Exposure period (days)	As in soil (mg/kg) 0.31	10	20
As in worm tissue (mg/kg)	28	0.82	4.46 [c]	12.2 [d]
	56	1.14	12.3 [d]	35.3 [d]
	84	1.25	36.2 [d]	50.9 [d]
Bioconcentration Factor	28	2.65	0.45	0.61
	56	3.68	1.23	1.77
	84	4.03	3.62	2.55
	Mean	3.45	1.77	1.64
	Std dev	0.72	1.65	0.97

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001
Difference between means compared with previous 28 d time period; [§] <0.05, [¶] <0.01, [*] <0.001, [#] <0.0001

As shown in **Figure 5.1**, worms exposed to As spiked soil showed a steady increase in concentrations of As in their tissues with time.

The amount of As in the worms is positively correlated ($R=0.792$, $n=120$, $p<0.01$) with As concentrations in soil. Worms in 10 mgAs/kg soils averaged 4.46 mg/kg of As in their tissue after 28 d. Over the second 28 d this increased by a further 7.84 mgAs/kg (**Figure 5.1**). Worms in 20 mgAs/kg soils showed an average of 12.2 mgAs/kg after first 28 d and 35.3 mgAs/kg after second 28 d period. Arsenic concentrations in worms living in 20 mgAs/kg soils were nearly three times higher than those of the worms in 10 mgAs/kg soils.

As accumulation was continuous throughout the experiment and an equilibrium plateau was not reached for As uptake during the experimental timeframe. In keeping with this several researchers also report As body burdens increased with increasing soil As concentrations (Romero-Freire et al., 2015, Anderson et al., 2013, Fischer and Koszorus, 1992, Lee and Kim, 2013). In a long-term (23 d) arsenate (40 mgAs/kg) accumulation experiment, Meharg *et al.*, (1998)reported that there was a steady increment of arsenate in the earthworm *Lumbricus terrestris* and after 12 d, the concentration in worm tissues were equivalent to the

soil As concentration (Meharg et al., 1998). However, another researcher reported that there is no direct relationship between As concentrations in the soil and those in the worm tissues (Geiszinger et al., 1998). Such results may reflect the influence of soil factors on As availability and uptake, as Geiszinger *et al.* (1998) collected worms from six As contaminated sites. In the natural environment worms can also discriminate to avoid contaminated soil (Langdon et al., 2003a). Therefore, it is difficult to predict that worms were living in the As contaminated soil throughout their lifetimes. However, in the experimental setup used in current work, OECD 'soil' conditions are constant and avoidance behaviour was not possible. Soil in each beaker had homogeneous levels of As and therefore worms could not avoid As exposure. These factors made it possible to detect an underlying relationship because variation of As concentration in the beaker soils and time was the only habitat-related variable to change.

In terms of relative bioconcentration factors (BCF values) (**Table 5.3**) two things stand out from the results:

1. The relative accumulation of As is slightly more efficient at natural As levels in soil than at either 10 mg/kg or 20 mg/kg As in soil. An effect like this was also seen for Cd. However, in the case of Cd the BCF from natural soils was much higher (up to 138 for Cd compared with 4.03 for As, both measured at 84 d).

2. In all cases, relative As uptake increased with exposure time. This result is most pronounced in the 10 mg/kg soil condition, as can also be seen in **Figure 5.1**.

Consistent with the first observation, Geiszinger *et al* (1998) reported the As bioconcentration factor for an uncontaminated site was much higher than for a contaminated site (Geiszinger et al., 1998). A possible reason for the first effect is that there may be a minimum amount of coincidental arsenate (AsO_4^{3-}) uptake that occurs along with normal uptake requirements of the major ion phosphate (PO_4^{3-}). This possibility comes about because arsenate and phosphate are isomorphous. Uptake of arsenate with phosphate is given as a reason why marine fish have developed mechanisms to detoxify As by conversion to organoarsenic species (predominantly arsenobetaine) that are virtually non-toxic (Lawrence et al., 1986, Edmonds and Francesconi, 1988). In that case, the mechanism has been linked to

low levels of phosphate in ocean waters driving the evolution of optimally-efficient means of harvesting it. This raises the question about whether earthworms may also require special mechanisms for concentrating phosphate, and whether (like ocean fish) they may be capable of detoxifying As through conversion of organic forms. A search for As speciation in earthworms confirms that this is likely to occur (Geiszinger et al., 1998, Langdon et al., 2002). (Langdon et al., 2002) reported presence of 17-23% arsenobetaine in worms (*L. rubellus*) from uncontaminated and mine sites, and noted presence of another As(III)-S species that may dominate but also disintegrate upon extraction. The picture is also more complex than metabolism within the worm alone because gut microbiota is also involved. (Wang et al., 2019) recently examined the effects of As exposure on As biotransformation genes in both soil and the earthworm (*Metaphire sieboldi*) gut. These authors reported considerable differences between soil and gut microbial communities, where the latter were capable of transforming arsenate (As(V)) to arsenite (As(III)).

The second effect is an increase in the efficiency of As uptake over time as exposure continued. For both exposure conditions, ~3 times as much As was taken up in the second 28 d period than the first. For the lower (10 mg/kg) exposure condition, this continues to 84 d, ie. ~3 times as much As again had been taken up by 84 d than had been taken up at 56 d. Whereas the curve for Cd uptake at lower exposure concentrations (30 mg/kg and 90 mg/kg) shows an initial rise then constant increase over time; the pattern for As at 10 mg/kg exposure was greater relative uptake with each 28 d step.

Results for the lowest Cd and As exposures are shown together in **Figure 5.2**

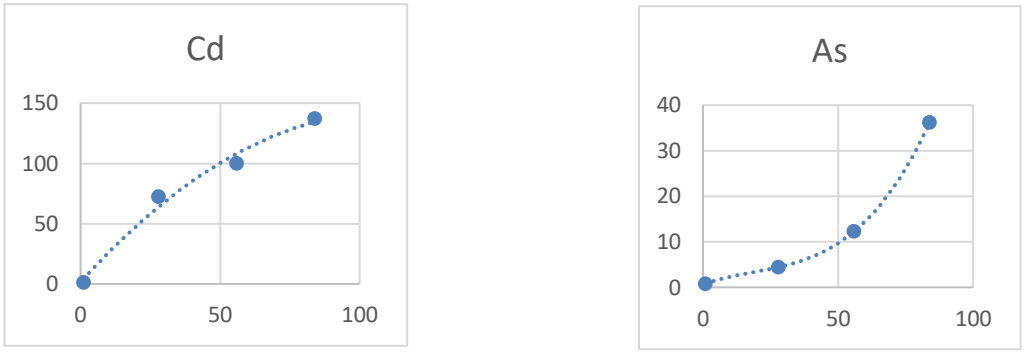

Figure 5.2: Comparison of mean uptake of Cd and As from soils containing 30 mgCd/kg (left) and 10 mgAs/kg (right). Horizontal axes are days, vertical axes are tissue concentrations in mg/kg.

One possible reason may be that worm growth, and /or metabolic repair mechanisms working to counter the impacts of As exposure, work to boost the uptake of phosphate, thus drawing up more As. This possibility seems reasonable from the point of view that all growth and repair processes will require additional metabolic energy, which requires phosphate for ATP, as well as P in new structural and other proteins. A second possible reason of contributing factor for growth is that low level As may suppress parasites in the worms, permitting increased growth (see **Section 5.2.1.3**).

This raises a potentially important connection to results of multi-element testing for the Cd exposure experiments (**Chapter 7**). In that case, increasing Cd was found to correlate ($p<0.0001$) with increasing As uptake, despite the fact that As was not spiked into the soils (**Section 7.2.2**). Following the reasoning above, the reason that As uptake increases with Cd exposure may be to maintain adequate levels of phosphate to enable operation of cellular repair mechanisms. It is noted that Cd and total P did not correlate, but this result is not inconsistent with an As-Cd correlation caused by increasing P uptake, because levels of P are subject to homeostatic regulation. With growth, P will be incorporated into tissue proteins. Even at a set body burden, uptake of P would still increase if net use and incidental losses of P increased due to increased use in cellular repair and/or growth.

These two results raise a wider question about all earthworm toxicity experiments based on natural or model soils containing some level of As. This is the possibility to some extent any contaminant exposure that causes an adverse effect and

triggers cellular repair processes may also involve some co-exposure to As, which may alter the outcome. Whether there is any significant impact on the outcome may depend on available As and P in the exposure medium, and the nature of the contaminant interaction (*e.g*. additive or synergistic).

It should be noted that As concentrations in the worm tissue were high compared with those that would be required to cause significant harm in humans. For example, they reached higher levels than those associated with a fatal poisoning by Moriya *et al*. (2006); who reported As tissue concentrations of 6.3-8.4 mg/kg in the lungs, 16.4 mg/kg in the kidney, and 23 mg/kg in the liver (Moriya et al., 2006).

5.2.1.3 Mortality and growth

Results for growth are provided in graphical form in **Figure 5.3** (see also **Table 5.1**).There was no significant mortality in this experiment at soil As concentrations up to 20 mg/kg.Only 5% of (2) worms from 10 mgAs/kg soils died during first 28 d and 2.5% (1 worm) died during the second 28 d. No mortality occurred worms in control and 20 mgAs/kg soils. The limited mortality observed at 10 mgAs/kg is unlikely to be related to As toxicity, as no mortality was observed at the higher 20 mg/kg concentration.

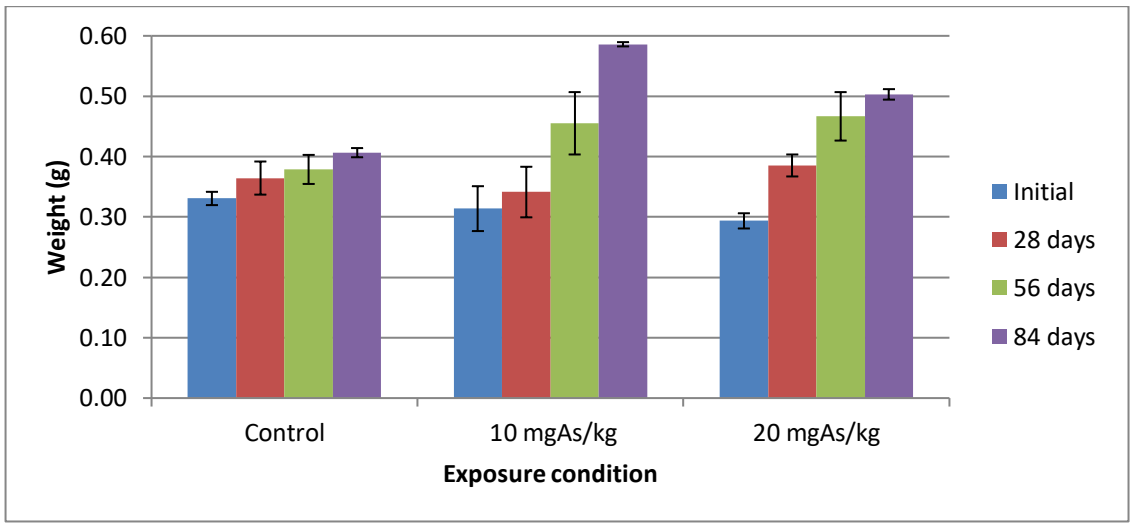

Figure 5.3: Changes in the fresh mean weight of *E. Fetida* in OECD artificial soil spiked with different As concentrations (Generation 1). Error bars represent standard deviations from the mean. The results are based on 40 worms. Refer to Table 5.1 for statistical significances of differences between means.

Worms in all samples including controls showed weight gain during the experiment. Initial weights of worms were similar in all beakers: 0.33±0.11 g for the control, 0.31±0.04 g for the 10 mgAs/kg soils and 0.29±0.01 g for 20 mgAs/kg soils. After the first 28 d the average weight of worms in 10 mgAs/kg soils was 0.0232 g lower than for controls and that of worms in 20 mg/kg was 0.0209 g higher than controls. After the second 28 d worms in both 10 mgAs/kg ($p=0.026$) and 20 mgAs/kg ($p=0.009$) soils showed significantly more growth than controls at 0.46±0.05 g, and 0.47±0.04 g in 10 mgAs/kg and 20 mgAs/kg soils, respectively. During the third 28 d period worms in 10 mgAs/kg soils showed more rapid growth than controls and those in 20 mgAs/kg soils. After 84 d, worms in 10 mgAs/kg soils showed the highest mean weight of 0.59 g while those in 20 mgAs/kg soils showed the second highest of 0.51 g.

The growth percentage of worms was calculated considering initial and final weights of worms in each time period. During the first 28 d worms in 20 mgAs/kg soils showed the highest growth percentage and worms in 10 mgAs/kg showed lowest. During second 28 d worms in 10 mgAs/kg soils showed highest growth percentage while controls showed lowest. The weight of worms also depends on the number of cocoons in the body. Worms in 20 mgAs/kg soils laid the most cocoons during second 28 d period compared to worms in 10 mgAs/kg soils. This could be a possible reason for the higher average weight of worms after the first 28 d period, and second lowest average weight of worms after second 28 d period in the 20 mgAs/kg soils.

In these experiments, and times of 56 d and longer, the presence of As in the soil appeared to increase the relative growth of worms (**Table 5.2**). This effect was particularly pronounced after 84 d, and strongest at the lowest As spike (10 mg/kg) where worms were 44% heavier than controls. Some researchers reported relationship between growth and As concentration in the soil is not always dose-related (Romero-Freire et al., 2015). However, in general, higher doses of As are associated with reduced weights and increased mortality. For example in a study done using earthworms in three As contaminated mining sites in Korea it was reported that As in mining soil caused weight loss of exposed earthworms (Shin et al., 2007a). Anderson *et al*. (2013) reported the average weight of *Lumbricus*

rubellus was negatively correlated with As concentrations in the soil and the worms exposed to the higher concentrations were significantly affected ($p<0.001$), especially at higher (36 and 125 mgAs/kg) concentrations (Anderson et al., 2013). The toxicity of As is known to vary substantially depending on soil physicochemical properties (Wijayawardena et al., 2012). Compared with literature accounts the current work differs in that As exposure concentrations are at the lower end, below any onset of mortality; and the exposure period was extended to give a longer timeframe of 84 d. The unexpected finding is that in this window of lower-exposures and longer times, As (as arsenate) exposure does appear to stimulate worm growth.

This finding builds on Lee and Kim (2013) reviewed in **Section 2.3.3** of this thesis, that under some conditions low-level As exposure was associated with increased growth compared with controls. A weakness of that work is that As concentrations identified by Lee and Kim (2013) as being linked with increased worm growth were below or near natural background values for As in soils. This work takes the finding (that moderate As exposure may stimulate the growth of worms) to higher As exposure concentrations, and longer timeframes.

Possible mechanisms behind the increased growth observed were not investigated as part of this work. However, one possible reason is that the presence of some As reduces the burden of parasites in the worms, permitting more resources to be used for growth. This explanation is consistent with the fact that As can be toxic to mites and was used for many years in sheep-dipping solutions to treat ectoparasites (NZ, 2006, Graham and Scott, 1948), and the organoarsenic compound Roxarsone has been widely used as a 'growth promotant' in poultry production (Garbarino et al., 2003) and is thought to work as both an antibacterial agent and a coccidiostat (Noack et al., 2019).

5.2.1.4: Sexual maturation and cocoon production

Cocoon production of the Generation 1 worms (**Table 5.1**) is shown in graphical form in **Figure 5.4** and tabulated in **Table 5.4**.

Table 5.4: Cocoon production by *E. fetida* (per 10 worms) exposed to As spiked soil expressed as both the cumulative total and increase over each 28 day exposure period.

Statistic	Exposure period (days)	Generation 1				
		Controls	10 mg/kg		20 mg/kg	
Cumulative total production (per 10 worms)	0	0	0		0	
	28	7.3	2.0		0	
	56	17.3	38.3[a §]		58.8 [§]	
	84	22.9	137 [e c]	61.3[f a]	137[ec]	86.9[f a]
New production during each 28 day exposure period (per 10 worms)	Exposure period (days)	Generation 1				
		Controls	10 mg/kg		20 mg/kg	
	0	0	0		0	
	28	7.3	2.0 [a]		0	
	56	10.0	36.3 [a]		58.8 [b §]	
	84	5.6	99.3 [e c]	23.0[f a]	78.0 [e c]	28.1[f a]

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001

Difference between means compared with previous 28 d time period; [§] <0.05, [¶] <0.01, [*] <0.001, [#] <0.0001

[e] from worms with continued As exposure; [f] from worms returned to clean soil

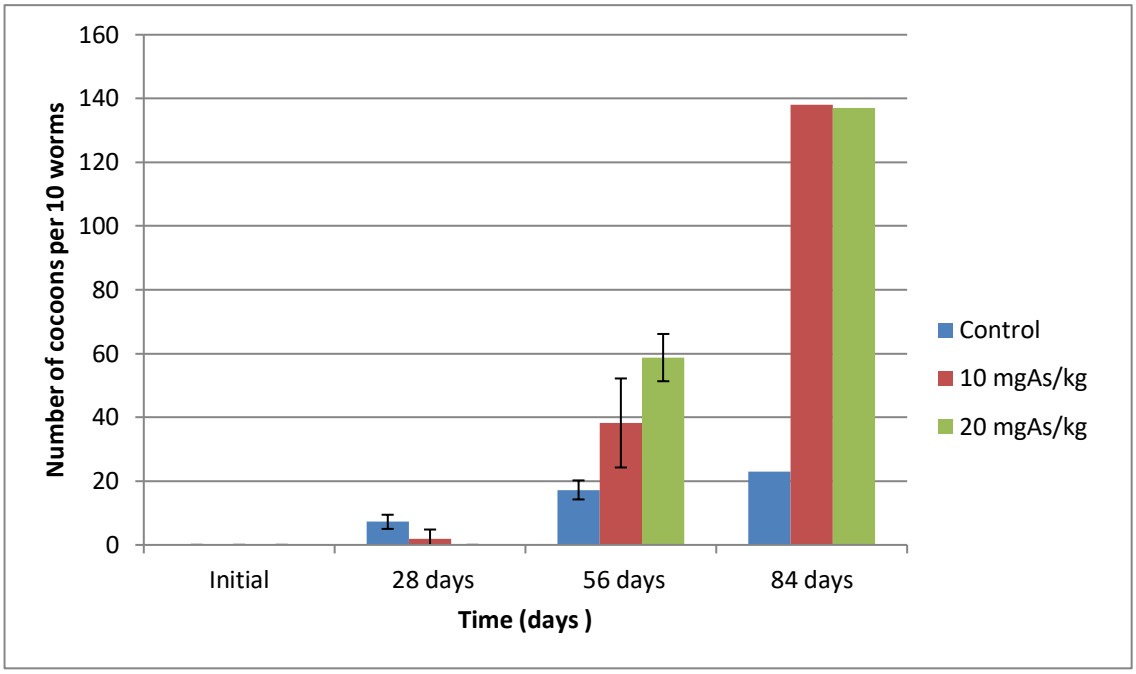

Figure 5.4: Cumulative cocoon production by *E. fetida* (per 10 worms) exposed to As spiked soil for 28, 56 and 84 days. Error bars represent the standard deviations from the mean. Refer to Table 5.1 for statistical significances of differences between means.

In **Table 5.4** the cocoon production figures are given as both the cumulative total, and new production over each subsequent 28 d period; along with cocoon numbers for the worms that were returned to clean soils for the final 28 days (from 56 to 84 d). (Other results for the recovery experiment are provided in **Section 5.2.1.5**.)

As is recognised as a reproductive toxicant in humans which causes negative effects such as stillbirth and spontaneous abortions. In these experiments a reduction in cocoon counts from worms in spiked soil (relative to controls) was observed in the first 28 d, which is the normal maximum length of time that these types of experiments are run with some exceptions. After 28 d worms in control soils had produced on average 7.25±2.22 cocoons per 10 worms, a result that was replicated in Generation 2 (6.0 cocoons per 10 worms). Worms in 10 mgAs/kg soils only produced 2.0±2.2 cocoons per 10 worms, and those in 20 mgAs/kg soils produced none. These results are consistent with literature reports in other 28 d experiments.

Unexpectedly however, after this first period, cocoon production showed dramatic increase relative to control soils. In the second 28 d period (to 56 d) the extent of this increase was proportional to the amount of As exposure, with new cocoon production (per 10 worms) of 10, 36.3 and 58.8 under the control, 10 mgAs/kg and 20 mgAs/kg exposure conditions, respectively. On average and to 56 d, worms in the two spiked soils therefore, showed about 3 (2.8) times more total cocoon production than those in the control soils.

By the end of the third period (to 84 days) total cocoon production of worms in the spiked soils had doubled again to reach a cumulative total that was 6 times that of control soils. By this stage, there was no difference between overall production in the 10 mg/kg and 20 mg/kg exposure conditions which had reached equal numbers of 137.6 and 136.9 cocoons per 10 worms, respectively. However, the relative increase in the final 28 day period (from 56 to 84 days) was 20% lower in the soil with the most As (**Table 5.4**).

This was recognised as a remarkable number of cocoons to be produced per worm. Although data is limited it could be speculated that the fact that the total numbers reached after 84 days in 10 mg/kg and 20 mg/kg soils may reflect some natural upper limit in production.

A relative reduction in cocoon production in the subset of (56 d) exposed worms that were returned to control soils over the final 28 day period (**Table 5.4**) appears to provide confirmation that As was a driving factor in stimulating excessive cocoon production. For these worms, the additional production (per 10 worms) from days 56-84 dropped back to similar values of 23.0 for the 10 mg/kg condition and 28.1 for the 20 mg/kg condition.

Therefore in these experiments, As appears to act as a reproductive stimulator to *E. fetida* over longer exposure periods than are typically used for toxicological testing and exposure concentrations that are elevated, but sub-lethal. Specifically, the cocoon production was significantly ($p<0.05$) increased when As exposure continued beyond 4 weeks. As noted above, this was also accompanied by a substantial increase in tissue levels of As.

These effects are unlikely to be linked to worm maturity. When the worms were introduced to the soil they were fully mature with well-developed clitellum. However, there is evidence that this may be a case where increased cocoon production should not be seen as a signal of reproductive success.

Hatchlings per cocoon were assessed at 56 days by dissecting 5 cocoons from each condition include from controls. Results are shown in **Table 5.5**.

Table 5.5: Average numbers of hatchlings per cocoon

As concentration in soil	Average hatchlings per cocoon(after 56 d)
Control	2.2
10 mg/kg	5.0
20mg/kg	2.6

In worms from control soils, out of five cocoons dissected 4 had 2 hatchlings per cocoon and 1 had 3 hatchlings. The average number of hatchings per one cocoon in control is 2.2 and this is the lowest average in the experiment. Cocoons sampled from 20 mgAs/kg soils did not largely differ from controls: out of five cocoons, 3 cocoons had 3 hatchlings and 2 had 2 hatchlings, giving an average of 2.6. All cocoons sampled from 10mgAs/kg soils had 5 hatchlings per cocoon, and the size of the cocoons were bigger than the cocoons in the control and 20 mgAs/kg soils.

Therefore at the lower of the two spiked concentrations (10 mg/kg) and at 56 d, the effect of As was to double the number of hatchlings in the cocoons. This finding complements the results for worm weights where it was found the lower of the two spikes was associated with the highest relative growth.

Results indicate that As may be stimulatory for both cocoon and hatchling **production**. As noted above, a possible factor in this which may also vary with soil type, bio-availability and chemical form is that up to a certain level As may act to suppress bacteria, parasites or other competing species.

Romero-Freire *et al* (2015) researched the influence of soil properties on effects of As on the juvenile production of *E. Andrei*. Seven different soils were used. Whereas 5 soils showed a decrease in cocoon production with increasing As exposure; in 2 of the soils there was an initial increase followed by a decrease only at higher concentrations. The exposure period was 28 days (Romero-Freire et al., 2015). These results and those of Wijayawardena *et al.* (2012) show that soil properties have a high influence of As availability to worms.

A second factor to consider is **viability** of the cocoons that are produced. Langdon *et al.* (2003) reported that As residues in a different species of worm (*L. rubellus*) increased cocoon production, and this was accompanied by a decrease in cocoon viability. In their study, worms were collected from a contaminated site (200 mgAs/kg) and from an uncontaminated site. Then they were kept in an uncontaminated soil for 9 weeks and cocoon production was observed. *L. rubellus* from the contaminated soil produced 301 cocoons in total and only 14% were viable; whereas the control worms produced 101 in total and 60% were viable (Langdon et al., 2003a). Even though the As exposure increased cocoon production, the ultimate result was decreased population size because of lower cocoon viability. Nevertheless, the picture is not straight forward, as other workers have reported that higher As concentrations such as 125 mg/kg were associated with significantly reduced cocoon production (Anderson et al., 2013). The authors note that the effect of As to worms depends on the availability of As and edaphic factors.

In Generation 1, four beakers of 10 worms each were maintained for each As concentration and control. Average numbers of cocoons per 10 worms after 56 d was 58.7±7.4, 38.2±13.9, and 17.2±2.99 in control, 10 mgAs/kg and 20 mgAs/kg soils respectively. These were incubated for three months under the same exposure conditions for use in Generation 2 experiments.

The worms hatched in As spiked soils had extremely low survival rates, with most of the worms found to be dead or dying soon after hatching.

- The 10 mgAs/kg soils averaged 5 hatchlings per cocoon. Allowing for worms removed for analysis the number of possible hatchlings across all

four beakers should have been ~764. However, after 3 months incubation there were only 26 hatchlings in total, or 3.4% of the possible amount. The 26 worms that survived grew fast and were healthy.

- The worms in 20 mgAs/kg soils produced 58.7 cocoons per 10 worms with 2.66 hatchlings per cocoon, giving a possible total of~627. Of these there were 14 surviving hatchlings after three months, giving a survival rate of 2.2%. In this case the surviving worms were small and immature with weights ranging from 0.051 g to 0.191 g (mean 0.124 g).

It is hard to distinguish between two possible causes of the high mortality, and both may contribute. One is that cocoons produced from As-exposed worms were less viable to begin with, and the other is that As toxicity has a greater impact on juveniles. When comparing toxicity to juveniles between studies, species and soil type also become variables. For example, (Anderson et al., 2013) reported that significant mortality was seen in the juvenile worms of L. *Rubellus* in under higher As concentrations (125 mg/kg). In this work juvenile worms showed high mortality at soil As concentrations as low as 10 mg/kg. Differences could be because of higher availability of As in the artificial OECD soil used in this work, or the species of worm.

Therefore, we can conclude that an effect of As exposure was to stimulate growth and cocoon production, but under conditions of ongoing exposure the reproductive success is very low. This may be due to the lower viability of cocoons or the sensitivity of juvenile *E. fetida* to As. It follows that even though As apparently acted as growth promotant and reproductive stimulator, ongoing exposure as happens in real-world settings is likely to lead to a decrease population size in the next generation.

It is unclear whether over longer time frames the ongoing presence of As in soil would create a selection pressure for evolution of As resistance in offspring of surviving worms. A potential avenue for future study could be to determine whether worms living through multiple generations on large-scale As contaminated areas (from which escape is less likely) have in fact developed As tolerance or resistance compared with unexposed worms. Old orchard areas

where lead arsenate had been used as a pesticide (and adjacent properties with the same soil type) may provide a suitable exposure and control sites for that type of study.

Hatchlings were taken across to the Generation 2 experiments (see **Section 5.2.2.1**).

5.2.1.5 Recovery tests

After 56 d of As exposure, worms in As spiked soil were randomly divided into two groups (8 worms each). One group was introduced to clean artificial soil and the other to soil with same As concentration as worms had been living in for the previous 56 d. After a further 28 d, weights of worms were measured, the number of cocoons in each beaker was counted, and RNA was extracted from 3 worms for further analysis.

Results are summarised in **Table 5.6**. Note that for the continued exposure conditions the 28 d results correspond to 84 d total exposure. Results for cocoons are also shown earlier in **Table 5.4** and discussed there in the context of the stimulatory effects of As exposure.

Table 5.6: Summary statistics relating to the 28 day recovery experiment. Results are reported as means with standard deviations in brackets.

Soil cadmium condition and concentration	Measured variable	Time (days)	
		Initial	28
Unspiked(control) soil: 0.011 mg/kg	Mean weight (g)	0.39 (0.01)	0.41 (0.01)
	Tissue [As] (mg/kg)	1.14 (0.49)	3.04 (3.89)
	Mass As per worm (µg)	0.44	1.25
	Cocoons/10 worms (summed)	0	5.62 (0.71)
	Percent fatalities (%)	0	0
10 mgAs/kg	Mean weight (g)	0.50 (0.01) [a]	0.59 (0.003) [a]
	Tissue [As] (mg/kg)	12.4 (1.7) [b]	36.2 (4.3) [c]
	Mass As per worm (µg)	6.2 [c]	21.3 [c]
	Cocoons/10 worms (summed)	0	97.5 (7.4) [c §]
	Percent fatalities (%)	0	0
20 mgAs/kg	Mean weight (g)	0.43 (0.01)	0.50 (0.01) [a]
	Tissue [As] (mg/kg)	35.3 (3.9) [c]	50.9 (5.4) [c]
	Mass As per worm (µg)	15.2 [c]	25.4 [c]
	Cocoons/10 worms (summed)	0	81.2 (5.6) [c §]
	Percent fatalities (%)	0	12.5
10-CS	Mean weight (g)	0.44 (0.01) [a]	0.45 (0.006)
	Tissue [As] (mg/kg)	12.4 (1.7) [b]	22.6 (9.7) [c]
	Mass As per worm (µg)	5.44 [c]	10.2 [c]
	Cocoons/10 worms (summed)	0	33.1 (6.3) [a §]
	Percent fatalities (%)	0	0
20-CS	Mean weight (g)	0.45 (0.002) [c]	0.48 (0.25)
	Tissue [As] (mg/kg)	35.3 (3.9) [c]	21.8 (8.6) [b]
	Mass As per worm (µg)	15.9 [c]	10.5 [c]
	Cocoons/10 worms (summed)	0	28.1 (3.5) [a §]
	Percent fatalities (%)	0	0

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001

Difference between means compared with previous 28 d time period; [§] <0.05, [¶] <0.01, [*] <0.001, [#] <0.0001

Losses of accumulated As

Figure 5.5 shows As concentrations in *E. fetida* before (56 days) and after (84 days) the 28 d recovery experiment.

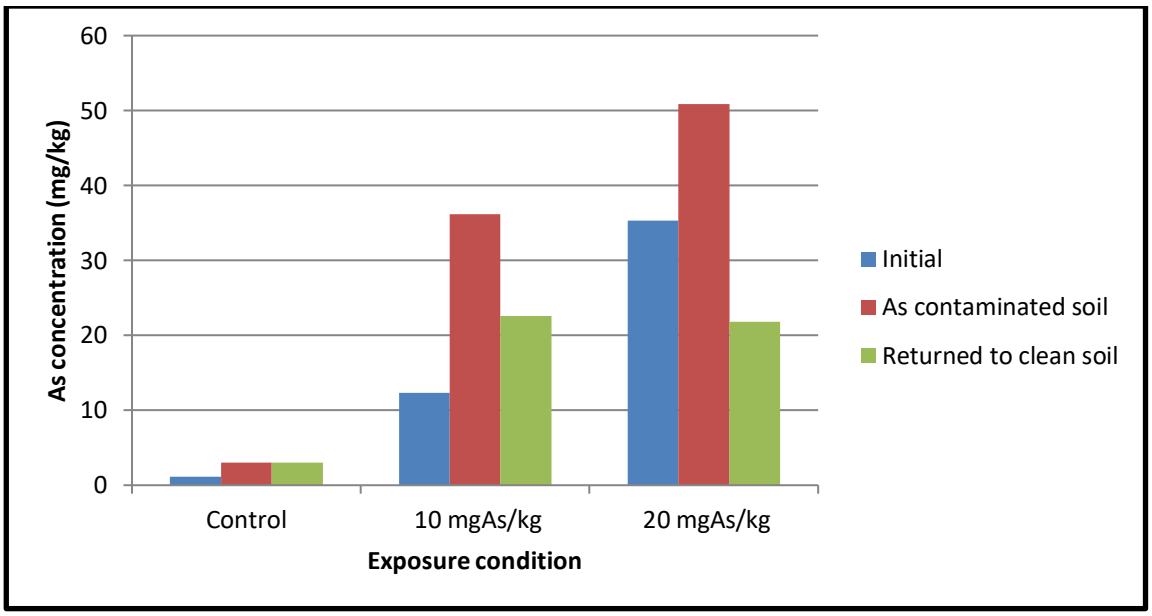

Figure 5.5: Average As concentrations in *E. fetida* (Generation 1) at 56 and 84 d, with and without ongoing exposure. Refer to Table 5.6 for statistical significances of differences between means.

Key:10-CS: worms exposed to 10 mgAs/kg for 56 d that were returned to clean soils for the final 28 d; 20-CS: worms exposed to 20 mgAs/kg for 56 d that were returned to clean soils for the final 28 d.

The assumption of the test was that after worms were moved to clean soil, they would show a decrease in tissue As concentrations and an increase in cocoon production and growth. A decrease in tissue As concentrations would be expected for two reasons:

1. The dilution effect of tissue growth. Tissue growth has no effect on the total amount (µg or mg) of As present, but having more tissue to distribute the As through reduces the As concentration expressed in units of mg/kg (mg of As per kg of tissue).

2. Elimination of As by worms. The total mass of arsenic directly reflects this effect. The biological half-life of As is expected to be approximately 15 days (Casado-Martinez et al., 2010), based on experiments such as relocated of a population of *L. rubellus* As contaminated sites (Langdon et al., 2003a). Some researchers have reported that worms do not eliminate As (Fischer and Koszorus, 1992, Lee and Kim, 2013), and that in at last some cases, As may be sequestered in worm tissues as a form of As-thiol complex (Meharg et al., 1998).

Results for worms exposed to 20 mgAs/kg soils were as expected. After these worms were returned to clean soils for 28 d, the total mass of As per worm decreased by 33% (from 15.9 µg/worm to 10.5 µg/worm) reflecting actual As loss. Over the same period the As concentration decreased by 38% (from 35.3 mg/kg to 21.8 mg/kg), with the difference between the two figures reflecting a slight increase in the mean weight of worms returned to control soils (Table5.7).

Once again, results for worms exposed to moderate As (10 mg/kg) were remarkably different to those that would normally be expected. After these worms were returned to clean soils for 28 d, the total mass of As per worm unexpectedly increased by 47% (from 5.44 µg/worm to 10.2 µg/worm) reflecting actual As gain, but from unspiked soils. The mean weight of these worms did not change significantly, so that over the same period the As concentration also increased by about the same amount (45%; from 12.37 mg/kg to 22.6 mg/kg) (**Table 5.6**). For reference, worms that remained in control soils also showed a relative As gain, but at much lower absolute amounts of As (0.44 to 1.25 µg/worm).The result needs to be viewed alongside the result for worms that were left in 10 mgAs/kg soils, in which concentrations of As almost tripled (**Figure 5.1**, **Table5.7**).

To summarise in terms of mass (µg/worm) of As taken up per worm from 56 to 84 d:

- Worms remaining in 20 mgAs/kg soils: factor of **1.7** *increase*
- Comparison set returned to control soils: factor of **0.7***decrease*
- Worms remaining in 10 mgAs/kg soils: factor of **3.4** *increase*
- Comparison set returned to control soils: factor of **1.9** *increase*

Results for 20 mg/kg worms returned to clean soils are as expected. Results for the 10 mg/kg worms returned to clean soils are not. They are consistent with removal from the spike soils in that relative uptake decreased. However to have any uptake increasing by so much under the 'recovery' condition strongly suggests the existence of a process in these worms that continued to result in increased As uptake or retention, despite their substrate having relatively low levels of As. It is speculated that this may represent these worms having already entered a phase where uptake up more phosphate has been triggered, causing an ongoing coincidental As uptake.

This result provides support for the idea that the reason that As can (apparently) promote its own uptake at modest exposures in the ongoing exposure experiments (**Figure 5.2**) is likely to be linked with phosphate utilisation. The biological half-life of As was calculated, based on the mass of As in worms in the recovery experiment, assuming first-order elimination kinetics. However, it was calculated only for 20-CS because in 10-CS the worms increased their level of tissue As within the 28 d period. The biological half-life of As in the experiment was 28 days.

Changes in mortality and growth

During the recovery period mortality only occurred in worms that had been exposed to 20 mgAs/kg soils and it was 14.3% (2 worms). Absolute weight changes are shown in **Figure 5.6**.

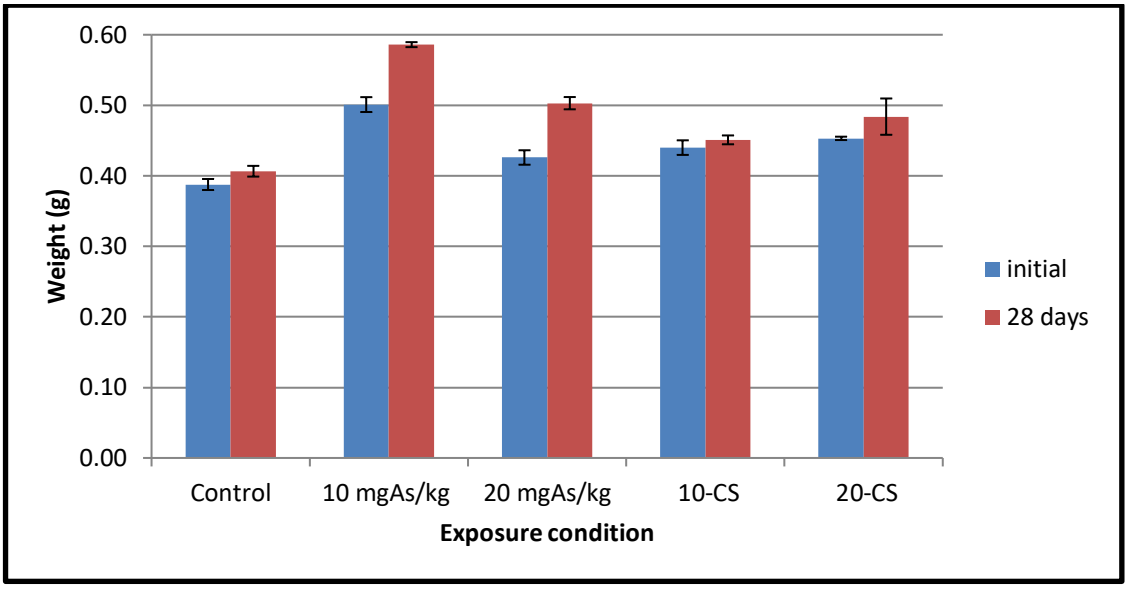

Figure 5.6: Average weight of *E. fetida* groups at beginning (56 d, 'initial weight') and end (84 d, 'final weight') of third 28 days period, for both continued exposure and worms returned to control soils (10-CS, 20-CS). The error bars represent standard deviation from the mean value**.** Refer to Table 5.6 for statistical significances of differences between means.

Some variations between matched initial conditions (**Figure 5.6**; **Table 5.6**) are probably due to limits from the sample sizes involved (n=8 worms for each condition). For this reason, relative weight changes are likely to be more reliable than cross-comparison of absolute weights. All worms showed positive growth. Relative weight gains as percentages followed the order 10 mg/kg (+18.0%) > 20 mg/kg (+16.3%) > 20-CS (+6.7%) > Control (+5.1%) > 10-CS (+2.3%).

Table 5.7: Weight changes of *E. fetida* in the recovery experiment

Condition	Initial mean weight (g)	Final mean weight (g)	Mean weight gained (g)
Control	0.39	0.41	0.02
10 mg/kg	0.5	0.59	0.09
20 mg/kg	0.43	0.5	0.07
10-CS	0.44	0.45	0.01
20-CS	0.45	0.48	0.03

Overall it is clear that worms that experienced continued As exposure continued to show the most growth. In contrast, worms returned to clean soils showed growth that was comparatively modest, and either lower or not significantly greater than the 5% growth seen in control worms. These results are consistent with the idea that As may promote growth, with the strongest effect seen under the 10 mg/kg ongoing exposure condition.

For the 10-CS worms there is a minor paradox. As noted above these worms did continue to extract As from control soils, but showed no significant growth. The difference may come down to the amount of As involved being lower than for 10 mg/kg worms. In the 10 mg/kg worms, 15 µg/worm of As was extracted over 28 d; for the matched set returned to control soils, the increase was one-third of that figure (4.8 µg/worm) (based on data in **Table 5.6**). It is also possible that the 10-CS worms may have also been using energy for that uptake process rather than for growth.

Cocoon production

Changes in cocoon production for worms experiencing continued exposure compared with those returned to control soil are shown in **Figure 5.7**.

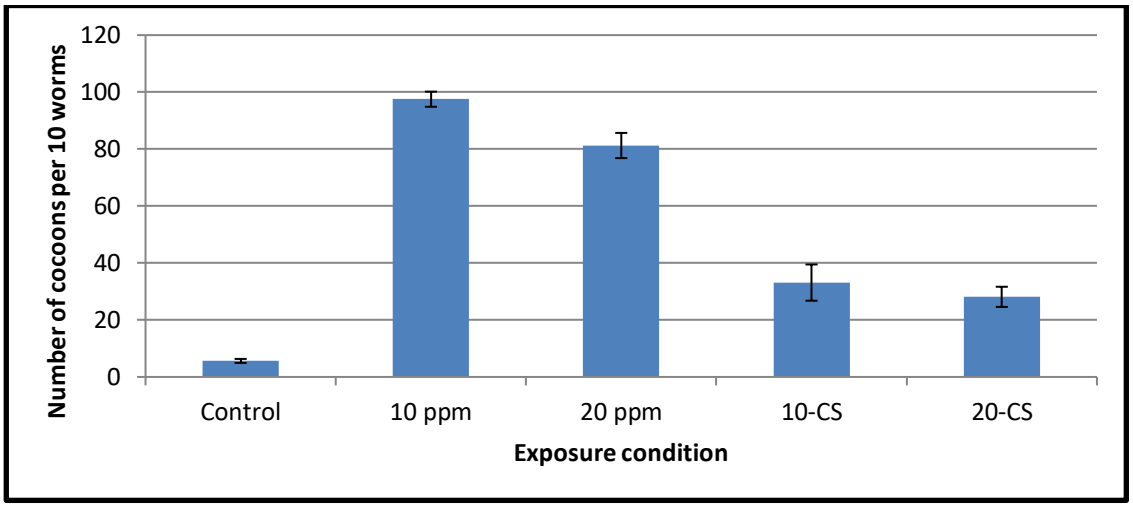

Figure 5.7: Average number of <u>new</u> cocoons produced after 28 days by *E. fetida* (per 10 worms) under continuation of the original exposure condition and return to control soil from 56-84 days. The error bars represent standard deviation from the mean value**.** Refer to Table 5.6 for statistical significances of differences between means.

Results for the ongoing exposure setting have been discussed above in **Section 5.2.1.4**, but are shown in **Figure 5.7** for comparison.

Results for equivalent worms returned to clean soils show two things:

1. When As exposure was reduced, cocoon production substantially reduced compared with worms that remained exposed to the As-spiked soils. Relative production dropped by similar figures of 66% for 10 mg/kg soils and 65% for 20 mg/kg soils.

2. Despite this, in both cases cocoon production in worms that had been exposed to As remained substantially higher than cocoon production in control worms. Relative production was still 489% higher in 10 mg/kg soils and 400% higher in 20 mg/kg soils. This could be because the worms had a substantial amount of As retained in their bodies as discussed above.

5.2.2 Generation 2

5.2.2.1 Overview

Hatchlings from first generation were taken to the Generation 2 experiments. As described in **Section 5.2.1.4**, for worms grown in 20 mgAs/kg soils, only 14 hatchlings survived, and these were very low weights. For 10 mgAs/kg worms, 26 hatchlings survived and these had good weights. These worms were the 2-3% that survived hatching in 10-20 mgAs/kg soils, so may have carried some genetic variation that put them at an advantage.

Experiments on Generation 2 worms were on these 40 worms, and controls. For 10 mg/kg exposure setting, the 26 worms were distributed across 2 replicate beakers. For the 20 mg/kg setting, the 14 hatchlings were 1 beaker. Experiments were to 56 d with measurements at 28 d and 56 d. Results are provided in **Table 5.8**.

Table 5.8: Summary statistics for effects of As on *E. fetida* of Generation 2. Results are reported as means with standard deviation in the brackets.

Soil As condition and concentration	Measured variable	Time (days) 1	28	56
Unspiked(control) soil: 0.011 µg/g	Mean weight (g)	0.33 (0.01)	0.38 (0.01)	0.39(0.0004)
	Tissue [As] (mg/kg)	2.13 (1.39)	1.00 (0.16)	0.53 (0.21)
	Mass As per worm (µg)	0.70	0.38	0.21
	Cocoons/10 worms (summed)	0	6.00 (1.41)	28.1 (2.7)
	Percent fatalities (%)	0	0	0
10 mgAs/kg	Mean weight (g)	0.41 (0.004)	0.45 (0.02)	0.39 (0.02)
	Tissue [As] (mg/kg)	9.88 (1.62)	38.9 (8.5) [d]	47.9 (9.2) [d]
	Mass As per worm (µg)	4.05	17.5 [d]	18.7 [d]
	Cocoons/10 worms (summed)	0	38.0 (8.4) [a §]	88.4 (2.90) [a]
	Percent fatalities (%)	0	0	0
20 mgAs/kg	Mean weight (g)	0.12(0.06)	0.15(0.07) [c]	0.23 (0.07) [a]
	Tissue [As] (mg/kg)	24.8 (6.6)	75.9 [a]	41.1 (7.3) [c]
	Mass As per worm (µg)	3.07	11.2[f]	9.41 [c]
	Cocoons/10 worms (summed)	0	0	26.7 [§]
	Percent fatalities (%)	0	40	0

[f] based on only 1 worm in this case.

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001

Difference between means compared with previous 28 d time period; [§] <0.05, [¶] <0.01, [*] <0.001, [#] <0.0001

5.2.2.2 As uptake

After three months in the old soil which are used for Generation 1 tests the worms were introduced to new sets of soil which has same As concentration for Generation 2 experiments. Average As concentrations in Generation 2 *E. fetida* initially and after 28 d and 56 d continued exposure to As (compared with controls) are shown in **Figure 5.8**.

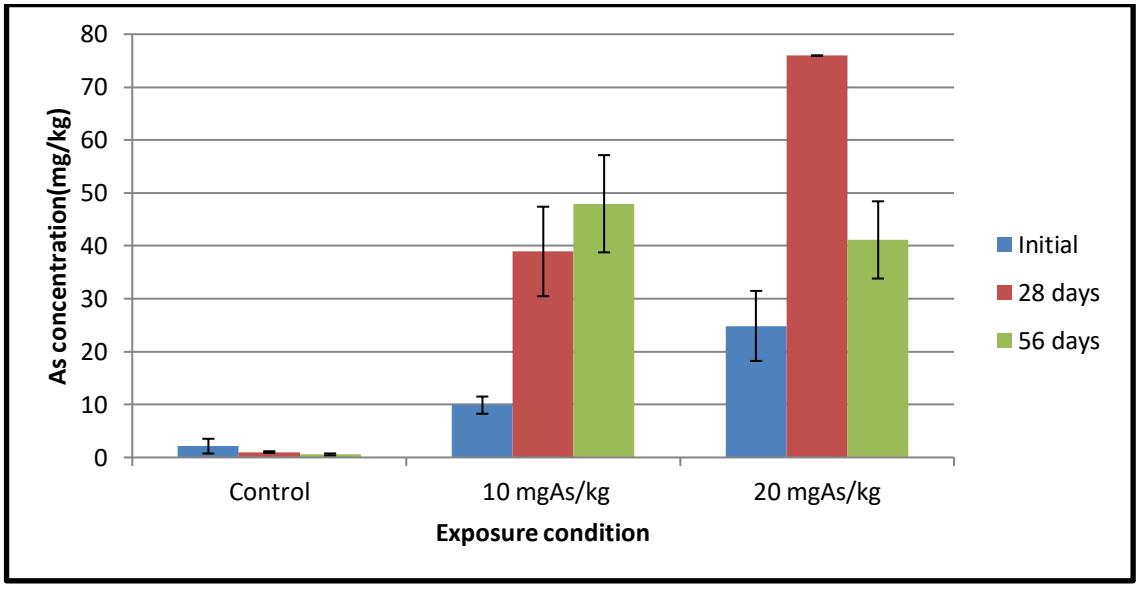

Figure 5.8: Average As concentration in *E. fetida* of generation two at 1, 28 and 56 days. Errors bars represent standard deviations. Note that the 28 day figure for 20 mg/kg is based on only 1 worm, as there was 40% mortality in that case (**Table 5.8**). Refer to Table 5.8 for statistical significances of differences between means.

This graph shows the As concentration in worm tissue at the beginning of the experiment, after 28 d and after 56 d. The worms used for this experiment were born and grown in the As contaminated soil. Therefore, compared with control worms, they carried a significant body burden of As at the beginning of the Generation 2 experiments (**Figure 5.9**). At the beginning of experiment As concentration in worms were 2.13±1.39 mgAs/kg, 9.88±1.62 mgAs/kg and 24.8±6.6 mgAs/kg in control, 10 mgAs/kg, and 20 mgAs/kg soils, respectively. Arsenic concentrations in worms were positively ($R=0.758$, $p<0.001$) correlated with those in soils. During first 28 d period the worms showed more rapid As accumulation than in the second 28 d period.

After first 28 d, As concentrations were 38.9±8.4 mg/kg and 75.9 mg/kg in worms living in 10 and 20 mg/kg As spiked soil, respectively. However, the high figure should be treated as an outlier. Due to lack of worms in 20 mgAs/kg soils, only one worm was sampled for metal analysis and this was small and under developed. All other averages are based on triplicates. Disregarding that sample, the overall pattern appears to be of As accumulation over time.

A key question with these results is whether there is evidence that surviving Generation 2 worms responded differently in terms of As uptake than the Generation 1 worms did under the same circumstances. The best reliability in comparing Generation 1 and Generation 2 results is for the worms in 10 mg/kg soils.

The bioaccumulation of As in *E. fetida* in 10 mgAs/kg soils shows both as an increase in concentration in worm tissue and as an increase in the total mass (**Tabe 5.9**) of As per worm. Focusing on total mass, it can be seen that 10 mg/kg Generation 2 worms began with about 10 µg of As per worm. This is equivalent to about 50 days of As accumulation in Generation 1 worms (based on a graph of the data in (**Table5.1**). From 50 to 84 days (34 days elapsed), Generation 1 worms accumulated an average of 26 µg/worm, or 0.77 µg/worm/day. By comparison, over the first 34 days and based on a simple least-squares plot (and fixing the origin to 9.88 µg/worm), Generation 2 worms accumulated 0.75 µg/worm/day.

On the basis of these results there is therefore, no evidence that the Generation 2 worms exposed to 10 mgAs/kg soils differed from Generation 1 worms in their rate of As uptake, when the results are viewed on the same basis. Despite being the 3.4% of hatchlings that were survivors from Generation 1 cocoons, the Generation 2 worms showed no evidence of any special ability to exclude As (or conversely, sequester more As in non-toxic forms, which could result in higher accumulation). If the surviving Generation 2 worms were more tolerant to As, alteration of As uptake could be ruled out as a mechanism.

5.2.2.3 Mortality and growth

Only worms in 20 mgAs/kg soils showed mortality during the first 28 d. There were only 10 worms for the beaker and 4 (40%) were dead after this time. No worms in the control and 10 mgAs/kg soils died during the experiment.

Weight of worms was measured at the beginning of the experiment, and after 28 d and 56 d. Because of different growth conditions and as discussed above, initial

weights of worms were noticeably different from each other to begin with (**Figure 5.9**). Initially, the worms in 10 mgAs/kg soils showed a higher mean weight than that of controls, and worms in 20 mgAs/kg soils were lower.

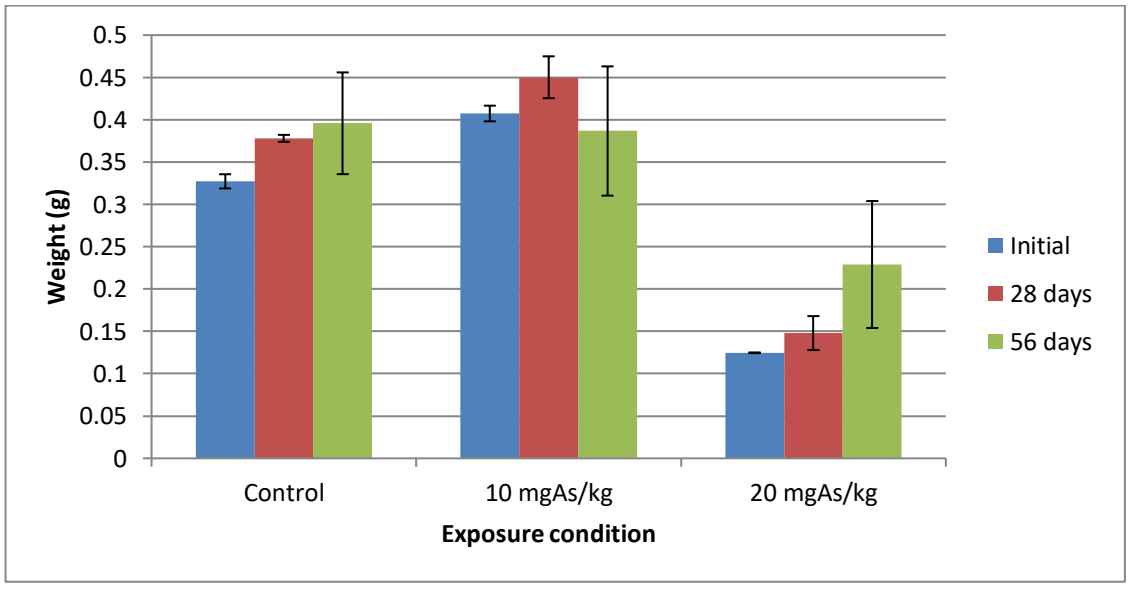

Figure 5.9: Changes in the fresh mean weights of *E. fetida* exposed to different As concentrations and clean soil after 28 d and 56 d. Errors bars represent standard deviations. Refer to Table 5.8 for statistical significances of differences between means.

Worms in clean artificial soil showed constant weight gain during the experimental period. Worms in 10 mgAs/kg soil showed an increase in mean weight during first 28 d and a slight loss during the second 28 d. This might be because some worms were developing cocoons at 28 d and had laid those by 56 d. The growth pattern of worms in 20 mgAs/kg soils was positive but slightly different than the other two conditions. These worms showed slower growth rate during first 28 d and comparatively faster growth rate during second 28 d.

The growth pattern differs from results from Generation 1, where worms in 10 mgAs/kg and 20 mg/kg soils showed very similar means weights up to 56 d (**Table 5.1**). In Generation 2 worms in the 20 mgAs/kg soil condition started with much lower weights to begin with, and this disadvantage persisted through to the end of the new 56 d term. In addition, the 10 mg/kg Generation 2 worms showed a lower weight gain compared with controls, and in fact had weights that were no different to controls at 56 d.

At 56 d, mean weights *relative* to controls were:

- 10 mg/kg condition: 1.21(Generation 1) compared with 1.0 (Generation 2)
- 20 mg/kg condition: 1.24 (Generation 1) compared with 0.59 (Generation 2)

5.2.2.4 Sexual maturation and cocoon production

Results for cocoon production are provided in **Table 5.9** and **Figure 5.10.**

Table 5.9: Cocoon production by Generation 2 *E. fetida* (per 10 worms) in As spiked soil and controls, expressed as both the cumulative total and increased over each 28 d period.

Statistic	Exposure period (days)	Controls	10 mg/kg	20 mg/kg
Cumulative total production (per 10 worms)	0	0	0	0
	28	7.50	50.7 [a]	0 [d]
	56	28.1	88.4 [a]	26.7 [§]
New production during each 28 day exposure period (per 10 worms)	**Exposure period (days)**	**Controls**	**10 mg/kg**	**20 mg/kg**
	0	0	0	0
	28	7.50	50.7 [a]	0 [c]
	56	20.6	37.7 [a]	26.7 [§]

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001

Difference between means compared with previous 28 d time period; [§] <0.05, [¶] <0.01, [*] <0.001, [#] <0.0001

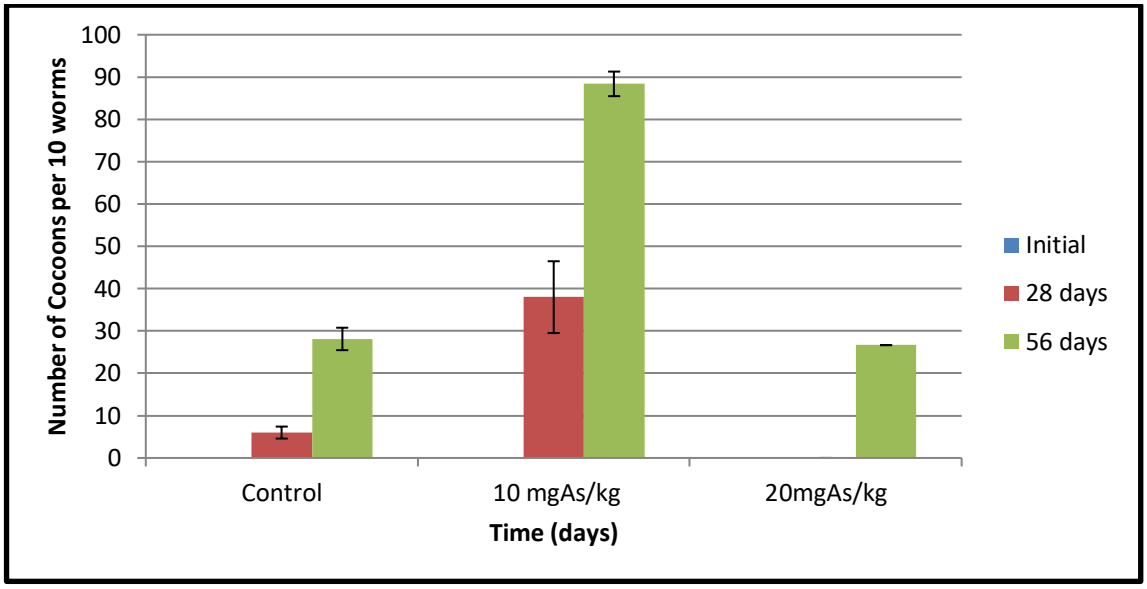

Figure 5.10: Cumulative cocoon production by Generation 2 *E. fetida*. Refer to Table 5.9 for statistical significances of differences between means.

At the beginning of the experiment the worms in control and 10 mgAs/kg soils were fully grown and mature. Numbers of cocoons per beaker increased with time in 10mgAs/kg and control soils (**Table 5.9**). After first and second 28 d worms in 10 mgAs/kg soils produced means of 28.1 and 88.4 cocoons per 10 worms, and these production rates are significantly (p=0.005) higher than those of worms in control soils. In this respect also results for Generation 2 worms are consistent with those from Generation 1.

In contrast, at the beginning of the experiment, the worms in the 20 mgAs/kg soils were small and no clearly visible clitella. These worms were slow to mature, with low weight gain and no worms had well-developed clitella even after 28 d. However, after the second 28 d a few worms in this exposure condition had a visible clitellum. In keeping with this, no cocoon production was observed after first 28 d in 20 mgAs/kg soils because the worms were not sexually mature. However, there were 10 cocoons after the second 28 d period.

Results for the 20 mg/kg soils, from the low initial weights to the lengthy maturation time, are likely to reflect the fact that As is more toxic to juvenile worms than to adults. Anderson *et al* (2013) reported that maturation of juvenile

worms is significantly dependent upon As concentration ($p<0.001$). In their work, worms exposed to 125 mgAs/kg failed to mature over the time of exposure (280 d) and juvenile exposure to 36 mgAs/kg resulted in a significantly lower proportion of mature adult worms relative to worms at lower As concentrations and in control soils (Anderson et al., 2013).

At 56 d, figures for cocoon production *relative* to controls were:

- 10 mg/kg condition: 2.22(Generation 1) compared with 1.35 (Generation 2)
- 20 mg/kg condition: 3.41(Generation 1) compared with 0.95 (Generation 2)

These results indicate that although As stimulated cocoon production in Generation 1 worms, when the exposure continued to a second generation this effect was diminished. On a 'cocoon per day' basis, by the second generation the toxicity of As worked to cause lower growth which substantially counteracted the stimulatory effect of As on cocoon production. In addition, only a small percentage (2-4%) of Generation 1 cocoons resulted in surviving offspring, potentially through reduced cocoon viability, the higher toxicity of soil As to juveniles, or a combination of both effects.

The conclusion is that although modest non-lethal levels of arsenic exposure (10-20 mg/kg) caused increased worm growth and greater cocoon production, the longer-term effects of that exposure were unquestionably negative. The negative impacts show up most strongly as a very low reproductive success rate, if that term is defined as the unsuccessful hatching and survival of the second generation offspring.

5.3 Changes in RNA expression

5.3.1 Generation one

5.3.1.1 Overview

As with the Cd experiments, samples for genomics testing were collected after the first 28 d exposure period, and the 28 d recovery period, from controls and the two exposure conditions. In the first set, samples from the 10 mgAs/kg condition

became unsuitable for sequencing because of an experimental error. Therefore, that analysis was only between controls and the 20 mgAs/kg exposure condition.

Differentially expressed transcriptomes can be either upregulated or downregulated compared with controls. Numbers of differentially expressed (p=0.001) transcripts are provided for the first 28 d exposure period in **Table 5.10**, and for the recovery experiment in **Table 5.11**.Compared with controls, large numbers of transcriptomes were 5-fold differentially expressed after 28 d As exposure, and also after the 28 d recovery period (**Table 5.10** and **Table 5.11**). As for Cd (**Section 4.4.1**) the numbers are largest for 5-fold differences.

5.3.1.2 Exposure condition

Table 5.10: Numbers of differentially expressed transcripts (p=0.001) in the Generation 1 *E. fetida* genome between the 20 mgAs/kg exposure condition and the control sample after 28 d.

5-fold differences		
	Control	20 mgAs/kg
Control	0	
20 mgAs/kg	8438	0
10-Fold differences		
	Control	20 mgAs/kg
Control	0	
20 mgAs/kg	1725	0
15-fold differences		
	Control	20 mgAs/kg
Control	0	
20 mgAs/kg	7	0

For the 28 d 20 mgAs/kg exposure condition (**Table 5.10**) over 8000 genes were ≥5-fold differentially expressed, which is similar to the result for Cd at a concentration of 30 mgCd/kg after the same time period (28 d) (**Table 4.12**).

A lower but still large number (1725) were ≥10-fold differentially expressed. However, of these, only 16 (under 1%) have had gene names assigned. Of these 1% with names, no gene was related to growth or reproduction.

Only 5 transcripts were ≥15-fold differentially expressed. For 30 mgCd/kg at 28 d, the comparable number was 7. Out of these 5 transcriptomes only one gene has a name assigned, and this is *LRR2.* This gene was extracted from *Plasmodium falciparum* and has no defined function identified. The protein produced by the gene is named as Protein PFF0380w (UniProt, 2019b). Genes showing ≥15-fold differential gene expression for Cd that were linked to reproduction (*CPG2, FST, CO4A1* and *MYSP*) were not present in the equivalent 10 mgAs/kg 28 d exposure data.

5.3.1.3 Recovery experiment

Table 5.11: Numbers of differentially expressed transcripts (p=0.001) in Generation 1 *E. fetida* allowed to recover in clean soils for 28 d (after 56 d of As exposure).

5-fold differences			
	Control	10 mgAs/kg-CS	20 mgAs/kg-CS
Control	0		
10 mgAs/kg -CS	10113	0	
20 mgAs/kg-CS	7972	10149	0
10-fold differences			
	Control	10 mgAs/kg-CS	20 mgAs/kg-CS
Control	0		
10 mgAs/kg-CS	2281	0	
20 mgAs/kg-CS	2582	2428	0
15-fold differences			
	Control	10 mgAs/kg-CS	20 mgAs/kg-CS
Control	0		
10 mgAs/kg-CS	14	0	
20 mgAs/kg-CS	22	23	0

As with the other experiments, a large number of transcriptomes were differentially expressed to the 5 fold cut-off and progressively fewer at 10 and 15-fold thresholds.

Table 5.10 and **Table 5.11** are not directly comparable, because exposure in **Table 5.10** was for 28 d, whereas recovery in **Table 5.11** follows an initial exposure period of 56 d. However, it would be expected that after 56 d exposure, more genes would have been differentially expressed than at 28 d (as seen in the Cd data), and that on recovery this number might substantially reduce.

This effect was seen in the Cd data. As an example for the 30 mgCd/kg condition, of 8272 genes were 5-fold differentially expressed after 28 d exposure, and this reduced to 93 genes after 28 d recovery (**Tables 4.14** and **4.18**). For Cd therefore, the number of differentially expressed genes dropped to 1.1% of their original value after 28 d recovery. This was despite the fact that the accumulated Cd remained in the worms, suggesting that it had been sequestered.

Relative to expectations the As results show three interesting features:

- **Observation 1**. After 28 d of recovery, large numbers of genes were still being differentially expressed in previously exposed worms, compared with controls.

- **Observation 2**. At the ≥5-fold level, there are *more* differentially expressed genes for the previous 10 mg/kg exposure than for the 20 mg/kg exposure situation. Numbers are similar to ≥10-fold differential expression, and drop back to a more expected pattern by ≥15-fold. (The expected pattern would be that higher pre-exposure levels would cause more differential gene expression, as seen for Cd.)

- **Observation 3**. For all levels, there are also substantial numbers of differentially expressed genes *between* the two exposure conditions (over 10,000 at 5-fold, 2400 at 10-fold, and 20 at 15-fold).

These results are likely to indicate the ongoing impact of the previous As exposure (**Observation 1**), and need to be set alongside the tissue As results (**Section 5.2.1.5**). There, it was found that contrary to expectations, the 10 mg/kg exposed

worms continued to extract As from clean soils, almost doubling their loadings of As over the 28 d recovery period. In contrast, the 20 mg/kg worms lost some of their accumulated As, but not as much as might have been expected. The tissue As results suggested that a different process had occurred in the worms exposed to 10 mg/kg As, which worked to promote ongoing As uptake. **Observations 2** and **3** are consistent with the idea that a range of additional metabolic processes may be operating in the 10 mg/kg worms that are inhibited at higher exposures. These could include genes regulating phosphate uptake, growth, cocoon production, and other biochemical pathways that are either upregulated by modest As exposure or able to operate at the lower levels but inhibited at higher exposures.

Gene identities

Information about gene identities is comparatively uninformative, partly because few of the genes have names assigned and can be annotated.

For the ≥15-fold differentially expressed genes, there were 44 trinity codes between the different conditions, but of these only 4 have gene names. These are shown in **Table 5.14**.

Table 5.12: Genes showing ≥15-fold differential gene expression after 28 recovery from As exposure, and their putative function.

Gene symbol	Protein	Putative function	Level of expression		
			Control	10-CS	20-CS
CNMD	Leukocyte cell-derived chemotaxin 1	Endothelial cell morphogenesis, cartilage development, negative regulation of angiogenesis	975	2665	0
PGPS1	CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase, mitochondrial	Biosynthesis of anionic phospholipids phosphatidylglycerol and cadiolipin.	841	78.6	0
RLA2	60S acidic ribosomal protein P2	Plays an important role in the elongation step of protein synthesis.	580	568	0
RL34	60S ribosomal protein L34	structural constituent of ribosome	776	1095	0

Comparing controls with the results for worms recovering from 10 mg/kg As, two genes were upregulated, one was down-regulated, and one stayed much the same. Moving to the 20 mg/kg recovery condition, expression of all four genes disappeared. Although limited to the expression of the four named genes, these results are consistent with the ideas that (a) significant activity occurs following the lower level exposure (10 mg/kg), and this is different to that of controls; and (b) a significant change occurs between the 10 mg/kg and 20 mg/kg exposures, whereby some biochemical pathways are effectively shut down.

In terms of their reported functions, all four genes are related to cell maintenance and/or growth. The *CNMD* gene is a bifunctional growth regulator, linked to growth of chondrocytes and thus contributing to growth of the organism (UniProt,

1998). The second function of the gene is negative regulation of angiogenesis (Yoshioka et al., 2006). *PGPS1* is involved with phospholipid production. The *RLA2* and *RL34* genes have both been linked to protein production. *RLA2* plays an important role in elongation of protein (UniProt, 1997) and 60S ribosomal protein L34 is a structural constituent of ribosome (UniProt, 1995).

For the ≥10-fold differentially expressed genes, there were 5408 trinity codes between the different conditions, of which 637 have gene names. Of these 637 named genes, 55 (8.6%) were related to growth and only 1 (0.15%) has been linked to regulation of reproduction. Identities of these 56 genes are shown in **Table 5.13**.

Table 5.13: Identities of the 56 named ≥10-fold differentially expressed genes related to growth and reproduction in Generation 1 As recovery experiment.

	Reproduction	**Growth**					
1	*MMP12*	*ABCA2*	*CGAS*	*GRN*	*LMNA*	*PFD1*	*STAR*
2		*AQP5*	*CLCN7*	*GRN*	*LRP2*	*PGM1*	*TFAP4*
3		*ATAD2*	*CNMD*	*GSTP1*	*LRP5*	*PRG4*	*UBP8*
4		*BAG1*	*CSRP2*	*HMCN1*	*MATN3*	*PRPH2*	*VWF*
5		*BMP1*	*DCLK1*	*HRH2*	*MMP12*	*PTBP1*	*XIAP*
6		*CAT8*	*DCLK1*	*IFI6*	*MYLK*	*RAN*	
7		*CCR4*	*EAF1*	*KLHL6*	*NUF2*	*RPM1*	
8		*CD109*	*FAT4*	*LAMP1*	*NUMB*	*SESN2*	
9		*CD36*	*FBN1*	*LBP*	*PACRG*	*SPAG6*	
10		*CELA1*	*FUT1*	*LDHB*	*PDIA3*	*SRPK2*	

5.3.2 Generation 2

In the Generation 2 worms were born in As-spiked soils, and RNA was extracted after 56 d of further exposure. In this case, samples were available for both the 10 mg/kg and 20 mg/kg conditions. Results for differential gene expression are provided in **Table 5.14**.

Table 5.14: Number of significantly differentially expressed (p≤0.001) genes in Generation 2 *E. fetida* after 56 days of As exposure.

5-fold differences			
	Control	10 mgAs/kg	20 mgAs/kg
Control	0		
10 mgAs/kg	8248	0	
20 mgAs/kg	8145	8141	0
10-fold differences			
	Control	10 mgAs/kg	20 mgAs/kg
Control	0		
10 mgAs/kg	1665	0	
20 mgAs/kg	1826	1895	0
15-fold differences			
	Control	10 mgAs/kg	20 mgAs/kg
Control	0		
10 mgAs/kg	4	0	
20 mgAs/kg	43	54	0

Results show a very similar pattern to the Generation 1 recovery experiment, which had involved Generation 1 worms being exposed for 56 d and then being allowed 28 d recovery (**Table 5.11**). Specifically:

- There as many differences between the 10 mg/kg and 20 mg/kg exposure conditions as there are between each of those and controls; and
- Relative differences between controls and each condition follow the same patterns, with the lower exposure showing a slightly higher number at a ≥5-fold cut-off, but the higher exposure showing a larger proportion of ≥15-fold differential expression.

Gene identities

At the ≥15-fold level, names could be assigned for 10 genes. These are identified in **Table 5.15**.

Table 5.15: Genes showing ≥ 15-fold differential expression compared with controls in Generation 2 As-exposed worms after 56 d, and their putative functions.

Gene symbol	Protein	Gene ontology	Level of expression		
			Control	10 mgAs/kg	20 mgAs/kg
CHIT1	Chitotriosidase-1	Chitinase activity	4.7	0	5234
BPI	Bactericidal permeability-increasing protein	Lipid and Lipopolysaccharide binding	0	0	767
LYS	Lysozyme	Bacteriolytic activity and host defence mechanism	0.22	0.13	7269
CL46	Collectin-46	Lipopolysaccharide binding	0	0	447
FIBG	Fibrinogen gamma chain	Metal binding has major function in homeostasis as one of the primary components of blood clots	0	1.0	469
SCP	Sarcoplasmic calcium-binding protein	Calcium ion binding	0.16	0	471
CPG2	Chondroitin proteoglycan 2	Embryo development, chitin binding, chitin metabolism process	0.65	0	427
ILEUC	Leukocyte elastase inhibitor C	Serine-type endopeptidase inhibitor activity	0	0.25	770
CLC5A	C-type lectin domain family 5 member A	Carbohydrate binding and innate immune activity	0.92	0	1026
GIG4	U-actitoxin-Avd12a	Has both toxic and EGF activity	12.1	0	1131

Reflecting the results in **Table 5.15**, all of the named genes were ones that were upregulated at exposures of 20 mgAs/kg. It is notable that activity of these genes is absent not only in the controls, but in the worms exposed to 10 mgAs/kg. This result provides further evidence for a qualitative difference between the two exposure levels in some of the biochemical pathways that are activated or inhibited.

The 10 identified genes have a range of different functions, and some of them were also differentially expressed in the Cd exposure experiments.

- *CHIT1* shows the consistency as a gene that is activated in response to both As and Cd toxicity. This gene was differentially expressed in Cd exposure experiments (Generation 1, Generation 1 recovery and Generation 2) as well. This gene is responsible for control chitinase activity as outlined in **Section 4.4.1**

- *FIBG* was also differentially expressed in the Cd exposure Generation 2 experiment. This gene is responsible of metal binding and homeostasis as outlined in **Section 4.4.1**

- The genes *SCP, ILEUC, CLC5A, BPI, CL46*and *GIG4* (functions outlined the **Table 5.15**) were not identified as differentially expressed in the previous experiments.

The gene *CPG2* plays a role in reproduction, and was also identified in the Cd Generation 1 exposure experiments (**Section 4.4.1**). This gene is responsible for polar body extrusion during cytokinesis in embryo development and effects cortical granule size (Olson et al., 2006, Lee and Schedl, 2001, Johnston et al., 2006). However, in this experiment there was no relationship with cocoon production and expression of *CPG2* as Cd generation one (**4.4.1**). In the As experiments, *CPG2* was not significantly expressed in the control sample, or the 10 mg/kg exposure condition (**Table 5.15**), despite the existence of cocoons at 56 d (**Table 5.11**). Results like this may reflect the variability in results caused by individual differences between worms given that experiments relied on small sample numbers. It is possible that in some samples the tested worm was not producing cocoons, while other untested worms were. This makes the result more

of a probability effect, where a positive result is probably more likely from worms sampled from beakers where cocoon production is higher, as it is more likely that the sampled worm would be producing cocoons. This is what was seen in these results, with positive expression showing up at the 20 mg/kg exposure condition.

For the ≥10-fold differentially expressed genes, there were 3219 trinity codes between the different conditions, of which 198 have gene names. Of these 198 named genes, 18 (9%) were related to growth and only 2 (1%) to reproduction. Identities of these 20 genes are shown in **Table 5.16.**

Table 5.16: Identities of the 20 named ≥10-fold differentially expressed genes related to growth and reproduction in Generation 2 As exposure experiment (56 d).

Reproduction	**Growth**	
MMP12	*ACE*	*FBP1*
ACE	*BIRC3*	*FCN2*
	CD109	*GSTA4*
	CETP	*ITLN1*
	CHIT	*LBP*
	CHIT1	*MATN3*
	ERAP1	*MMP12*
	FAT1	*PLAC8*
	FAT4	*SNED1*

5.4 Findings and wider implications

The most notable findings of this part of the work are as follows.

[1] Worms exposed to As-spiked soil continually accumulated As in their bodies. Relative accumulation was slightly more efficient at natural As concentrations than from spiked soils, but the difference was not so marked as was seen for Cd.

[2] Under modest sub-lethal chronic exposures for longer than 28 d, As stimulated growth and cocoon production.

A possible reason is that As reduces the burden of parasites, allowing more energy for growth. However, the large number of genes showing changes in RNA expression at the lowest As exposure level (Finding **[5]**) suggests that it is more likely that As is triggering internal metabolic changes which drive these effects.

The stimulatory effect of low level As exposure on growth and cocoon production has been largely overlooked and is likely to be missed in standard 28 d exposure trials.

[3] Although modest As exposure promotes worm growth and extreme cocoon production, by Generation 2 the effects of that are unquestionably negative, in terms of both overall reproductive success and the retarded growth rate of surviving offspring.

[4] At sub-lethal exposure levels, there are circumstances where arsenate appears to promote its own uptake in a positive feedback loop.

This effect was so powerful that significant As uptake persisted in worms exposed to 10 mgAs/kg even after they were returned to un-spiked OECD soil with only natural levels of As. Although their rate of As uptake decreased, As was still being taken up, so that their tissue As load was doubled after 28 d.

It is speculated that this effect may relate to the increased uptake and utilisation of phosphate for cellular repair and the additional growth noted above.

At the higher 20 mg/kg As setting this effect was not seen, presumably as the direct impact of toxicity was felt more strongly. Worms exposed to the 20 mg/kg condition returned to clean soils did what was expected and lost some of their accumulated As.

[5] Results of RNA transcriptome testing show that substantial changes occur to expression of many genes as a result of sub-lethal As exposure. Such changes are likely to reflect a significant 'whole-organism' response in earthworms experiencing sub-lethal As. In addition, there seems to be a qualitative difference between responses of many genes between the 10 mg/kg and 20 mg/kg exposure levels. It is speculated that the lower exposures promote metabolic pathways (or permit metabolic processes to occur), that are then inhibited by additional As toxicity at the higher exposure level.

[6] Offspring of exposed worms did not show any ability to moderate their As uptake, but take up as much as their parents, when other factors are standardised. There was no evidence that surviving offspring had greater resistance to As; in fact by most measures they performed more poorly.

Implications

In terms of wider implication for ecosystems the results in this chapter support the argument that longer term (>28 d) chronic exposure experiments taken across more than one generation are essential for understanding the impacts of As on invertebrate populations. From Findings **[2]** and **[3]** it can be seen that an effect that initially seems beneficial may result in a strongly negative impact on the worm population.

Finding **[4]** is essentially that modest As exposure appears to trigger one of more biochemical pathways that increase its own uptake, and continue to operate even after intentional exposure ceases. This links to findings in **Chapters 6** and **7**. In **Chapter 7** tentative correlative evidence is presented suggesting that Cd exposure

also increased the uptake of As from natural soils. If so (and if a common mechanism is involved), the possibility exists that many contaminants may trigger this response, effectively turning a single exposure into an As co-exposure. This may be important because as shown in **Chapter 6**, impacts of Cd exposure are made significantly worse in the presence of additional As.

Negative impacts of modest and even apparently beneficial As exposure on the second generation may have implications for other modest As exposure situations, whether or not exposure is intentional. Unintentional cases relate to human populations that are chronically exposed to elevated As in drinking water. Significant numbers of people live in areas with elevated As levels in groundwater. According to the literature, these exposures cause negative effects on reproduction such as low birth weight (Yang et al., 2003, Nordström et al., 1979), preterm delivery (Yang et al., 2003, Ahmad et al., 2001), spontaneous abortion (Nordström et al., 1979, Ahmad et al., 2001, Aschengrau et al., 1989, Milton et al., 2005) and stillbirth (Ahmad et al., 2001). (see also **Chapter 2**).

Intentional exposures may relate to cases where As or its compounds are still used (a) as insecticides (*e.g.* in cotton production, or for CCA-treated wood) where As ends up in soil and it likely to impact non-target soil-dwelling organisms or(b) as growth promotants (*e.g.* as Roxarsone in poultry-meat production) (**Chapter 2**).

The implication of Finding **[6]** is that invertebrates do not find it easy to adapt to As exposure. From first-to-second generation there was potential for As to exert a selection pressure, and it was possible that the 2-4% of surviving offspring were worms with genetics that made them more tolerant to As. This did not happen; and in fact the surviving worms performed more poorly. A finding like this may apply to the environmental fate of other contaminants of concern such as neonicotinoids which are used as pesticides, for which non-target impacts are substantial and concerning (Dülger, 2018, EFSA, 2018). Absence of evidence that non-target species could adapt highlights the importance of better controls on use and stewardship of intentionally toxic substances.

Chapter 6: Co-exposure of *E. fetida* to both As and Cd

6.1 Introduction

In previous chapters the focus was exposure of *E. fetida* to either Cd (**Chapter 4**), or As (**Chapter 5**). However contaminated soil typically contains a mixture of various metals. As examples mining (Hutchinson and Whitby, 1974, El Khalil et al., 2008) and metal smelting (Luo et al., 2018, Kachenko and Singh, 2006) release more than one heavy metal or metalloid contaminant at a time.

Adverse effects of individual metal exposures are most commonly researched and best understood (**Chapter 2**). Combined effects of co-exposure to a mixture of contaminants are more poorly characterised, especially for chronic exposure.

One of the problems with studying co-contamination is that there are many individual contaminants that could be studied, and many more possible pairs, and even more combinations of three or more co-contaminants (**Table 6.1**).

Table 6.1: The number of binary and ternary combinations possible in systems containing between 2 to 20 distinct contaminants. (Data determined as part of this work.)

Number of distinct contaminants	Number of possible binary combinations	Number of possible ternary combinations
2	1	
3	3	1
4	6	4
5	10	10
6	15	20
7	21	35
8	28	56
9	36	84
10	45	120
11	55	165
12	66	220
13	78	286
14	91	364
15	105	455
16	120	560
17	136	680
18	153	816
19	171	969
20	190	1140

The range of possibilities increases further when allowance is made for the fact that in real-life situations, concentrations of one or more of the contaminants may vary significantly across a site. These problems represent a significant challenge for researchers studying the effects of multiple contaminant mixtures. Nevertheless, some researchers who have focused on key contaminants have determined interesting features that help to map out some fundamental principles of how multi-contaminant exposures may differ from exposures to single contaminants. Wijayawardena *et al.*(2018) examined chronic and reproductive toxicity of cadmium, zinc, and lead in binary and tertiary mixtures to *E. fetida*, and determined that the combined effect of Zn and Pb exposure was substantially more toxic than either contaminant alone, to the extent that worms experienced

60% mortality at levels still below Canadian and Dutch guideline values for protection of ecological receptors (Wijayawardena et al., 2018).

Both As and Cd are common soil contaminants, and there has been a wide range of studies examining soil invertebrate exposures to each of these for characterising toxicological endpoints (Cavanagh and Munir, 2016). However, as far as the author is aware, there have not been any previous studies examining co-exposure of earthworms to both As and Cd. This work may add a new dimension to previous co-exposure experiments on the basis that Cd and As differ from each other in both their soil chemistry and their biochemistry and biological effects. Cadmium is a divalent cation which accumulates in tissue, whereas inorganic As exists as one of two oxyanions (As(V) or AsO_4^{3-} as the usually dominant 'arsenate' form analogous to phosphate, and As(III) as AsO_2^- as the lower 'arsenite' oxidation state) and behaves in fundamentally different ways to Cd in organisms. Without experiments is it unclear whether the two contaminants may interact in when earthworms are exposed to both As and Cd together, compared with the equivalent As-only or Cd-only single-exposure experiments.

Consequently, in this part of the work experiments were designed to explore the effects of co-exposure to Cd and As. This chapter provides results and discussion relating to how co-exposure of *E. fetida* to As and Cd affected growth, reproduction and gene expression over 56 days. For practicality, and matching with previous results, exposure concentrations for each were pre-set at one level only: 90 mg/kg for soil Cd, and 20 mg/kg for soil As. These corresponded to levels of low or no mortality in the Cd-only and As-only experiments, where worms were still capable of growth and reproduction.

Methodological approaches to the co-exposure experiments are outlined in **Section 3.6**. Genomic tests involved RNA transcriptome measurements as outlined in **Section 3.2.3**. The aims and objectives of this experiment are outlined in **Section 1.2**. Objectives sets to achieve the aims were:

1. to quantify how combined exposure of *E. fetida* to 20 mgAs/kg and 90 mgCd/kg affected the worms' growth, reproduction and gene expression; and

2. to determine how impacts of this co-exposure differ from those caused by exposure to the same soil concentrations of As and Cd individually.

6.2 Phenotypic effects

6.2.1 Summary statistics

Summary results of phenotypic measurements are provided in **Table 6.2**, and full results in **Appendix 4**

Table 6.2: Summary statistics relating to co-exposure of Cd and As effect on *E. fetida*.

Soil cadmium condition and concentration	Measured variable	Time (days) 1	28	56
Unspiked (control)	Mean weight (g)	0.21(0.003)	0.32 (0.009)	0.37(0.02)
	Tissue [Cd] (µg/g)	4.44 (1.22)	5.01(1.16)	4.40(1.08)
[Cd]- 0.011 mg/kg	Tissue [As] (µg/g)	0.95 (0.33)	0.82(0.14)	0.45(0.01)
[As]- 0.31 mg/kg	Mass Cd per worm (µg)	0.93	1.62	1.63
	Mass As per worm (µg)	0.20	0.26	0.16
	Cocoons/10 worms (summed)	0	4 (0)	36 (7)
	Percent fatalities (%)	0	0	0
Experiment	Mean weight (g)	0.20(0.005)	0.21(0.01) [b]	0.24(0.02) [b]
[Cd]- 90 mg/kg	Tissue [Cd] (µg/g)	4.44 (1.22)	81.2(15.5) [c *]	109(0.5) [c]
[As]-20 mg/kg	Tissue [As] (µg/g)	0.95 (0.33)	12.3(5.2) [b ¶]	11.5 (3.0) [c]
	Mass Cd per worm (µg)	0.89	17.0 [c *]	26.6 [c]
	Mass As per worm (µg)	0.19	2.58 [¶]	2.80
	Cocoons/10 worms (summed)	0	0	0
	Percent fatalities (%)	0	0	0

Difference between means compared with control condition; [a] <0.05, [b] <0.01, [c] <0.001, [d] <0.0001

Difference between means compared with previous 28 d time period; [§] <0.05, [¶] <0.01, [*] <0.001, [#] <0.0001

6.2.2 Cd accumulation in *E. fetida* with and without As exposure

In the co-exposure environment, worms continuously accumulated Cd (**Figure 6.1**). However, the rate of accumulation was more rapid during the first 28 d (81.2±15.5) mg/kg) and comparatively lower in the second 28 d. Cadmium concentrations in control worms remained low and did not show evidence of Cd accumulation.

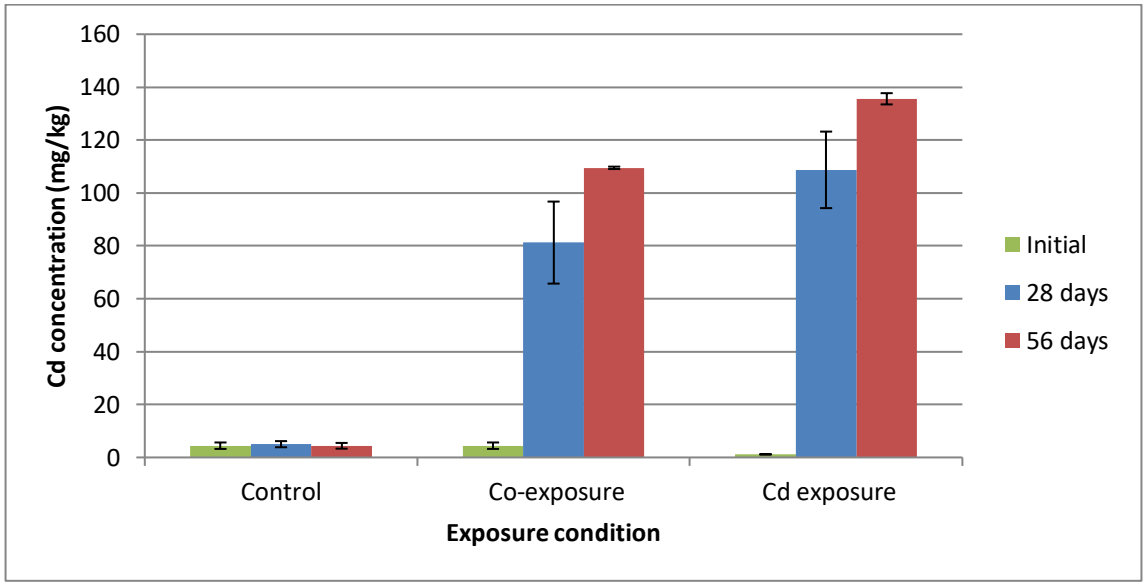

Figure 6.1: Average Cd concentrations in *E. fetida* tissue after being co-exposed to 90 mgCd/kg and 20 mgAs/kg soils for 28 and 56 d, and comparison with results from exposure to equivalent Cd in the absence of As. Error bars show standard deviations. Refer to Table 6.3 for statistical significances of differences between means.

As with the individual Cd exposure experiments (at the same soil Cd concentration), the worms accumulated Cd throughout the experimental period and absorption was faster during first 28 d than the second 28 d period. However average concentrations of Cd in the worm tissues were lower under co-exposure conditions.

Table 6.3: Comparison of mean Cd concentrations and masses in *E. fetida* in the co-exposure environment compared with the Cd-only exposure experiment. (Soil Cd concentration = 90 mg/kg; soil As concentration = 20 mg/kg.)

Time (days)	Cd concentrations in worm tissue (mg/kg)		Ratio
	Cd+As	Cd-only	Cd+As / Cd-only
1	4.44	1.17	[3.8*]
28	81.2	109	0.75
56	109	136	0.80
	Cd masses in worm tissue (µg/worm)		Ratio
	Cd+As	Cd-only	Cd+As / Cd-only
1	0.89	0.34	[2.6*]
28	17.0	24.4	0.70
56	26.6	43.9	0.61

*the day 1 ratio is considered uninformative as it is a ratio of two low numbers

In the Cd-only exposure tests in the worm tissue after 28 d from 90 mgCd/kg soils was (108.7±14.5) mgCd/kg, which is 27.5 mgCd/kg higher than worms in co-exposure experiments. After 56 d the Cd concentration in the worm tissues of co-exposure experiments was still 26.1 mgCd/kg lower than for the Cd-only exposure experiment (**Table 6.3**).

When viewed as a mass of Cd per worm, results are consistent. From 28 to 56 d worms continued to grow, but not excessively, so that tissue dilution would not be a factor. Over this period total Cd in the worms increased from 17.0 µg per worm to 26.6 µg per worm (**Table 6.2** and **6.3**).

Results show that mean uptake of Cd over 28 and 56 d *decreased* in the presence of As, and this is true whether results are expressed on a concentration or mass basis (**Table 6.3**). On a mass basis, the presence of As caused a 39% reduction in Cd uptake over 56 d. Factors other than As remained constant as the co-exposure experiment used the same OECD model soils and edaphic factors, the same species of worm and incubated them under the same conditions.

6.2.3 As accumulation in *E. fetida* with and without Cd

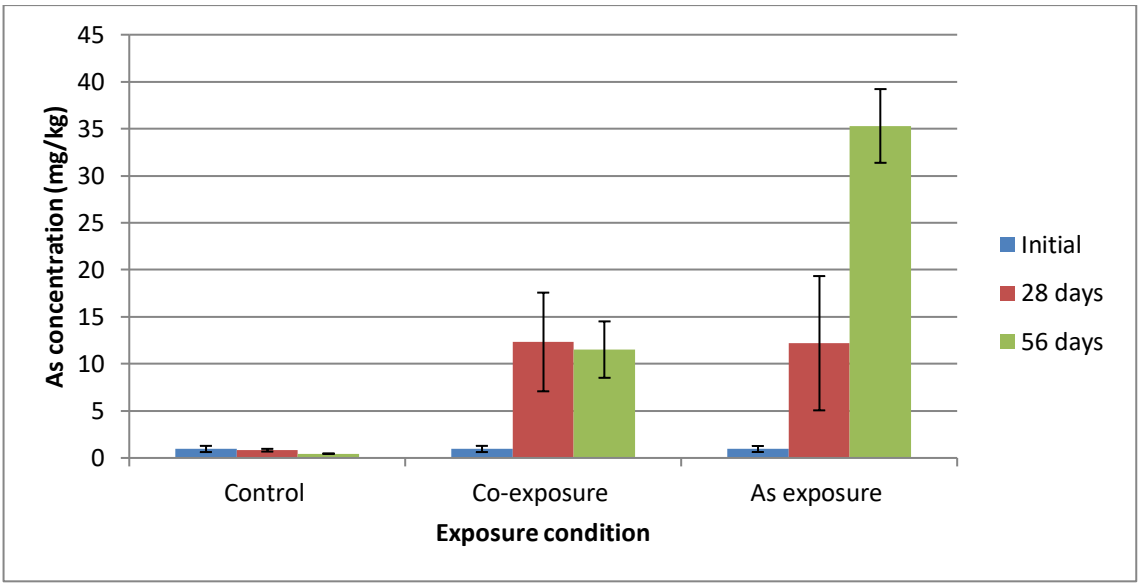

Figure 6.2: Average As concentrations in *E. fetida* after being co-exposed to 90 mgCd/kg and 20 mgAs/kg soils for 28 and 56 d, and comparison with results from exposure to equivalent As in the absence of Cd. Error bars show standard deviations. Refer to Table 6.4 for statistical significances of differences between means.

In the co-exposure environment, worms rapidly absorbed As from soil during the first 28 d period and showed a slight (non-significant) decrement in the level of As after the second 28 d period. This might be interpreted as a plateau. Average As concentrations of worm tissues were (12.33 ± 0.33) mg/kg and (11.52 ± 3.0) mg/kg in first and second 28 d respectively. Worms in control soils showed no evidence of As accumulation (average measured As concentrations in worm tissues were (0.82±0.15) mg/kg and (0.45±0.02) mg/kg in first and second 28 days respectively).

Table 6.4: Comparison of mean As concentrations and masses in *E. fetida* in the co-exposure environment compared with the As-only exposure experiment. (Soil Cd concentration = 90 mg/kg; soil As concentration = 20 mg/kg.)

Time	As concentrations in worm tissue (mg/kg)		Ratio
(days)	As+Cd	As-only	As+Cd / As-only
1	0.95	0.95	1.0
28	12.3	12.2	1.0
56	11.5	35.3	0.33
	As masses in worm tissue (µg/worm)		Ratio
	As+Cd	As-only	As+Cd / As-only
1	0.19	0.27	[0.7*]
28	2.58	4.63	0.55
56	2.80	16.6	0.17

*the day 1 ratio is considered uninformative as it is a ratio of two low numbers

Results show that mean uptake of As over 28 and 56 days *decreased* in the presence of 90 mg/kg Cd, and this is true whether results are expressed on a concentration or mass basis (**Table 6.4**). This difference was particularly marked at 56 days, where the total mass of As taken up in an As-only experiment was about 6 times more than the mass taken up in the presence of 90 mg/kg soil Cd.

The pattern of As absorption by *E. feti*da in co-exposure is clearly different from individual As exposure test at the same soils As concentration (20 mgAs/kg) (**Figure 6.2**). In the As-only experiment, As concentrations in worm tissues continued to increase with the time of exposure (**Section 5.2.1.2**) and did not reach an equilibrium plateau.

When viewed in mass of As per worm, results show a modest increase in total As, but only from 2.58 µg per worm to 2.80 µg per worm (**Table 6.4**). This result shows that a slight decrease in mean As concentrations (**Figure 6.2**) was caused by tissue dilution associated with modest growth. The overall picture for the second 28 d period under the co-exposure is that As accumulation had decreased and was reaching a plateau. This contrasts with the behaviour of Cd which continued to accumulate.

6.2.4 Considering both together

For comparing uptake, tissue mass (µg/worm) may discriminate better than tissue concentration (µg/g) because of the way the former corrects for changes in tissue weight.

Overall (and on this basis) the results show that after 56 d at the soil As and Cd concentrations examined (20 mgAs/kg and 90 mgCd/kg): Cd uptake is moderately (-39%) decreased in the presence of the As, and As uptake is significantly (-83%) decreased in the presence of the Cd (figures based on mean µg/worm, **Table 6.3** and **6.4**). Results for both As and Cd can also be viewed in terms of simple bioconcentration factors (**Table 6.5**).

Table 6.5: Comparison of bioconcentration factors (BCF values) in the single and co-exposure experiments. BCFs are simple ratios of tissue concentration to soil concentration.

Arsenic	**20 mgAs/kg alone**	**20 mgAs/kg in presence of 90 mgCd/kg**
As BCF at 28 d	0.61	0.62
As BCF at 56 d	1.77	0.58
Cadmium	**90 mgCd/kg alone**	**90 mgCd/kg in presence of 20 mgAs/kg**
Cd BCF at 28 d	1.2	0.9
Cd BCF at 56 d	1.5	1.22

Bioconcentration factors are another way of looking at the same data, but some further points can be made.

Compared with the metal-only results, in the co-exposure experiments:

- Cd bioconcentration appears to be 'retarded', by about 28 d. This was lower in the presence of As than without it, but still occurs (**Table 6.5**). By 56 d, Cd accumulation in the co-exposure setting had reached the same level as Cd-alone accumulation after 28 d (BCFs 1.22 c.f. 1.20). Accumulation of Cd here is likely to be linked to the ongoing production of metallothionein

and/or other retentive proteins, which appears to continue as long as the worms survive.

- As bioconcentration is initially equivalent (BCFs of 0.61 and 0.62 at 28 days), but As does not continue to accumulate in the co-exposed worms. This contrasts strongly with results from the As-only exposure experiments where the uptake of As uptake actually increased over time (and also with increasing concentration). Here it does appear that some primary or secondary effect of the Cd exposure is having a dramatic impact on As uptake and/or retention.

6.2.5 Growth and relative growth

Figures for mean growth and relative growth of the worms are compiled in **Table 6.6**, where data for Cd and As are from the results presented in **Chapters 4** and **5**, respectively. **Figure 6.3** shows changes in mean weights under different conditions, including standard deviations.

Table 6.6: Absolute and relative mean weights of *E. fetida* co-exposed to 90 mgCd/kg and 20 mgAs/kg, compared with equivalent individual Cd and As exposures and controls.

Days	Mean weights (g)							Relative weights (relative to controls)		
	Controls				Exposed conditions					
	Cd 90	As 20	Cd+As	Means	Cd 90	As 20	Cd+As	Cd 90	As 20	Cd+As
1	0.270	0.330	0.210	0.270	0.293	0.290	0.200	1.09	0.88	0.95
28	0.303	0.360	0.324	0.329	0.224	0.380	0.210	0.74	1.06	0.65
56	0.394	0.380	0.370	0.381	0.324	0.470	0.240	0.82	1.24	0.65

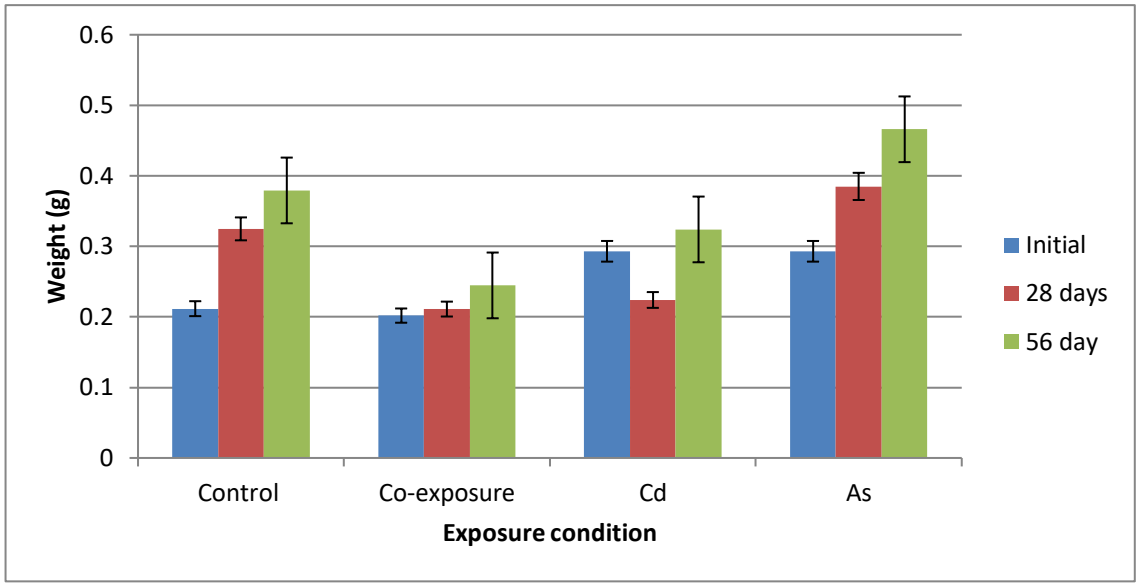

Figure 6.3: Changes in fresh mean weight of *E. fetida* co-exposed to 90 mgCd/kg and 20 mgAs/kg, compared with equivalent individual Cd and As exposures and controls. Error bars represent standard deviations. Refer to Table 4.1, Table 5.1 and Table 6.1 for statistical significances of differences between means.

In the co-exposure experiment, weights were measured on days 1, 28 and 56. Worms showed positive growth, but this was only modest. During the first 28 d worms in contaminated soil increased their weight on average by 0.01 g while worms in control increased by 0.114 g. During the second 28 d period worms in contaminated soil showed slightly faster growth; but mean weights remained at only two-thirds (0.65) of those of control worms. These results indicate that the Cd+As co-exposure was inhibiting worm growth, but not to a point that it ceased.

Effects of co-exposure on worm weights were quite different ($p=0.002$) to those of the individual exposures. Relative to controls (**Table 6.6**):

- worms exposed to 90 mg/kg soil Cd only showed reduced growth (0.82 at 56 d); and
- worms exposed to 20 mg/kg soil As only showed increased growth (1.24 at 56 d)

The average of these two changes is 1.03. Therefore, if the growth-promoting effect of As 'cancelled' the growth inhibiting effect of Cd in an additive way, we would

expect to see that that growth of co-exposed worms after 56 d would be the same as controls. This is not the effect which occurred. Instead, the co-exposed worms showed significantly reduced growth (0.65 at 56 d), which was a stronger impact than that seen in the Cd-only exposure experiments. This result indicates that the adverse impact of Cd exposure on growth was made worse in the presence of As. This is despite the facts that (a) As-alone exposures from the OECD soils seem to promote worm growth at the longer timescales, and (b) with co-exposure, lower amounts of Cd and As were taken up.

6.2.6 Cocoon production

Results for cocoon production in the co-exposure experiment (control and exposed worms) are provided in **Table 6.2**. These are compared with results from the As and Cd-only exposure experiments in **Figure 6.4**.

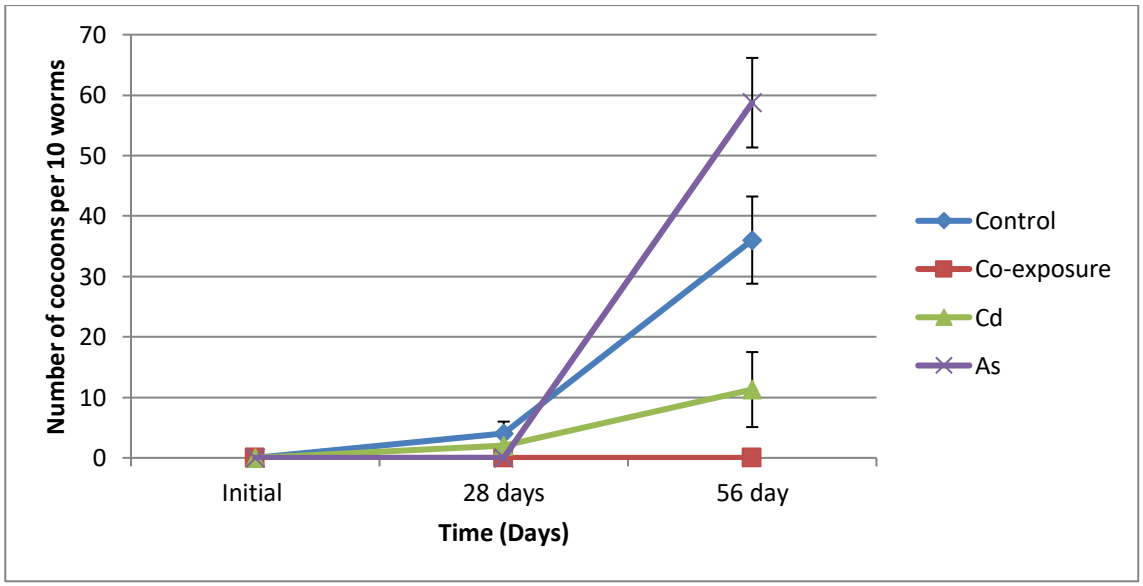

Figure 6.4: Cocoon production by *E. fetida* co-exposed to 90 mgCd/kg and 20 mgAs/kg, compared with equivalent individual Cd and As exposures and controls. Error bars represent standard deviations. Error bars represent standard deviations. Refer to Table 4.1, Table 5.1 and Table 6.1 for statistical significances of differences between means.

This graph shows cocoon production of worms in the co-exposure environment and individual Cd and As exposure experiments.

In the co-exposure experiment, worms in control soils produced an average of 4.0 cocoons during first 28 d and 36±7.2 cocoons during second 28 d period. However, in contrast to the equivalent Cd-only and As-only experiments, there was no cocoon production in Cd+As co-exposed worms.

These results are likely to reflect the inability of the worms to gain significant weight and attain reproductive maturity. Results for cocoon production also reflect those for weights in another way, in showing that the effect on worms from co-exposure was negative like Cd, but more drastic than seen with exposure to Cd-only.

Worms in 90 mgCd/kg soils produced 2±4 and 11.3±6.2 cocoons during the first and second 28 d periods (**Section 4.2.1.5**). Worms in the 20 mgAs/kg soils did not produce cocoons during the first 28 d but produced 58.7±7.4 cocoons during the second 28 d period (**Section 5.2.1.4**). For those experiments, after 56 d the conclusion was that exposure to 90 mgCd/mg causes a decrease in cocoon production (but with cocoons still being produced), whereas 20 mgAs/kg causes an increase. Therefore, in the co-exposure experiments, the assumption was that worms would produce cocoons by 56 d.

6.2.7 Mortality

Worm mortality during the Cd-As co-exposure experiment was zero. This is same as the equivalent 20 mg/kg As-only exposure experiment. However there, was 7.1% (2 worms) mortality recorded during the second 28 d period in 90 mgCd/kg soils. This difference is not thought to be particularly significant and may reflect unknown random factors in the Cd-only experiment.

6.3 Changes in gene expression

6.3.1 Differential gene expression

In the co-exposure experiment changes to gene expression in *E. fetida* were based on samples extracted after 56 d of exposure. The number of differentially expressed genes between control and the co-exposure (90 mgCd/kg and 20 mgAs/kg) condition were as follows: 5-fold differences: 7531; 10-fold differences: 1777; 15-fold differences: 5. As with the Cd and As-only experiments, large numbers of genes showed 5-fold differential expression between the exposure and control condition, fewer were in the 10-fold category, and under 10 transcriptomes were ≥15-fold differentially expressed. These results are shown together with those of the comparable As-only and Cd-only Generation 1 experiments in **Table 6.7**.

Table 6.7: Numbers of differentially expressed transcriptomes in Cd exposure Generation 1, As exposure Generation 1 and Co-exposure Generation 1.

	90 mgCd/kg	20 mgAs/kg	Cd + As Co-exposure
5-fold differences	7462	8438	7531
10-fold differences	1119	1725	1777
15-fold differences	7	7	5

Before the experiment it was speculated that the number of differentially expressed transcriptomes under co-exposure conditions would be higher than sole Cd and As exposure experiments. However, the numbers in each category do not differ significantly, and could be regarded instead as remarkably similar. This is despite the fact that in the phenotypic data, *E. fetida* showed greater negative impacts from the co-exposure than the individual exposure conditions.

Of course, the absolute numbers of differentially expressed genes may hide substantial differences in the identities of the genes being expressed under each condition. Out of 5 transcripts that were 15-fold differentially expressed with co-exposure, only two had identities. The first is *CBS2* and the putatively-linked protein is serine hydroxyl methyl transferase (mitochondrial) which is responsible

for inter conversion of serine and glycine (UniProt, 2019a). The second gene is *CYP26B1* which codes for protein cytochrome P450.

For the ≥10-fold gene expression analysis, out of 1776 differentially expressed transcriptomes only 16 (under 1%) had gene names. None of these was related to growth or reproduction of *E. fetida*. This is a reduction compared to individual Cd exposure experiment **(Section 4.4.1.1)**. In those trials, the number the ≥10-fold differentially expressed genes that were related to growth and reproduction were 151 and 5 respectively.

The following Venn diagrams (**Figure 6.5**) show overlaps of differentially expressed genes in the co-exposure experiment compared with the individual Cd (90 mg/kg) and As (20 mg/kg) experiments.

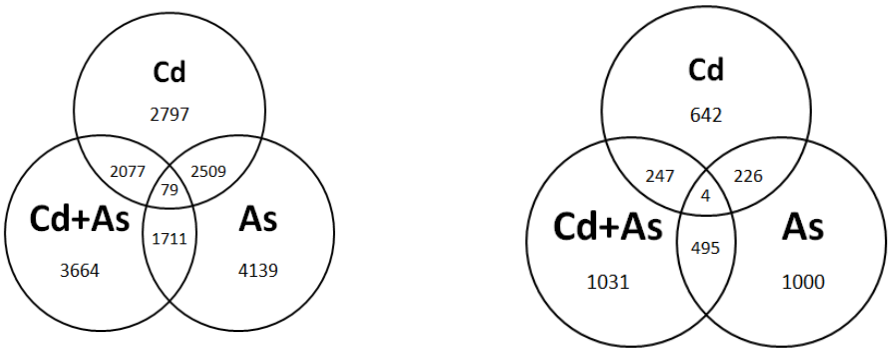

Figure 6.5: Venn diagrams showing overlaps of the same differentially expressed genes in co-exposure and individual Cd (90 mg/kg) and As (20 mg/kg) experiments. Left: based on ≥5-fold differentially expressed genes. Right: based on ≥10-fold differentially expressed genes (right).

The left-hand diagram in **Figure 6.5** relates to numbers ≥5-fold differentially expressed genes. At this cut-off, 79 genes were differentially expressed in all three conditions. Considering the binary pairs, in the Cd-only and As-only experiments, there were 2509 genes in common between As and Cd. For co-exposure, these numbers dropped, but not substantially. Comparing Cd-only with co-exposure, there are 2077 genes in common, which is an 83% (2077/2509) match.

Comparing As-only there are 1711 genes in common, which is a 68% (1711/2509) match.

The right-hand diagram in **Figure 6.5** relates to numbers ≥10-fold differentially expressed genes. At this cut-off, only 4 genes were differentially expressed in all three conditions. Considering the binary pairs, there is now a turn-around. In the Cd-only and As-only experiments, there were 226 genes in common that were ≥10-fold differentially expressed. For co-exposure compared with individual exposure, these numbers increased. Comparing Cd-only with co-exposure, there are 247 genes in common, which is a 9% increase. Comparing As-only there are 495 genes in common, which is over double (495/226, ratio = 2.19).

This significance of these numbers is unclear, and they need to be interpreted in relation to the both the lower overall Cd and As uptake (**Section 6.2.4**) and more serious adverse effects that occurred with co-exposure (**Sections 6.2.5** and **6.2.6**). However, the gene expression differences that are seen are consistent with the idea that at a molecular biology level, co-exposure to As and Cd trigger both similar and distinctly different responses to those seen for exposures to either contaminant alone.

6.4 Discussion

As with individual As and Cd exposure experiments, first generation *E. fetida* accumulated Cd and As under equivalent co-exposure conditions. However, the absorption pattern of As and Cd under co-exposure environment was different from the individual exposure conditions. Arsenic absorption approached equilibrium during the exposure period, and the Cd concentrations reached in worms were not as high as Cd concentrations reached in individual Cd exposure experiments. Worms showed slow growth in the co-exposure experiment, and did not produce cocoons, in contrast to the individual As exposure experiments where As appeared to act as a reproductive stimulator.

As far as the author is aware there is no previous literature on co-exposure of *E. fetida* to As and Cd. However, there are some other significant co-exposure studies. Wijayawardene *et al*(2018), examined chronic exposure of *E. fetida* to binary and

ternary mixtures of Cd, Zn and Pb. These authors found for an acidic soil environment Zn and Cd are the most toxic metal combination for the earthworms out of Zn, Cd and Pb. *E. fetida* reproduction was more significantly impacted by Cd and Pb co-exposure than for either metal alone in the akaline soil; as seen with Cd and As co-exposure results in the current research in slightly alkaline soil.

Several studies have been carried out to examine effects of co-exposures of earthworms to specific pesticides, or particular metal and pesticide(s) (Uwizeyimana et al., 2018, Stepić et al., 2013, Lister et al., 2011, Liang and Zhou, 2003, Van Gestel and Hensbergen, 1997, Wu et al., 2016b). Vellinger et al (2013) examined co-exposure of Cd and As in the freshwater grammarid *Gammarus pulex*, focusing on shorter-term biochemical response markers. These authors found the interactions to be complex and 'difficult to unravel' but noted the importance of such studies to better assess the effects of contaminant stressors on ecological receptors (Vellinger et al., 2013).

Outside well-controlled 'systematic' experiments, a range of studies have been carried out looking at phenotypic and biomolecular aspects of earthworms already living in contaminated soils (Ray et al., 2019, Beaumelle et al., 2015, Kim et al., 2016), or added to soils made with metal mixtures similar to field-contaminated soils (Sivakumar, 2015) where exposure is commonly to two or more contaminants.

6.5 Findings and implications

The most notable findings of this part of the work are as follows.

[1] Presence of As reduced Cd uptake and *vice versa*. At soil concentrations of 20 mg/kg As and 90 mg/kg Cd, and over 56 d: the presence of As caused a 39% reduction in Cd uptake, and the presence of Cd caused an 83% reduction in As uptake.

[2] Co-exposure to As and Cd produced more serious impacts on growth and reproduction than exposure to either alone, despite their differing chemistries, and the fact that less Cd and As were taken up (**Finding [1]**). Under the co-exposure condition, worm growth was inhibited and no reproduction occurred.

[3] Co-exposure to As and Cd produced similar numbers of differentially expressed genes as exposure to either contaminant alone. This reflects a similar whole-organism response to the exposure, again despite the fact that less As and Cd had been taken up (**Finding [1]**).

[4] Results for comparison of identities of differentially expressed genes between the different conditions suggest that co-exposure to As and Cd trigger both similar and distinctly different responses to those seen for exposures to either contaminant alone.

[5] As with **Chapters 4** and **5**, when it comes to identifying named genes and linking those to phenotypic outcomes, the results of RNA transcriptome testing were uninformative. This is mainly because only a small percentage of genes showing the most differential expression have yet been annotated and had putative functions assigned, reflecting the developing state or inherent nature of this complex area.

Implications

Results of this work show that co-exposure to two dissimilar inorganic contaminants cationic Cd^{2+} and anionic AsO_4^{3-}had a combined adverse effect that was greater from exposure to either contaminant alone. Despite Cd and As having different chemistries, their impacts to *E. fetida* were not independent, but (in some sense) additive. Exposure to As worsened the impacts of Cd, and *vice versa*. Whereas individual exposures still allowed reproduction to continue, the equivalent combined exposures did not. These findings build on those of Wijayawardena et al (2018) who reported that effects of combined exposures to Zn^{2+} and Pb^{2+} (both cationic heavy metals) were significantly greater than those of either contaminant alone.

In terms of wider implications for ecosystems the results add support for the argument that soil guideline values derived from single-contaminant toxicity experiments may be insufficiently protective in important real-life cases where ecological receptors are exposed to two or more contaminants at a time, such as in contaminated agricultural soils or in soils of contaminated sites. This in turn

suggests a need for synthesis of our understanding of the biochemical pathways of toxicity to allow stressors to be assessed from a whole-organism perspective.

The results may also have significant implications for human health, on the basis that there are many examples of populations who are exposed to elevated As (especially in drinking water), and Cd in modern diets is known to be near the tolerable limits for first onset of adverse effects (EFSA, 2011). On this basis there may well be a subset of the wider human population who are exposed to elevated levels of both contaminants on a daily basis. Such populations may be more vulnerable than would otherwise be anticipated. Moderns intakes of Cd in some well-characterised western populations are known to be close to the onset threshold for a change in kidney function over the long-term, which is taken as the first reliable toxic effect from dietary Cd (EFSA, 2009, EFSA, 2012).

On this basis, there is potential for adverse effects to be experienced from co-exposures to As and Cd even under conditions where tolerable daily intake limits for each contaminant is being met. This suggests an urgent need for policymakers or regulatory agencies to consider the wider implications of human co-exposures, prioritising cases where one contaminant is known to be already near its threshold for the onset of toxic effects.

Chapter 7: Behaviour of other trace elements

7.1 Introduction

This part of the work was a preliminary assessment of whether exposure to Cd or As was associated with changes in tissue concentrations of other selected elements in exposed worms. The rationale for this was based on the possibility that some effects caused by high levels of Cd or As in soil may come about through alteration of uptake, absorption and/or metabolism of other major and trace elements in worms. Although many authors have reported tissue concentrations for elements that worms have been intentionally exposed to, few reports examine the potential for alteration of uptake or interactions between elements that might be induced by the high-dose exposure.

Earthworms have the ability to take up both essential and non-essential elements when living in both non-contaminated and contaminated environments. Uptake efficiency depends on the element, its concentration and chemical form, and soil (edaphic) factors. Under some conditions earthworms can tolerate high environmental concentrations of toxic heavy metals and accumulate some toxic metals in non-toxic forms or excrete them from the body efficiently.

Interactions between metals can influence the amount of specific metal accumulation in worms. As an example, high soil copper (Cu) and calcium (Ca) can reduce the uptake of lead (Pb) (Ireland, 1983). Ireland (1983) reported that Pb concentrations in the tissue of *L. Rubellus* at Borth (site 1) were 400 times lower than those in the same species at Cwmystwyth (site 2), even though Pb concentrations in the soil at the two sites only differed by only a factor of ~2 . The reason was that Ca and Cu concentrations were much higher at site 1 than at site 2 (Ireland, 1983). Similarly, exposure to high levels of Cd lead to Cu deficiency in grazing animals (Blanco-Penedo et al., 2006). As mentioned in the methodology (**Section 3.2.2**), 9 elements were targeted in this part of the research. These were Cd and As themselves (where Cd concentrations were also measured in As exposed worms and *vice versa*), and 7 essential elements that might be affected by Cd or As. These included divalent (M^{2+}) cations that may be affected by Cd^{2+} exposure (Ca, Mg, Cu, Zn, and to some extent Fe), other elements involved with metallo enzyme

functioning and repair (Fe, Se); and the macronutrient P, which in its phosphate form is isomorphous with arsenate.

7.2 Results and discussion

Concentrations of the 9 elements detected in worm tissue under different exposure conditions and generations are provided in **Table 7.1**

Table 7.1: Concentrations of As, Cd, Ca, Mg, Fe, P, Zn, Cu, Se in worm tissue under different exposure conditions and generations.

Sample	Gener-	Time	Element concentration in worm tisssues (mg/kg)								
Identity	ation	(days)	As	Cd	Ca	Mg	Fe	P	Zn	Cu	Se
Cd-Control	1	28	0.745	0.200	616	124	35.1	1240	25.4	1.84	<0.1
Cd-30	1	28	2.57	75.2	532	133	78.0	1580	26.6	1.31	<0.1
Cd-90	1	28	2.87	111	556	138	106	1611	32.3	1.43	<0.2
Cd-270	1	28	4.23	166	529	129	116	1600	29.6	1.15	<0.2
Cd-control	1	56	1.96	0.287	490	129	51.8	1470	21.0	2.03	<0.1
Cd-30	1	56	3.50	106	399	128	95.3	1581	29.2	1.19	<0.1
Cd-90	1	56	1.83	148	442	117	61.7	1421	21.7	0.98	<0.1
Cd-270	1	56	7.39	379	693	143	263	1640	35.6	1.34	<0.2
Cd- control	1	84	0.872	0.212	592	149	37.5	1400	23.0	1.58	<0.1
Cd- 30-CS	1	84	1.79	65.7	614	138	52.9	1500	28.6	1.43	<0.1
Cd-90-Cs	1	84	3.53	135	738	143	86.3	1520	28.1	1.35	<0.1
Cd-270-CS	1	84	4.19	280	934	157	142	1420	44.9	1.54	<0.3
Cd-Control	2	56	0.130	0.579	488	130	25.4	1370	21.5	1.63	<0.1
Cd-30	2	56	<0.1	150	463	150	13.0	1500	24.8	0.95	<0.1
Cd-30-CS	2	56	0.103	38.2	522	140	23.7	1335	21.2	1.40	<0.1
Cd-90	2	56	<0.1	199	629	125	15.5	1160	24.3	0.85	<0.1
Cd-90-CS	2	56	<0.1	102	316	76.5	8.7	842	14.8	0.49	<0.1
Cd-Control	3	56	<0.1	0.132	452	113	19.9	1170	18.0	1.41	<0.1
Cd-30	3	56	<0.2	106	399	120	16.5	1210	26.0	1.63	<0.1
Cd-30-CS	3	56	0.401	27.7	610	141	48.8	1380	34.7	2.09	<0.2
As-Control	1	28	<0.2	0.095	648	188	28.3	1600	33.0	1.94	<0.2
As-10	1	28	4.13	0.058	363	111	64.4	1240	29.4	2.01	<0.2
As-20	1	28	12.0	0.076	679	196	163	2120	37.2	2.88	<0.3
As-Control	1	56	2.05	0.356	644	123	147	1370	45.2	5.07	<0.3
As-10	1	56	15.3	0.093	810	241	60.2	1670	49.1	3.56	<0.5
As-20	1	56	36.3	0.050	398	137	83.0	1560	34.4	1.70	<0.2
As-Control	1	84	8.89	0.144	926	219	40.7	1960	42.6	3.26	<0.4
As-10-CS	1	84	12.1	0.026	151	48.5	10.7	473	8.58	0.55	<0.1
As-20-CS	1	84	15.5	0.020	213	62.9	16.6	651	13.9	0.79	<0.1
As-Control	2	56	0.483	0.651	349	128	24.2	1210	22.7	2.53	<0.2
As-10	2	56	47.8	0.074	362	129	37.5	1330	28.4	2.02	<0.1
As-20	2	56	41.4	0.048	569	114	27.6	1220	31.6	1.26	<0.2
Co-ex- test	1	56	7.77	74.8	440	104	38.1	909	29.2	3.37	<0.1
Co-ex-control	1	56	0.504	0.439	1160	327	87.7	3180	96.5	6.58	<0.2
Minimum			<0.1	0.020	151	48.5	8.67	473	8.58	0.495	<0.1
Maximum			47.8	379	1162	327	263	3180	96.5	6.58	<0.5
Mean			7.1	63.7	551	140	62.5	1420	30.4	1.92	-
Standard deviation			11.9	91.5	206	49.7	54.5	448	14.7	1.25	-
Percent RSD			168	144	37.4	35.6	87.2	31.4	48.4	65.4	-
95% confidence error			4.15	31.90	71.80	17.30	19.00	156.1	5.13	0.44	-

*Element concentrations in *E. Fetida* tissue reported by (Babić et al., 2015)

7.2.1 Comparison to expected ranges in general

The reference values are shown in **Table 7.1** were taken from a study carried out in 2015 on metal homeostasis disturbance in *E. fetida* after exposure to sludge (Babić et al., 2015). The values were from the control samples which involved the worms being exposed to clean soil from a worm farm.

With the exception of Cd (in Cd exposure experiments), and As (under As exposure conditions), element concentrations in worm tissues were in ranges and magnitudes that have previously been reported (**Table 7.1**). This gives some confidence in the general validity of the results.

Natural concentrations of selenium (Se) (given as just over 0.1 mg/kg by Babic *et al.* 2015) are at the detection limit of the method employed. Results for submitted samples were consistent with this, in that Se was not detected in any submitted sample to detection limits ranging from 0.1-0.5 mg/kg (**Table 7.1**). Se is not included in further discussion.

7.2.2 Inter-relationships between variables - Generation 1

Correlation matrices

Results of Pearson's correlations between variables in Generation 1 worms (for all conditions) are shown in **Table 7.2** for Cd, and **Table 7.3** for As.

Table 7.2: Correlation coefficients (R-values) and probabilities for relationships between variables in the Cd-exposed Generation 1 worms overall exposure conditions. Number of pairs = 12 (see Table 7.1)

	Wt	CC	%F	As	Cd	Ca	Mg	Fe	P	Zn	Cu
Wt	1										
CC	0.670	1									
%F	-0.517	-0.137	1								
As	-0.799	-0.322	0.757	1							
Cd	-0.867	-0.497	0.690	0.912	1						
Ca	-0.579	-0.542	-0.070	0.342	0.495	1					
Mg	-0.396	-0.425	-0.117	0.329	0.372	0.804	1				
Fe	-0.826	-0.393	0.780	0.969	0.930	0.384	0.368	1			
P	-0.379	-0.088	0.449	0.675	0.454	-0.187	0.106	0.586	1		
Zn	-0.845	-0.521	0.159	0.658	0.754	0.739	0.661	0.686	0.261	1	
Cu	0.292	0.420	-0.322	-0.354	-0.444	0.226	0.187	-0.286	-0.492	-0.123	1

	Wt	CC	%F	As	Cd	Ca	Mg	Fe	P	Zn	Cu
Wt	1										
CC	0.01	1									
%F	0.02		1								
As	0.01		0.01	1							
Cd	0.001		0.02	0.0001	1						
Ca	0.01		0.05			1					
Mg						0.01	1				
Fe	0.01		0.01	0.0001	0.0001			1			
P				0.02				0.05	1		
Zn	0.001	0.05		0.02	0.01	0.01	0.02	0.02		1	
Cu					0						1

Table 7.3: Correlation coefficients (R-values) and probabilities for relationships between variables in the As-exposed Generation 1 worms over all exposure conditions. Number of pairs = 9 (see Table 7.1)

	Wt	CC	%F	As	Cd	Ca	Mg	Fe	P	Zn
Wt	1									
CC	0.878	1								
%F	-0.294	-0.125	1							
As	0.825	0.834	-0.305	1						
Cd	-0.168	-0.131	-0.327	-0.529	1					
Ca	0.170	-0.235	-0.395	-0.294	0.273	1				
Mg	0.350	-0.031	-0.243	0.061	-0.236	0.840	1			
Fe	-0.077	-0.080	-0.255	-0.162	0.412	-0.125	-0.290	1		
P	0.231	-0.204	-0.540	0.135	-0.233	0.650	0.732	0.171	1	
Zn	0.465	0.232	-0.243	-0.177	0.533	0.739	0.546	0.171	0.255	1

	Wt	CC	%F	As	Cd	Ca	Mg	Fe	P	Zn
Wt	1									
CC	0.01	1								
%F			1							
As	0.01	0.01		1						
Cd					1					
Ca						1				
Mg						0.01	1			
Fe								1		
P						0.05	0.02		1	
Zn						0.02				1

Across all exposure conditions, Cd is negatively correlated with the weight ($p<0.001$) and mortality ($p<0.05$). The negative relationship with cocoon production is weaker ($p<0.1$) and does not reach the normal cut-off for statistical significance of $p<0.05$. A likely reason for this is due to the inclusion of 30 mgCd/kg exposure conditions in the overall relationship as Generation 1 cocoon production did not decrease substantially under these exposures (**Section 4.2.1**).

Of more interest in this data is relationships between Cd and other elements, in Cd exposed worms. In these experiments, only the concentration of Cd was changed in the artificial soil. Concentrations of all other metals in artificial soil remained constant. Despite this, three metals showed a positive relationship with Cd uptake in worm tissue.

These were the positive relationships between Cd-Fe ($R=0.930$, $p<0.0001$), Cd-As ($R=0.912$, $p<0.0001$), and Cd-Zn ($R=0.754$, $p<0.01$). Only one element, Cu, showed an apparently negative association with Cd; but this did not reach statistical significance (Cd-Cu; $R = -0.444$, $p<0.1$). A first tentative finding is therefore that Cd exposure did not appear to *reduce* the uptake of other elements. This was a possibility for divalent metals in particular because Cd^{2+} may compete for their uptake.

Cadmium and arsenic

Cd and As showed a highly ($p<0.0001$) significant positive correlation in Generation 1 Cd exposure experiments (**Table 7.2**). This is noteworthy because As was not being added to the OECD soils, and they had a low concentration of natural As (1.31 mg/kg).

There are two possible reasons for these results: that Cd causes increased phosphate uptake with co-uptake of arsenate; and that upregulation of proteins that also bind As may occur as defence mechanism above certain As exposure levels.

As outlined in Section 4.4.1 the first possible reason for this relationship is that worms subjected to chronic wounding will be utilising more phosphate (PO_4^{3-}) for repair. Phosphorus is important to the body because it is an essential element of ATP and DNA, among numerous other biochemicals. According to literature Cd exposure causes internal and external lesions and haemorrhage in *E. fetida* (Rodriguez et al., 2013). Under these conditions, the worms are likely to draw more heavily on phosphate to recover.

Unlike humans (Hibberd et al., 1985), worms are unable to mobilise phosphate reserves from an internal skeleton. It is therefore probable that they instead take

more phosphate up from the soil. This in turn, would be likely to cause higher uptake of arsenate (AsO_4^{3-}), which is isomorphous with phosphate. Arsenate is the dominant form of As in soils (Mandal and Suzuki, 2002).

Increased utilisation of phosphate for repair is unlikely to increase the concentration of phosphorus in the worm (or human) tissue because P is homeostatically regulated. However, it is likely to increase P turnover, incidental losses and uptake requirements. For this reason, a correlation between Cd and P would not be expected, but a correlation between Cd and As would be plausible. In these results, therefore, As uptake may have acted as a marker for P utilization.

Co-uptake of these two chemical species is well known in marine fish for which phosphate is in limited supply and is considered to be the primary reason that fish have evolved a mechanism to detoxify inorganic As by conversion to arsenobetaine (Neff, 1997).

If the above mechanism is significant, the same effect should be caused by exposures to other contaminants. This leads back to the As exposure experiments themselves, where it was found that >28 days, As exposure was associated with an increase in relative As uptake, as measured by the simple tissue/soil As BCF values (**Table 5.2**).

As a final comparison, the correlation would not be expected to work in reverse. In the As exposure experiments, we would not expect to see an increase in the uptake of Cd. The results of this work are also consistent on this point. In the Generation 1 As exposure data, there is no significant relationship between As and Cd ($p>0.1$, and the R-value is in fact, negative) (**Table 7.3**).

A second possibility that could explain the results of the As exposure experiments is that at high As concentrations (or long exposures) a specific resistance mechanism is upregulated which promotes more As retention. For example, it is possible that above a certain threshold the worm may upregulate metallothionein (MT) synthesis as a detoxification pathway, which would have a side-effect of causing more As to be retained (Langdon et al., 2005). This explanation would require there to be a substantial difference in how the worms use retention proteins for Cd exposures, compared with As exposures. In this explanation,

results for Cd exposure experiments (where more As was also taken up at higher Cd levels) would be interpreted as increasing retention of As on MT-like proteins that have been induced by the Cd exposure. Expression of MT may occur at lower exposure concentrations for Cd than for As (Del Razo et al., 2001).

Of course, both processes may work together. A possible avenue for future research would be to investigate the importance of each possibility.

Other elements

Essential dietary minerals such as Zn, Fe and Ca perform key functions in cellular metabolism, repair, and functioning of an animal's body systems. For example, Zn is considered to be one of the most important nutrients for the immune system because it is essential for hormone function and formation of antibodies and white blood cells.

In the Generation 1 Cd results, Zn was negatively correlated with weight ($p<0.001$), cocoon count ($p<0.05$) and mortality ($p<0.01$), and positively correlated with Cd ($p<0.01$), Ca ($p<0.01$), Mg ($p<0.02$), Fe ($p<0.02$) and As ($p<0.02$) (**Table 7.2**). Fe was correlated with Cd and (therefore) As, both at $p<0.0001$ (**Table 7.3**)

In the Generation 1 As results, Zn was positively correlated with Ca ($p<0.02$) (**Table 7.2**), and no significant correlations were found for Fe (**Table 7.3**).

Given the relatively low number of pairs in each case (12 for Cd and 9 for As), results for apparent relationships at $p<0.001$ and below might be of most interest. However, a possible reason for the overall pattern of Cd correlation results is that increasing concentrations of Cd^{2+} in soils may act to displace the exchangeable fraction of other divalent metals (Zn^{2+}, Ca^{2+}, Mg^{2+}, and $Fe^{2+/3+}$) into soil pore water, making them more available for uptake by the worms. This explanation would also fit with a lack of any similar correlations in the As exposure experiments because inorganic As is an oxyanion (AsO_4^{3-} as arsenate or AsO_2^- as arsenite) and will not act as a cation exchange agent.

The Cd-Fe ($p<0.0001$) and Cd-Zn ($p<0.001$) relationships may be linked with cellular repair or (for Zn) upregulation of metallothionein which would help worms to limit Cd toxicity while regulating Zn (Demuynck et al., 2007a,

Subramanian Vignesh and Deepe Jr, 2017)However, the same relationships are not seen in the As data (**Table 7.3**). It may therefore, be more likely that these relationships reflect higher levels of available Zn and Fe in soil porewater caused by displacement on soil binding sites by some of the spiked Cd. Both Zn and Fe are homeostatically regulated, but when uptake is higher for these elements, part of this regulation will involve storage and accumulation (Roh et al., 2012, Procházková et al., 2011). The Cd-Zn and Cd-Fe results are therefore consistent with more Cd promoting higher Zn and Fe uptake and storage.

7.3 Findings and implications

There are two main findings in this part of the work, each with its own implications. In this section each finding and its implication is considered separately.

Finding [1]. Results suggest that in *E. fetida*, exposure to Cd may work to potentiate As uptake. This may come about through an increase in phosphate uptake (with concurrent uptake of more arsenate), and/or upregulation of proteins that also bind As. This finding sits alongside **Finding [4]** in **Chapter 5** (**Section 5.4**) where it appeared that As may also potentiate its own uptake in a positive feedback loop.

Although tentative, these results for Cd possibly increasing As uptake have some implications for interpretation of some ecotoxicology trials on earthworms and other invertebrates, because inter-element interactions may work to increase or reduce toxicity (Ireland 1983).

Most notably, the results suggest that if the response to a contaminant includes an increase in phosphate uptake (e.g. for cellular repair), this may also cause an increase in the uptake of As. This raises the *possibility* that some contaminants may work to induce an As co-exposure situation for soil-dwelling organisms even from settings where As is not especially elevated. Together with the results of **Chapter 6** this implies that some single-contaminant exposure experiments in which As uptake is negligible may significantly underestimate the toxicity in real-life settings where 4-5 mg/kg As forms a natural part of the soil matrix.

Secondarily, in cases where a Cd contaminated site also contains elevated Zn, additional available Zn in pore water may work to reduce Cd uptake and/or effects of Cd toxicity. Such effects would be case-specific but may need to be considered more frequently.

Potential implications for human health settings relate to the causes and significance of co-exposures as outlined in **Section 6.5** for populations that may experience higher than average levels of both Cd and As.

A phosphate uptake mechanism of the type proposed above should not apply to human exposures. This is because phosphorus (P) is mobilised from bone reserves as part of Ca and P homeostasis (Shaker and Deftos, 2018), and dietary P (as a sum of all foods eaten) is likely to be reasonably independent of local soil contaminant levels.

Finding [2]. Results of this Chapter suggest that some spiking experiments may distort the natural picture by causing a release of other elements. In this case the possibility existed that spiking of soils with Cd may have resulted in increased uptake and release of other cationic metals, including Fe, Zn, Mg and Ca.

An implication of this finding is that even in controlled toxicology experiments (*e.g.* run in model OECD soils) the impact of the spiking agent on the release of other elements should be considered as a possible confounding variable in future. It can be argued that this same situation would also exist in actual anthropogenically contaminated soils, where another layer of complexity comes about through differences in the soil types and properties (edaphic factors).

More generally for ecotoxicology experiments with trace elements as contaminants it would be useful to start routinely testing for other elements, in addition to the contaminant(s) of interest. This would enable a better picture to be developed of the range of inter-relationships involved with anthropogenic contamination, including its effect on soil chemistry and uptake of other elements. It is possible that a range of potentially important modifying factors may have been missed by having a narrow focus on the behavior of the spiking agent or anthropogenic contaminant alone.

Chapter 8: Summary and Recommendations

The focus of this thesis was on uptake and effects of Cd and/or As exposures on *E. fetida* on three consecutive generations, including recovery potential. Key variables examined included contaminant concentrations in tissues, growth, reproduction, levels of gene expression, and associated trace elements.

Most notable findings for each area are listed at the end of each applicable result chapter. Specifically, key findings relating to Cd are provided in **Section 4.5**, to As in **Section 5.4**, to Cd and As co-exposure in **Section 6.5**, and to relationships with other elements in **Section 7.3**.

Of these, a number of key themes come through.

Contaminant uptake and loss

Cd shows continuous accumulation in the worm tissue and this continues until lethal levels in worm tissue are reached. Moreover, the efficiency of uptake (as measured by the relative BCF) increases with decreasing Cd concentrations in the soil. Therefore, it may not be valid to rely on standard 28 d exposure experiments to derive guideline values that would be genuinely protective of worms and other soil invertebrates. Instead of single soil concentrations (such as EC_{10} or EC_{50}), thresholds for the onset of effects and minimum lethal dose (MLD) would be better viewed as an envelope defined by soil [Cd], BCF, and exposure time. Accumulation of Cd was a faster process in exposed worms than Cd loss when exposure ceased. Based on first-order elimination kinetics, the biological half-life for Cd loss was 198 days or 6.5 months. This implies that worms that have been exposed to elevated Cd for more than a few weeks would be unlikely to be able to eliminate much of the accumulated Cd burden over their normal lifetimes.

Like Cd, worms living in As-spiked soil continuously accumulated As into their tissues. However, unlike Cd at sub-lethal exposure levels, there are circumstances where arsenate appears to promote its own uptake in a positive feedback loop. This was most noticeable at the lower 10 mg/kg soil As exposure scenario and is most likely to be linked to increase phosphate (PO_4^{3-}) uptake, with which arsenate

(AsO_4^{3-}) is isomorphous, as occurs also in marine fish where phosphate is limited. The fact that significant As uptake and similar levels of differential gene expression persisted even after worms were returned to un-spiked soils (containing only natural As) suggests that the newly upregulated pathways remained active for some time after exposure.

In the Cd exposure results, a correlation between Cd and As (**Chapter 7**) adds another facet to this picture, because it suggests that potentiation of As uptake may also be caused by Cd, and therefore by other contaminants as well. This would again make sense if one of the general responses to toxicity is upregulation of phosphate for protection, cell-repair or new growth. A remarkable implication of this finding is that it is possible that for worms (and perhaps other soil invertebrates), single contaminant exposures may actually become co-exposures with both the contaminant of interest, and As. Such an effect may be extremely important at many sites with moderately elevated soil As levels, because the impacts of Cd and As co-exposure were more severe than exposure to either contaminant alone (**Chapter 6**).

Impacts of Cd or/and As on worms

Cd negatively affected worm growth, cocoon production and sexual maturity. In-fact the time needed to reach a sexually mature state increased with increasing Cd, and at the higher concentrations, cocoon production reduced from generation to generation. However, phenotypic and gene expression differences were evident worms exposed to the two higher Cd levels and those experiencing the lowest 30 mg/kg exposure; for example at the lowest exposure, cocoon production showed a recovery by Generation 3, and worms returned to clean soil showed a large rebound effect to well above performance of controls, suggesting that resources being used to counter toxicity were redirected when the Cd stressor was removed. For higher exposure conditions and longer exposure times these effects disappeared and worms performed more poorly suggesting that there is a tissue Cd threshold beyond which recovery from Cd exposure becomes challenging. This threshold is most likely to be the point where metallothionein (MT) (and/or other

proteins) produced to sequester Cd reach tissue saturation, as can also occur in kidney tissue in cases of chronic human poisoning.

Modest As exposure that extended longer than 28 d had the unusual effect of stimulating growth and cocoon production, with the scale of genomic results suggesting this was a biochemical response to toxicity rather than a beneficial effect of As exposure such as suppression of parasites. This effect may be important in real-life settings and is likely to have been missed in most 28 d exposure experiments, indicating another limitation of the standardised approach. Stimulation of growth and cocoon production initially appeared to be positive; however, by Generation 2 it was clear that the effects of the exposures were overwhelmingly negative, in-terms of both the low survival percentages and the retarded growth of surviving offspring.

Co-exposure to As and Cd together produced more serious impacts on growth and reproduction than exposure to either contaminant alone. Even though less Cd and As were taken up compared to individual exposure conditions, under co-exposure conditions worm growth was inhibited and no reproduction occurred. This result supports an argument that soil guideline values derived from single contaminant toxicity experiments may be insufficiently protective in many real-life settings.

Across all of these experiments another theme emerged about the significance of (and distinction that should be made between) ongoing exposures, and one-off (e.g. 28 d) exposure experiments.

In real-life situations, the offspring of exposed invertebrates are most likely to be born into the same contaminated soil environment as their exposed parents. Under these conditions, subtle inherited effects from the parental generation which may cross generations are probably less important than the burden of ongoing exposures. In this work it was found that the impact of ongoing contamination can easily compound across generations. Viable offspring of exposed parents began life with a tissue contamination burden and were slower to grow and reach sexual maturity. A key finding therefore, might be that inherited genetic effects might sometimes be over-rated when it comes to real-life ecotoxicology, where the contaminant exposure is most likely to be multi-generational and ongoing. This

lesson could also apply to wide-scale human exposure cases, including the issues around large populations who are exposed to elevated As in drinking water.

Interpretation of gene expression

Results of gene expression tests provided a number of insights, but two key themes were those exposures to Cd, or As or both together triggered large-scale changes in gene expression indicating an 'organism-wide' response; and that differential expression results tracked along with phenotypic responses.

In some cases the gene expression results added to the circumstantial evidence supporting a particular interpretation. For example, at Cd exposures under the presumed MT-saturation threshold, differential gene expression reduced substantially when the exposure ceased, whereas at longer exposure times of toxicity were also still reflected in the high numbers of differentially expressed genes even after exposure ceased. For As, large numbers of differentially expressed genes *between* the two exposure conditions suggested the existence of substantive biochemical changes between lower and higher exposures. These might reflect upregulation of defence responses and/or inhibition of various metabolic pathways as exposure levels increase. Similarly, numbers of differentially expressed genes in the co-exposure experiment were not very different from those seen from individual Cd and As exposures, in line with the serious adverse phenotypic effects that were seen with the co-exposures (despite less Cd and As being taken up).

Identification of specific differentially expressed genes was not particularly useful as an interpretive tool. This is both because (a) only a small proportion of expressed genes are annotated with putative functions assigned to them, and (b) most responses probably involve many genes working together, both named and unnamed, reflecting biochemical complexity. These factors mean that the approach of identifying specific genes is unlikely to be particularly useful for interpreting toxicity responses except in special cases where the researcher is targeting simple and well-defined pathways.

A separate finding was made during preparation of the artificial soils, that after wetting, the pH of the mixture progressively increases for several days before reaching pseudo-equilibrium (**Section 3.1.3.3**; **Table 3.3**). In this work, artificial soils were allowed to reach this pH equilibrium and trial went for extended times. In shorter toxicity trials, this effect may represent an uncontrolled variable at the outset of experiments. It is recommended that further work be undertaken to investigate the significance of this effect in relation to the availability of cations like Cd and oxyanions like As.

Recommendations for future research

Based on the findings of this work, the following areas would be useful areas for future research:

1. Exploration of the relationship between Cd concentrations in soil and relative uptake factors (BCF values), including identification of pathways that drive higher uptake at lower concentrations;

2. More long-term and lifespan experiments on earthworms exposed to a range of soil Cd concentrations, to determine the boundaries of the toxicity envelope for Cd as a model cumulative and persistent contaminant;

3. Further exploration of the possibility that contaminant exposure triggers an increase in phosphate uptake and utilisation in earthworms and related invertebrates, and if so:

    - The toxicological implications of that mechanism, including the feedback effects on arsenate uptake and resultant As co-exposures;

    - Investigation of the potential for use of arsenate as a biochemical marker for phosphate uptake in invertebrates.

4. Further work on the impacts of contaminant co-exposures on invertebrates with regard to commonly occurring pairs and ternary combinations of contaminants that can occur in agricultural and other soils (e.g. Cd and Fe);

5. Exploration of the relative importance of genetic effects in situations where exposures are ongoing and multi-generational;
6. Method development of standardised toxicity tests that allow for ongoing multi-generational exposures; and
7. The potential significance of human co-exposures to Cd and As.

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10: Appendices

Appendix 1: Appendices for Chapter 2

Published paper

Title: Maternal cadmium exposure and impact on foetal gene expression through methylation changes

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Maternal cadmium exposure and impact on foetal gene expression through methylation changes

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ABSTRACT

Cadmium (Cd) exposure is not easily avoidable; it is a common contaminant found in many food sources, accumulates throughout life and, in high doses, is a significant health hazard for humans. Women are highly vulnerable to Cd because of their relatively higher absorption rate than men. High levels of Cd accumulated in the mother could potentially cause harm to both the mother and new-born child. The foetal genome is vulnerable to external signals; Cd partially crosses the placental barrier and can impact on foetal development, potentially, through epigenetic mechanisms causing changes to foetal gene expression. This review explores current research on Cd induced methylation changes to maternal and foetal genomes. Cd is significantly associated with differential methylation of both maternal and foetal genomes. Some studies have described infant sex-specific changes in DNA methylation in association with maternal Cd burden. However, research on methylation changes to the foetal genome due to prenatal Cd exposure is scarce. More research is required to explore the impact of maternal Cd accumulation on differential methylation of the foetal genome.

1. Introduction

Cadmium (Cd) is a naturally occurring transition element which is found ubiquitously in the Earth's crust. It is present in all environmental compartments at low levels with background concentrations determined by natural biogeochemical cycling. However, since industrialisation, anthropogenic activities have caused the release of more Cd to surface environments, in particular to agricultural soils (WHO, 2016).

Cd, like other trace elements, can be absorbed into the blood stream via three main routes; ingestion (via the gastrointestinal tract), inhalation/pulmonary (through the lung surface) and dermal absorption (through the skin). The digestive tract absorbs approximately 5% of the ingested Cd (Godt et al., 2006), with the exact proportion absorbed depending on a range of factors (WHO, 2011) including iron content in the body (Kippler et al., 2009) and age (Kello and Kostial, 1977). A significant proportion of absorbed Cd is retained in the body, with approximately 50% being distributed between the liver and kidneys where it is usually complexed with metallothionein. Because of its long biological half-life (10–35 years) the body burden of Cd increases with age (WHO, 2011). Research carried out in 1997–1998 in Australia (Satarug et al., 2002) studied the level of Cd exposure in the general population. The data were interpreted according to age groups and Cd concentrations in liver, kidney and lungs (Table 1).

Food is the main source of Cd exposure in non-smokers. Although Cd is found in most foods consumed its concentration varies greatly depending on the type of food and level of environmental contamination (Ysart et al., 2000). Higher concentrations of Cd are commonly seen in molluscs and crustaceans such as bivalves and crabs (Järup and Åkesson, 2009). Dark meat and offal products also contain higher levels of Cd. Several plant-derived foods contain more Cd than meat, eggs and dairy products (Ysart et al., 2000). Across a typical diet, leaf and root vegetables (including potatoes) and grains account for a substantial part of total cadmium intake, with lower contributions spread across other food groups such as muscle meats, eggs, dairy products and fruits. Cd is readily absorbed by plants via the roots, with availability and uptake increasing as pH decreases (Olsson et al., 2002). Grains such as rice and wheat, green leafy vegetables, potatoes and root vegetables such as carrot and celeriac contain higher concentrations of Cd than other plants (Järup and Åkesson, 2009). Total Cd intake depends on both the concentration of Cd in the food as well as the amount of each food type eaten. In general, vegetarians consume more Cd than others. Smoking is also an important exposure route to Cd (Järup et al.,

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Table 1
Mean Cd concentration (μg/mg) in lung, liver and kidney (Satarug et al., 2002).

Age group (years)	Kidney	Liver	Lungs
1–10	1.63	0.21	0.01
11–20	5.44	0.71	0.04
2130	9.80	0.65	0.22
31–40	17.72	1.01	0.26
41–50	25.93	1.44	0.16
51–60	22.48	0.91	0.12
>60	21.27	1.46	0.10

1998) with Cd concentration in a cigarette dependent on the country of manufacture and brand, typically between 0.19 and 3.0 μg/g dry weight (Elinder et al., 1983) an average of about 10% of which is inhaled during smoking (Elinder et al., 1983).

It has been known for half a century that women may be more vulnerable to Cd than men. This is well illustrated by Cd induced *itai-itai* disease reported in Japan. In this case exposure to Cd was through consumption of rice contaminated by Cd which was emitted from upstream mines (Ogawa et al., 2004). *Itai-itai* disease caused severe pain to the affected people, resulting from fractures and distortions to the long bones of the skeleton (Järup et al., 1998), and most commonly affected elderly multiparous women, who, on a rice diet, and as a group, tended to have low calcium (Ca) status (Vahter et al., 2007a). This was a significant factor contributing to the development of the osteotoxic aspects of *itai-itai* disease, as Cd replaced Calcium in the bone tissue of the sufferers. In a study investigating comparative Cd levels in an Australian population, comprising 43 males and 18 females (Satarug et al., 2002), it was found that the average Cd level in the liver was 74% higher in females than in males. The same study found that the average liver Cd concentration in females was 100% higher than males of the same age.

Generally, concentrations of Cd in blood, urine and kidney are higher in women than in men (Vahter et al., 2007a; Bæcklund et al., 1999) especially in women of a younger age (Bæcklund et al., 1999). The absorption rate (Satarug et al., 2002) and retention (Vahter et al., 2007b) of Cd are also both higher in women than in men. Blood Cd levels tend to be elevated in people with low body iron stores (Järup et al., 1998). In mammalian cells the active cellular mechanisms utilised for absorbing cations have low discrimination capability between some of the divalent cations, including Fe^{2+} and Cd^{2+} (Gunshin et al., 1997). It is thought that depletion of iron through menstruation and associated iron deficiency results in increased absorption of the other divalent cations, including Cd, and contributes to the increased Cd concentration found in women. Furthermore, women with low body iron stores showed greater Cd adsorption than those with normal body iron stores (Vahter et al., 1996).

Studies on rats have clearly confirmed that gender and diet influence the level of Cd adsorption. Specifically, female rats retain more Cd than male rats (Kello et al., 1979). Vidal et al. stated that Cd in maternal blood does not vary with maternal age. However, other studies have found contrary results and have concluded that age is in fact a significant factor affecting absorption of Cd by the female body (Horiguchi et al., 2004). Cd absorption rate for females aged 20–39 was found to be 44%, this is nearly six times higher than typical Cd absorption rates found in older women aged 40–79 (Horiguchi et al., 2004). It was also found that when dietary calcium, iron and zinc levels were low, the Cd absorption rate tended to increase (Reeves and Chaney, 2001). During pregnancy the maternal gastrointestinal tract undergoes physiological and biochemical changes in order to expedite absorption of essential nutrients for the foetus (Leazer et al., 2002). The efficiency in uptake of dietary iron is increased by divalent metal transporter 1 (DMT1) and, as a consequence, the rate of uptake for non-essential divalent metal cations, such as Cd, is also accelerated (Leazer et al., 2002). The reasoning for this is that, as mentioned above, in mammalian cells the active cellular mechanism governing the absorption of dietary divalent cations does not appear to discriminate between the different cations (Gunshin et al., 1997).

The Cd which accumulates in the maternal internal environment has an adverse effect on the fetoplacental unit. The chorionic villus is undoubtedly important for the protection of the foetus from external bodies such as xenobiotics and toxic elements (Knipp et al., 1999). However, the placenta is only a partial barrier to Cd and Cd tends to accumulate in the placenta throughout the gestational period. A considerable amount of Cd is transferred to the foetus from the mother and this Cd is then accumulated in the new-born's liver and kidney (Nakamura et al., 2012b). A study on rats revealed that the concentration of Cd in the placenta increases in a dose dependent manner (Nakamura et al., 2012a). According to a study of 106 Swedish women, the concentration of Cd in the placenta is typically found to be higher than the Cd concentration in the blood. Cd concentrations in the placenta ranged from 10 to 170 nmol/kg whereas the range found in maternal blood was between 0.12 and 18 nmol/L (Osman et al., 2000). Women who smoked were found to have significantly higher Cd concentrations in the placenta ($p = 0.001$) and the blood ($p < 0.001$) (Osman et al., 2000). The percentage increase in Cd due to smoking was found to be 32% in the placenta (Kuhnert et al., 1982). However, it was also dependent on the number of cigarettes smoked.

The genome of the mature gametes of mammals is highly methylated when compared to that of somatic cells (Reik et al., 2001). During the pre-implantation development of early embryos the genome undergoes methylation reprogramming. However, some methylated imprinted genes and some repeated LINES (Long Interspersed Nuclear Elements) do not undergo this process (Reik et al., 2001). During this period, whereby methylation reprogramming occurs, it is more desirable to maintain an optimal internal maternal environment. If Cd concentration is high in the maternal body this could interfere with the demethylation and re-methylation process which occurs during pre-implantation.

Recent studies have reported a negative correlation between maternal Cd levels and the birth-weights of their neonates (Kippler et al., 2012a, 2012b; Ikeh-tawari et al., 2013, Menai et al., 2012). Cd concentrations in cord blood have also been negatively correlated with the length of the new-born child (Zhang et al., 2004; Dahaghin et al., 2010; Nishijo et al., 2004) and both maternal urinary and cord blood Cd levels have been negatively correlated with small head circumference of the infant (Kippler et al., 2012a; Lin et al., 2011). Sex-specific phenotypic differences found in infants linked to the maternal body burden of Cd have also been described in several studies (Kippler et al., 2012a, 2012b).

Prenatal Cd exposure represents a particular period of vulnerability because during early developmental periods the epigenome of the foetus is more pliable to change. Genome-wide epigenetic reprograming occurs at two main developmental stages: during gametogenesis, and in early embryogenesis (Inbar-Feigenberg et al., 2013), specifically during the pre-implantation period of the embryo (Reik et al., 2001). Soon after fertilization maternal and paternal pronuclei undergo genome-wide demethylation by passive and active mechanisms respectively and are then both re-methylated at the time of implantation (Reik et al., 2001). These processes are vulnerable to disruption from increased Cd levels in the maternal environment during the period of fertilization and implantation.

The placenta is an efficient but partial barrier for Cd (Sakamoto et al., 2013). According to the literature very little Cd is transferred across the placenta, and the placenta Cd level is about 10% of that

found in the maternal blood (Lin et al., 2011; Osman et al., 2000). However, Cd has the ability to be retained by the placenta and accumulate to relatively high levels (Ji et al., 2011). In a study on pregnant mice, 18 pregnant females were injected daily with 0.5 mg/kg $CdCl_2$ during days 12–17 gestation. Consequently the placental Cd level was increased 750 fold (from 1.55 ± 0.61 ng/g to 1160 ng/g) in Cd-treated mice compared to controls (Ji et al., 2011). Epigenetic mechanisms, in particular DNA methylation, have been proposed as a route through which Cd appears to interfere with foetal development. Several studies have investigated Cd-related DNA methylation in the foetus (Sanders et al., 2014; Boeke et al., 2012; Kippler et al., 2013; Mohanty et al., 2015; Vidal et al., 2015). Cd has been reported to cause both hypermethylation- (Kippler et al., 2013; Mohanty et al., 2015) and hypomethylation (Mohanty et al., 2015; Sanders et al., 2014; Kippler et al., 2013; Boeke et al., 2012), both impacting upon foetal growth. In a recent study (Everson et al., 2016) further evidence of Cd disrupting the placental function is provided whereby maternal Cd levels were inversely associated with expression levels of PCDHAC1 in the placenta and positively associated with an increased risk of having a small infant. Further studies are still required to investigate whether DNA methylation is the mechanism linking cadmium exposure and altered gene expression.

Females are more vulnerable to Cd accumulation and they are the child bearing gender therefore it is pertinent to explore the potential impact of Cd on foetal development. The aim of the this review is to summarize and provide a critical examination of reported DNA methylation changes potentially caused by Cd exposure, specifically in relation to maternal and foetal health.

2. Method

We conducted a bibliographic search on PubMed (National Library of Medicine, Bethesda, MD, USA). The literature was searched in order to identify original research relating to maternal Cd accumulation and its effect on the foetus. The time frame for inclusion of articles was those published between 1st of January 2006 and 31st of December 2016. The topic was then further refined to Cd and its effect on DNA methylation. Experiments on animal models were excluded and only experiments on humans were taken to review. The key words and their combinations used in the search were; cadmium, epigenetics, methylation, human, foetus, prenatal, in utero, and maternal. The aim of this review is to summarize and critically analyse the current state of knowledge on Cd induced differential methylation in mothers and their infants.

3. Results

Five original research papers met the inclusion criteria for this review. All articles concerned the human epigenome, specifically methylation, and the impact of maternal and prenatal cadmium exposure. The findings of these studies are summarised in Table 2 and discussed further below.

3.1. Cadmium exposure and the epigenome: exposure-associated patterns of DNA methylation in leukocytes from mother-baby pairs

This research focused on the relationship between Cd consumption during the pregnancy period and the level of methylation in CpG islands in 17 mother and baby pairs at birth (Sanders et al., 2014). Women who received prenatal care from health clinics were recruited for the study. Maternal blood samples and new-born cord blood samples were collected at delivery (Sanders et al., 2014). DNA was extracted and methylated CpG areas were located using the MethylCollector Ultra kit. Finally, the enriched DNA was amplified using the Genome Plex WGA3 genome amplification kit (Sanders et al., 2014). In this study the average level of Cd found in the maternal blood was less than 1.05 μg/L. Key findings from this research were that maternal DNA methylation was associated with maternal age (n = 596 genes) and that foetal gene specific DNA methylation levels were also associated with maternal age (n = 39 genes) as well as ethnicity (n = 949 genes) and infant gender (n = 176 genes) (Sanders et al., 2014). In total there were 61 differentially methylated genes associated with Cd levels identified in the new-born genome and 92 in the maternal genome. The majority of differentially methylated genes had increased promoter methylation (hypermethylation) associated with increased Cd levels in both new-born and maternal DNA (Sanders et al., 2014). Out of the 61 differentially methylated genes identified in the foetal genome, only one gene was hypomethylated in association with elevated Cd levels, similarly, in the maternal genome, only 11 of the 92 differentially methylated genes were hypomethylated in conjunction with elevated Cd concentrations. Furthermore, there was no overlap found between maternal and foetal Cd-associated genes (Sanders et al., 2014).

3.2. Infant sex-specific placental cadmium and DNA methylation associations

Mohanty et al. conducted research to investigate whether there was a sex specific association between placental Cd concentration and placental genome wide methylation (Mohanty et al., 2015). 24 mothers participated in the study. Placentas were collected at delivery and Cd levels were measured using Inductively Coupled Plasma Mass Spectrometry (ICP-MS). DNA was extracted, treated with bisulphite and the global methylation was measured using an Infinium Human Methylation 450K BeadChip (Mohanty et al., 2015).

The median placental Cd concentration was higher in female infants (5 ng/g) compared to male infants (2 ng/g) in wet weight. High placental Cd levels were associated with differential methylation. Among female infants all differentially methylated genes were hypomethylated. Three hypomethylated CpG islands were identified as ARL9, SIAH3 and HS3ST4 by individual probe analysis and one genomic region (region 86974674 to 86975244 on chromosome 7) by genomic region analysis, this region is associated with two genes; CROT and TP53TG1 (Mohanty et al., 2015).

In male infants both high and low placental Cd concentrations were associated with differential methylation. Three specific CpG sites were identified as differentially methylated; these sites were closest to the two genes MECOM and SALL1. Genomic region analysis found that high placental Cd levels were associated with hypomethylation of two genomic regions; one on chromosome 3 (region 169379554 to 169380078) and one on chromosome 8 (region 1792758 to 1792758). Two genes that are located in this region are MECOM and ARHGEF10 (Mohanty et al., 2015).

3.3. Maternal cadmium, iron and zinc levels, DNA methylation and birth weight

This experiment was conducted to study the association between maternal Cd levels during the early pregnancy period and the methylation of regulatory imprinted genes in new-born babies. 319 pregnant mothers participated in the study; the level of Cd was measured in the maternal blood at, or before, 12 weeks gestation. DNA was extracted from the umbilical cord blood and analysed using bisulphite pyrosequencing. The percentage methylation was analysed at multiple methylation sites for nine imprinted genes (5 maternally imprinted genes and 4 paternally imprinted genes). The results indicated that high blood Cd concentrations were associated

Table 2
Summary of research articles meeting inclusion criteria for review.

First author and year of published	Population size, age and ethnicity (human subject)	Cd exposure-	Type of methylation and markers used analysis	Major finding
Cadmium exposure and the epigenome: Exposure-associated patterns of DNA methylation in leukocytes from mother-baby pairs (Sanders et al., 2014)	17 mother and baby pairs from North Carolina Average maternal age was 28 and most mothers had more than one child.	<1.05 μg/L in maternal blood	Methylated cytosine – guanine (CpG) island recovery assay	New-born DNA methylation levels were associated with maternal age, race and infant sex. Identified Cd associated genes in foetal (61 genes) and maternal (92 genes) genome. No overlap between maternal and foetal genes.
Infant sex-specific placental cadmium and DNA methylation associations (Mohanty et al., 2015)	24 maternal-infant pairs	Median placental Cd – 5 ng/mg (wet weight) for female and 2 ng/g for male. Lower limit of detection is < 2 ng/g	Individual probe or CpG sites analysis and Genomic region analysis	Female infant Individual probe analysis - high placental Cd (≥5 ng/g) was associated with hypomethylation of three CpG sites closest to ARL9, SIAH3 and HS3ST4 genes. Genomic region analysis showed hypomethylation of one genomic region on chromosome 7 (region 86974674 to 86975244) Male infant Individual probe analysis- placental Cd caused differentially methylation of three CpG sites which are closest to two genes (MECOM and SALL1). Genomic region analysis - high Cd associated with hypomethylation of two genomic regions in chromosome 3 (region 169379554 to 169380078) and 8 (region 1792758 to 1792758).
Maternal cadmium, iron and zinc levels, DNA methylation and birth weight (Vidal et al., 2015)	319 mother and child pairs	Maternal blood Cd level- from 1.84 ng/g to 6.48 ng/g	Pyrosequencing of sodium bisulphite treated genome	Elevated maternal blood Cd levels were associated with lower birth weight and lower offspring methylation at PEG3 DMR in female and MEG3 and MEST DMRs in males.
Sex-specific effects of early life cadmium exposure on DNA methylation and implications for birth weight (Kippler et al., 2013)	127 mother-child pairs and 56 children at 4.5 years old	Maternal blood concentration in gestation week 14–0.38–5.4 μg/kg	Measured Cd concentration of maternal blood and urine of children using ICPMS. Global DNA methylation was analysed by infinium human methylation 450K beadchip in cord blood	Early life Cd exposure results to sex specific changes in DNA methylation. In boys 96% of the top 500 CpG sites showed positive relationship where girls were 29% positive. In girls over expression of differentially methylated genes were associated with embryonic and organ development (especially connective tissues and skeletal development) and morphology. In boys over expressed genes involved in cell death. In both girls and boys blood Cd was inversely correlated with birth weight.
Gestational intake of methyl donors and global LINE-1 DNA methylation in maternal and cord blood (Boeke et al., 2012)	516 individuals for periconceptional diet with first trimester analysis, second trimester and cord blood methylation 840 and 484 mother and infant pairs respectively.	Mean Cd intake is 15.4 (±3.9) μg/day assessed from FFQ	Measured percentage of methylated cytosine in Long Interspersed Nuclear element –1 (LINE-1) in first trimester and second trimester in maternal blood and cord blood at delivery.	Maternal Dietary Cd intake directly associated with first trimester methylation and inversely associated with cord blood LINE-1 global DNA methylation. Cord blood methylation is higher for male than female infants.

DMR-differentially methylated regions, DNMT- DNA methyl transferase.

with lower birth weight infants ($p = 0.03$). The level of Cd in the maternal blood did not vary significantly with maternal age, obesity, gestational age at delivery, pre-pregnancy obesity, or sex and birth weight of the offspring. However, the Cd level was significantly associated with ethnicity. The level of Cd was higher among Hispanic and African American women compared with Caucasian women ($p = 0.03$). Variation with respect to ethnicity and toxin induced methylation has been found in other epigenomic studies that have investigated maternal antidepressant use and cord blood methylation in new-borns (Viuff et al., 2016), and could be reflective of genetic variation governing drug and toxin metabolism. Maternal blood Cd concentration was significantly associated with differential methylation of the genomic region regulating the PEG3 gene ($p = 0.03$). The study found that three differentially methylated regions varied with respect to fetal gender, with PEG3 more specific to females ($p = 0.05$) and MEG3 ($p = 0.08$) and MEST ($p = 0.10$) specific to males (Vidal et al., 2015). Interestingly, the study also found that the association between maternal Cd and decreased DNA methylation of the PEG3 and PLAG1 genes was also stronger in offspring born to mothers with lower Fe levels.

3.4. Sex-specific effects of early life cadmium exposure on DNA methylation and implications for birth weight

This study evaluated the association between prenatal Cd exposure, DNA methylation and birth weight of new-borns and children (4.5 years old) using 127 mother-child pairs and 56 children aged 4.5 years from rural Bangladesh. The concentration of Cd was measured in maternal blood and from urine samples taken from the children. DNA was isolated from cord blood and childrens blood for global methylation analysis and was carried out using the Infinium HumanMethylation450K BeadChip (Kippler et al., 2013).

The Cd concentration in maternal blood at gestational week 14 ranged between 0.38 and 5.4 μg/kg. The results showed that Cd concentration in maternal blood was associated with CpG methylation in new-born DNA ($p < 10^{-16}$). The effect of Cd exposure on DNA methylation was more prominent in new-born boys than girls. The relationship between maternal blood Cd concentration and DNA methylation is slightly stronger in new-born boys ($p = 6.3 \times 10^{-7}$) than new-born girls ($p = 0.72 \times 10^{-7}$). Amongst the top 500 CpG sites analysed 96% of the CpG sites in boys showed a positive correlation with maternal Cd concentration whilst only 29% of CpG sites showed a positive relationship in new-born girls. In terms gene function of the CpG sites identified there were some overlap between girls and boys such as; cell morphology, cell cycle, cellular growth, and proliferation. However, there were also sex specific differences in regards to some functions; in females CpG sites identified related to genes involved in embryonic and organ development ($p = 1.5 \times 10^{-5}$). In males the strongest association was found with genes involving cell death ($p = 3.9 \times 10^{-6}$). Cd concentration in maternal blood and methylation of foetal DNA showed the strongest correlation, there was a similar relationship found between Cd concentration in the urine of the children (4.5 years old) and the methylation of CpG sites (Kippler et al., 2013).

3.5. Gestational intake of methyl donors and global LINE-1 DNA methylation in maternal and cord blood

This study focused on the impact of the maternal diet on DNA methylation, specifically nutrients containing methyl donors (betaine, choline, folate and vitamin B12) ingested periconceptionally and during the second trimester. The study used food frequency questionnaires to obtain data on the maternal intake of cadmium, iron, zinc, protein and vitamins E and C. The association between methylated cytosine levels in the Long Interspersed Nuclear Element-1 (LINE-1) and dietary intake was explored. This study found that periconceptional Cd intake was positively associated with first trimester methylation and inversely associated with cord blood methylation (Boeke et al., 2012).

4. Discussion

The association of prenatal Cd exposure and DNA methylation is demonstrated by several studies (Gona et al., 2015; Sanders et al., 2014; Mohanty et al., 2015; Vidal et al., 2015; Kippler et al., 2013). General findings from the five studies suggest that Cd levels in the maternal blood did not vary with respect to maternal age, pre-pregnancy obesity or gestational age at delivery. However, they did vary with respect to ethnicity (Vidal et al., 2015). The absorbed Cd partially crosses the placental barrier from mother to foetus and was associated with negative physiological effects to the foetus such as decreased head circumference, height and birth-weight (Lin et al., 2011).

4.1. Differential DNA methylation in foetal and maternal genome

All of the studies reviewed concluded that early life Cd exposure resulted in epigenetic dysregulation. However, different studies followed different DNA recovery methods, analysis techniques and were conducted under different Cd concentrations. The association between Cd levels and differential methylation of CpG islands was reported by two studies (Mohanty et al., 2015; Kippler et al., 2013). One study focused on the association between Cd concentration of maternal blood and DNA methylation of new-borns and the other one used median placental Cd concentration. The two concentrations were different from each other. However, both studies found sex-specific CpG methylation changes which have also been reported in other studies on rat foetuses (Castillo et al., 2012). In the study by Kippler et al. 96% of the top 500 CpG sites identified in new-born boys showed a positive relationship with maternal Cd exposure whilst in girls 71% of CpG sites were inversely associated with maternal Cd exposure. Animal studies have shown similar results under high Cd concentration (50 ppm); with male rat foetuses showing significant hypermethylation and female rat foetuses showing significant hypomethylation when compared to controls (Castillo et al., 2012).

De novo DNA methylation is essential for human embryogenesis and it is regulated by DNMT3a and DNMT3b. The activity of these two enzymes has been found to be increased in a dose dependent manner to Cd in cell culture experiments (Jiang et al., 2008). However, Doi et al. demonstrated Cd downregulated the expression level of DNMT3a and DNMT3b genes in chick embryo (Doi et al., 2011). Another study in rat liver cells in both in vitro and ex-vivo systems found that short term exposure (24 h) to Cd led to decreased DNMT activity and long term exposure (10 weeks) led to increased activity and methylation (Takiguchi et al., 2003). Several in vitro and ex vivo studies showed that chronic Cd exposure led to hypermethylation of DNA (Yuan et al., 2013; Zhang et al., 2013; Wang et al., 2012) and acute toxicity led to hypomethylation (Takiguchi et al., 2003). However, Hossain et al. found that low level environmental Cd exposure was associated with global hypomethylation in peripheral blood DNA (Hossain et al., 2012). More recently murine studies have found up regulation of both DNMT3B and DNMT3L in Cd-exposed placenta in conjunction with methylation and downregulation of GLUT3 and propose this as a potential mechanism for fetal growth restriction (Xu et al., 2016).

4.2. Differentially methylated genes and enriched function in maternal genome

The above results add evidence to suggest that Cd does induce

alterations to DNA methylation. The accumulated Cd in a woman's body affects both the mother and their in utero baby. Sanders et al. (2014) found 92 Cd associated differentially methylated genes. Out of the 92 genes 11 showed hypomethylation under higher Cd concentrations. Gene regulation and transcription, apoptosis and neuronal quality were identified as enriched biological functions of differentially methylated regions of maternal DNA. All these genes were significantly differentially methylated. Boeke et al. stated that maternal dietary intake of Cd during the first trimester period was directly associated with maternal LINE 1 methylation (Boeke et al., 2012). However, no data were provided on specific genes that were affected.

4.3. Differentially methylated genes and enriched function in foetal genes

Accumulated Cd in the maternal body could affect foetal health through differential methylation (Sanders et al., 2014; Mohanty et al., 2015; Vidal et al., 2015; Kippler et al., 2013; Boeke et al., 2012). The majority of research analysed found that increased Cd in the maternal body resulted in DNA hypomethylation (Sanders et al., 2014; Mohanty et al., 2015; Kippler et al., 2013; Boeke et al., 2012) however two studies found the converse in that it resulted in hypermethylation (Kippler et al., 2013; Mohanty et al., 2015). The enriched biological functions within differentially methylated regions were identified in two studies; however, the identified functions were different in both; those identified in the study by Sander et al. were predominantly gene regulation and transcription, adipose tissue quality, hyperplasia, lipid accumulation and apoptosis, where as those identified in the study by Kippler et al. were cell morphology, cell death, cell growth and cell proliferation. The Cd affected differentially methylated genes (gender specific and non-gender specific) identified by three of the studies reviewed (Kippler et al., 2013; Vidal et al., 2015; Mohanty et al., 2015) are summarised in Table 3. As identified through Uniprot, the putative functions of the genes named in the table below include metal ion binding, signal transduction, transcriptional regulation, neuropeptide signalling, apoptosis and mesoderm development. Both genes and associated putative function varied with respect to gender possibly reflecting the temporal developmental differences between male and female foetuses.

4.4. Conclusion

Cd is a heavy metal which can pose a serious risk to human health. DNA methylation is the most widely studied epigenetic mark. High levels of Cd accumulated in maternal blood and placenta has potential to impact upon methylation of the foetal genome. The research analysed in this review generally found that high maternal Cd levels resulted in hypomethylation of the fetal genome, however, some studies also identified hypermethylation occurred. The literature regarding maternal Cd exposure and the effect to the foetus is scarce and so it is challenging to conclude precisely what the effects are on the embryonic methylation process. It is also important to note that the majority of DNA methylation studies, including those reviewed here, focus on CpG methylation and not CpH (CpA, CpT and CpC) methylation contributing in particular to embryonic stem cell and brain tissue methylome (Jang et al., 2017) and could contribute to lack of correlation in some studies. Non-CpG methylation, although rare in differentiated cells, has also been found to have a regulatory role and a potential contributory role to disease progression, particularly neurological disease (Guo et al., 2014).

Different studies arrive at different conclusions; that Cd exposure increases methylation in a dose dependent manner (Jiang et al., 2008), high maternal Cd is associated with lower offspring methylation (Sanders et al., 2014), Cd associated methylation is fetal sex-dependent (Kippler et al., 2013; Mohanty et al., 2015), and that Cd is directly associated with first trimester methylation and negatively associated with LINE1 methylation (Boeke et al., 2012). A common theme to all the studies analysed is that women are more vulnerable to Cd, because of their higher rate of Cd absorption, especially during the pregnancy period. The existing experiments provide interesting data concerning maternal Cd concentration levels and the differential methylation of foetal DNA but there is insufficient data to confidently make conclusions, especially concerning the natural exposure doses of Cd. From the limited data available the impact associated with higher Cd intake potentially has a toxicoepigenomic effect to both mother and foetus and therefore urgently warrants further research.

Table 3
Cd affected differentially methylated genes (gender specific and non-gender specific).

Girls	Boys	Non gender specific
HDAC4	STK10	GAP43
KLHL29	STX7	C3orf23 (TCAIM)
SORCS2	CDH17	APBB3
RBM33	HRASLS2	HIST1H4L
SLC45A4	MYO1H	RAET1G
GPR123	TBCD	PTPRN2
ADAMTS8	CD70	PAX9
GTF2A1	MATK	SRP14
PEG3	MEG3	POLR2E
ARL9	MEST	NNAT
SIAH3	MECOM	
HS3ST4	SALL1	
CROT	ARHGEF10	
TP53TG1		

Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.fct.2017.09.002.

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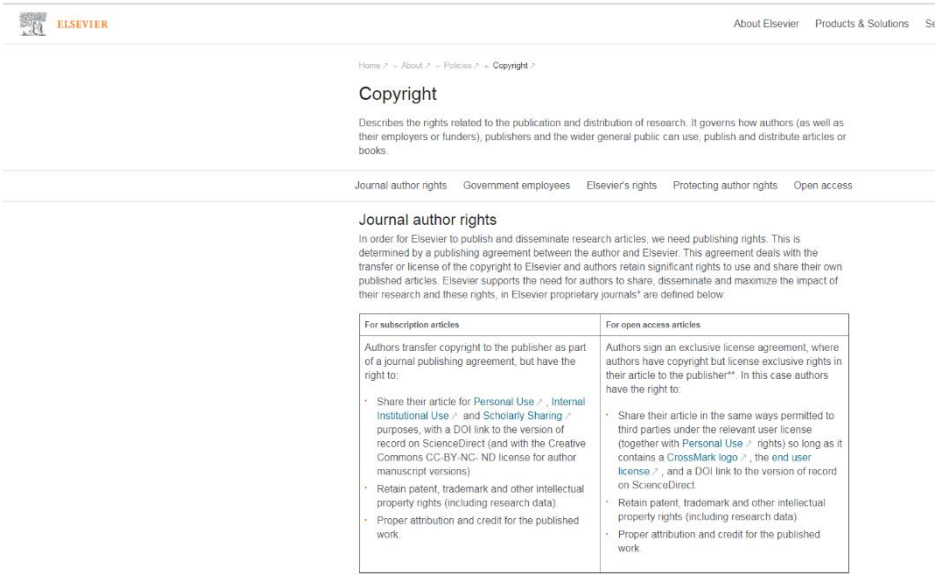

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Appendix 2: Appendices for Chapter 4

2.1 Generation one

Initial weights of worms and weights of worms of generation 1 in Control, 30 mgCd/kg, 90 mgCd/kg, 270 mgCd/kg after 28 days, 56 days and 84 days period. all weights are in grams.

	Initial				28 DAYS				56 DAYS				84 DAYS			
	Control	**30**	**90**	**270**	**Control**	**30**	**90**	**270**	**Control**	**30**	**90**	**270**	**Control**	**30**	**90**	**270**
1	0.25	0.20	0.18	0.28	0.33	0.32	0.17	0.15	0.40	0.43	0.24	0.12	0.52	0.30	0.16	0.04
2	0.26	0.39	0.33	0.37	0.41	0.26	0.16	0.14	0.50	0.35	0.36	0.11	0.42	0.22	0.16	Dead
3	0.34	0.36	0.28	0.33	0.32	0.35	0.11	0.12	0.59	0.50	0.33	0.15	0.59	0.30	0.18	Dead
4	0.22	0.29	0.22	0.28	0.34	0.42	0.27	0.23	0.57	0.37	0.28	0.11	0.47	0.32	0.23	Dead
5	0.31	0.27	0.26	0.32	0.31	0.26	0.18	0.10	0.34	0.31	0.36	0.11	0.57	0.32	0.23	Dead
6	0.41	0.34	0.16	0.23	0.40	0.29	0.18	0.19	0.46	0.38	0.29	0.08	0.45	0.22	0.28	-
7	0.39	0.20	0.24	0.28	0.30	0.35	0.12	0.15	0.42	0.30	0.32	0.12	0.44	0.28	0.16	-
8	0.32	0.22	0.19	0.26	0.38	0.29	0.17	0.11	0.37	0.35	0.41	0.07	0.32	0.44	0.19	-
9	0.26	0.22	0.23	0.37	0.19	0.33	0.26	0.17	0.33	0.41	0.24	0.08	0.32	-	-	-
10	0.23	0.27	0.25	0.26	0.24	0.30	0.19	0.22	0.48	0.26	0.39	0.09	0.46	-	-	-
11	0.24	0.22	0.23	0.25	0.30	0.35	0.27	0.21	0.41	0.28	0.53	0.11	0.30	-	-	-
12	0.26	0.14	0.28	0.27	0.26	0.32	0.34	0.14	0.56	0.33	0.39	0.10	0.31	-	-	-
13	0.39	0.24	0.40	0.33	0.26	0.36	0.24	0.19	0.27	0.32	0.20	0.13	0.36	-	-	-
14	0.34	0.15	0.34	0.24	0.44	0.29	0.25	0.15	0.31	0.40	0.31	0.09	0.25	-	-	-
15	0.19	0.13	0.25	0.23	0.34	0.22	0.26	0.20	0.31	0.27	0.48	0.09	0.29	-	-	-
16	0.29	0.23	0.24	0.30	0.30	0.31	0.30	0.16	0.49	0.38	0.34	0.15	0.21	-	-	-
17	0.19	0.21	0.39	0.36	0.26	0.30	0.14	0.16	0.34	0.34	0.12	0.09	0.35	-	-	-
18	0.28	0.29	0.31	0.31	0.27	0.27	0.16	0.12	0.29	0.36	0.26	0.13	0.32	-	-	-

19	0.24	0.20	0.27	0.38	0.29	0.35	0.21	0.21	0.30	0.38	0.26	Dead	-	-	-	-
20	0.20	0.26	0.26	0.28	0.38	0.34	0.24	0.18	0.34	0.41	0.51	Dead	-	-	-	-
21	0.28	0.28	0.31	0.21	0.35	0.26	0.33	0.11	0.37	0.40	0.45	Dead	-	-	-	-
22	0.27	0.28	0.37	0.22	0.20	0.29	0.26	0.11	0.33	0.20	0.39	Dead	-	-	-	-
23	0.32	0.23	0.40	0.37	0.19	0.23	0.17	0.09	0.35	0.20	0.34	Dead	-	-	-	-
24	0.20	0.25	0.22	0.22	0.33	0.34	0.14	0.22	0.43	0.32	0.17	Dead	-	-	-	-
25	0.25	0.36	0.39	0.30	0.29	0.33	0.22	0.13	0.38	0.21	0.21	-	-	-	-	-
26	0.27	0.24	0.28	0.22	0.29	0.34	0.25	0.15	0.26	0.30	0.25	-	-	-	-	-
27	0.29	0.24	0.33	0.24	0.31	0.29	0.26	0.19	0.42	0.27	Dead	-	-	-	-	-
28	0.35	0.23	0.28	0.33	0.28	0.30	0.32	0.15	Dead	Dead	Dead	-	-	-	-	-
29	0.26	0.21	0.38	0.25	0.28	0.34	0.22	0.09	-	-	-	-	-	-	-	-
30	-	0.29	0.32	0.25	0.29	0.28	0.26	0.14	-	-	-	-	-	-	-	-
31	-	0.22	0.26	0.23	-	0.22	0.15	0.13	-	-	-	-	-	-	-	-
32	-	0.22	0.33	0.22	-	0.18	0.21	0.16	-	-	-	-	-	-	-	-
33	-	0.31	0.34	0.29	-	0.17	0.23	0.11	-	-	-	-	-	-	-	-
34	-	0.15	0.23	0.35	-	0.29	0.22	0.10	-	-	-	-	-	-	-	-
35	-	0.28	0.33	0.24	-	0.30	0.32	0.12	-	-	-	-	-	-	-	-
36	-	0.14	0.30	0.39	-	0.32	0.26	0.16	-	-	-	-	-	-	-	-
37	-	0.19	0.28	0.26	-	0.27	0.27	Dead	-	-	-	-	-	-	-	-
38	-	0.12	0.43	0.29	-	0.38	0.26	Dead	-	-	-	-	-	-	-	-
39	-	0.32	0.37	-	-	0.24	-	Dead	-	-	-	-	-	-	-	-
40	-	0.18	-	-	-	-	-	Dead	-	-	-	-	-	-	-	-

Weight of worms in recovery test after 28 days. All weights are in grams

	84 DAY (Cd spiked soil)				84 DAY (Clean artificial soil)			
	Control	**30**	**90**	**270**	**Control**	**30-CS**	**90-CS**	**270-CS**
1	0.52	0.30	0.16	0.04	0.40	0.38	0.28	0.07
2	0.42	0.22	0.16	Dead	0.50	0.41	0.36	0.08
3	0.59	0.30	0.18	Dead	0.59	0.40	0.29	0.09
4	0.47	0.32	0.23	Dead	0.57	0.20	0.32	0.11
5	0.57	0.32	0.23	Dead	0.34	0.32	0.39	0.10
6	0.45	0.22	0.28	-	0.46	0.21	0.20	-
7	0.44	0.28	0.16	-	0.48	0.30	0.31	-
8	0.32	0.44	0.19	-	0.41	0.27	0.28	-
9	0.32	-	-	-	0.26	-	-	-
10	0.46	-	-	-	0.37	-	-	-
11	0.30	-	-	-	0.26	-	-	-
12	0.31	-	-	-	0.29	-	-	-
13	0.36	-	-	-	0.34	-	-	-
14	0.25	-	-	-	0.42	-	-	-
15	0.29	-	-	-	0.30	-	-	-
16	0.21	-	-	-	0.34	-	-	-
17	0.35	-	-	-	0.43	-	-	-
18	0.32	-	-	-	0.35	-	-	-

Cocoon production by worms in generation one during Cd exposure experiment

Sample	28 days	56 days
Control 1	10	54.3
Control 2	10	39.3
Control 3	5	38.6
30 (1)	9	31.9
30 (2)	12	13.4
30 (3)	8	18.7
30 (4)	4	26.9
90(1)	0	4.3
90(2)	8	18
90(3)	0	5.7
90(4)	0	10
270 (1)	0	0
270 (2)	0	0
272 (3)	0	0
273 (4)	0	0

Cocoon production by worms in Cd recovery experiment

After separation	Cocoon	Cocoons per 10 worms
Control (1)	8	13.3
Control (1)	5	12.5
Control (1)	5	6.25
30 (1)	2	5
30(2)	1	2.5
30 C(1)	6	15
30 C(2)	3	5
90(1)	1	2.5
90(2)	0	0
90C(1)	2	3.33
90C(2)	3	6
270(1)	0	0
270C(1)	0	0
270C(2)	0	0

Cd concentration (mg/kg) measured in E. fetida samples collected from generation one (Cd exposure and recovery test).

Sample	Time (days)	[Cd]
Control -1	28	0.93
Control -2	28	0.48
Control -3	28	0.52
Control -4	28	0.43
30 mgCd/kg - 1	28	0.65
30mgCd/kg - 2	28	0.51
30 mgCd/kg - 3	28	0.55
30 mgCd/kg - 4	28	0.48
90 mgCd/kg -1	28	0.28
90 mgCd/kg -2	28	0.41
90 mgCd/kg -3	28	0.52
90 mgCd/kg -4	28	0.46
270 mgCd/kg - 1	28	0.28
270 mgCd/kg - 2	28	0.52
270 mgCd/kg - 3	28	0.36
Control -1	56	0.01
Control -2	56	0.02
Control -3	56	0.01
30 mgCd/kg - 1	56	1.18
30mgCd/kg - 2	56	1.02
30 mgCd/kg - 3	56	1.13
90 mgCd/kg -1	56	1.81
90 mgCd/kg -2	56	2.02
90 mgCd/kg -3	56	1.63
270 mgCd/kg - 1	56	1.02
270 mgCd/kg - 2	56	1.52
Control -1	84	0.02
Control -2	84	0.02
Control -3	84	0.02
30 mgCd/kg - 1	84	1.54
30mgCd/kg - 2	84	1.55
90 mgCd/kg -1	84	1.54
90 mgCd/kg -2	84	1.93
30-Cs - 1	84	0.89
30-CS -2	84	0.91
90 -CS-1	84	1.19
90 -CS -2	84	1.20
270 -CS -1	84	0.86
270-CS -2	84	1.09

2.2 Generation 2

Initial weight and weights of worms in generation 2 in Control 30 mgCd/kg, 90 mgCd/kg, 30-CS and 90-CS after 28 days and 56 day of the experiment. All

Initial					28 days					56 days				
Control	**30-CS**	**30**	**90-CS**	**90**	**Control**	**30-CS**	**30**	**90-CS**	**90**	**Control**	**30-CS**	**30**	**90-CS**	**90**
0.38	0.47	0.29	0.15	0.28	0.48	0.38	0.38	0.28	0.19	0.45	0.56	0.36	0.15	0.19
0.32	0.46	0.24	0.34	0.31	0.39	0.29	0.42	0.23	0.23	0.55	0.39	0.37	0.35	0.14
0.32	0.38	0.23	0.18	0.31	0.38	0.37	0.40	0.33	0.19	0.39	0.48	0.61	0.23	0.24
0.30	0.47	0.31	0.23	0.23	0.35	0.37	0.33	0.18	0.25	0.53	0.57	0.40	0.39	0.27
0.34	0.37	0.46	0.19	0.23	0.47	0.37	0.41	0.29	0.31	0.62	0.48	0.48	0.49	0.08
0.49	0.33	0.36	0.08	0.08	0.25	0.32	0.32	0.29	0.04	0.42	0.50	0.43	0.34	0.24
0.35	0.28	0.58	0.40	0.45	0.36	0.38	0.37	0.26	0.06	0.33	0.51	0.46	0.22	0.33
0.37	0.39	0.54	0.39	0.16	0.34	0.31	0.21	0.17	0.19	0.56	0.40	0.39	0.37	Dead
0.31	0.30	0.31	0.12	0.06	0.39	0.33	0.22	0.10	Dead	0.25	0.50	0.43	0.35	-
0.45	0.24	0.29	-	-	0.43	0.32	0.26	-	-	0.32	0.54	Dead	-	-
0.38	0.27	0.16	-	-	0.35	0.46	0.38	-	-	0.48	0.47	0.50	-	-
0.29	0.34	0.40	-	-	0.44	0.37	0.20	-	-	0.55	0.47	0.29	-	-
0.39	0.36	0.29	-	-	0.30	0.43	0.26	-	-	0.48	0.38	0.38	-	-
0.40	0.48	0.42	-	-	0.29	0.42	0.29	-	-	0.35	0.36	0.49	-	-
0.45	0.21	0.27	-	-	0.37	0.36	0.27	-	-	0.47	0.58	0.55	-	-
0.28	0.32	0.21	-	-	0.36	0.33	0.38	-	-	0.40	0.46	0.52	-	-
0.35	0.24	0.09	-	-	0.35	0.36	0.28	-	-	0.44	0.54	0.46	-	-
0.35	0.30	0.25	-	-	0.29	0.35	0.30	-	-	0.49	0.22	0.47	-	-
0.28	0.28	0.18	-	-	0.42	0.33	0.28	-	-	0.32	0.25	0.29	-	-
0.38	0.34	0.20	-	-	0.35	0.41	0.37	-	-	0.19	0.28	0.35	-	-

weights are in grams.

Cocoon production Cd 2nd generation

Sample	Number of cocoon	
	28 days	**56 days**
Control 1	4	28
Control 2	6	27
30 (1)	2	6
31 (1)	1	4
30-CS (1)	9	57
30-CS (2)	9	55
90	0	0
90-CS	3	5

Cd concentration(mg/kg) in worm tissue of E.fetida in generation 2 Cd exposure experiments

Sample ID	**Time**	**Concentration in tissue**
G2-0d-Cont-1	0	5.48
G2-0d-Cont-2	0	4.74
G2-0d-Cont-3	0	3.09
G2-0d-30ppm-1	0	65.56
G2-0d-30ppm-2	0	86.86
G2-0d-30ppm-3	0	70.12
G2-0d-90ppm-1	0	113.11
G2-28d-Cont-1	28	2.01
G2-28d-Cont-2	28	2.61
G2-28d-30ppm-1	28	72.11
G2-28d-30ppm-2	28	96.28
G2-28d-90ppm-1	28	122.24
G2-28d-90ppm-2	28	133.40
G2-28d-30ppm-csoil-1	28	68.11
G2-28d-30ppm-csoil-2	28	71.61
G2-28d-90ppm-csoil-1	28	99.55
G2-56d-Cont-1	56	2.49
G2-56d-Cont-2	56	3.74
G2-56d-30ppm-1	56	124.75
G2-56d-30ppm-2	56	134.45
G2-56d-90ppm-1	56	178.83
G2-56d-90ppm-2	56	167.99
G2-56d-30ppm-csoil-1	56	44.08
G2-56d-30ppm-csoil-2	56	35.38
G2-56d-90ppm-csoil-1	56	89.14

2.3 Generation 3

Initial weight and weights of worms in generation three in Control 30 mgCd/kg and 30-CS after 28 days and 56 day of the experiment. All weights are in grams.

Initial			28 days			56 days		
Control	**30**	**30-CS**	**Control**	**30**	**30-CS**	**Control**	**30**	**30-CS**
0.25	0.31	0.43	0.32	0.39	0.26	0.57	0.33	0.42
0.34	0.24	0.30	0.31	0.22	0.30	0.55	0.44	0.56
0.29	0.26	0.16	0.36	0.24	0.15	0.61	0.32	0.56
0.21	0.28	0.38	0.32	0.30	0.37	0.57	0.36	0.59
0.32	0.28	0.23	0.29	0.21	0.39	0.40	0.38	0.75
0.34	0.14	0.32	0.35	0.12	0.34	0.46	0.38	0.43
0.26	0.26	0.38	0.22	0.36	0.35	0.50	0.53	0.50
0.24	0.14	0.17	0.40	0.24	0.34	0.48	0.40	0.42
0.26	0.32	0.13	0.28	Dead	0.24	0.42	0.34	0.60
0.35	0.32	0.26	0.29	Dead	0.38	0.52	0.39	0.55
0.25	0.25	0.17	0.22	0.31	0.28	0.54	0.44	0.54
0.25	0.23	0.34	0.28	0.25	0.29	0.43	0.34	0.50
0.23	0.26	0.41	0.24	0.32	0.32	0.55	0.55	0.47
0.23	0.35	0.20	0.21	0.22	0.39	0.54	-	0.49
0.23	0.33	0.29	0.21	0.24	0.31	0.45	-	0.42
0.19	0.19	0.20	0.34	0.23	0.31	-	-	-
0.20	0.17	0.22	0.19	0.40	0.18	-	-	-
0.20	0.25	0.19	0.22	0.24	0.19	-	-	-
0.21	0.37	0.15	0.31	0.29	0.18	-	-	-
0.24	0.12	0.17	0.24	0.24	0.17	-	-	-

Cocoon count

Concentration	Day 28	Day 56	Equivalent estimate cocoons for 10 worms
Control 1	5	15	21
Control 2	6	17	21.25
30-C1	8	38	54
30-C2	11	41	51
30-30(1)	3	6	10
30-30(2)	2	8	11.4

Cd concentration (mg/kg) in E. fetida in generation 3

Sample ID	Time (days)	Cd concentration mg/kg
G3-0d-Cont-1	0	1.22
G3-0d-Cont-2	0	1.29
G3-0d-Cont-3	0	22.20
G3-0d-30ppm-1	0	62.22
G3-0d-30ppm-2	0	61.14
G3-28d-Cont-1	28	3.30
G3-28d-Cont-2	28	3.37
G3-28d-Cont-3	28	2.34
G3-28d-30ppm-1	28	83.80
G3-28d-30ppm-2	28	72.17
G3-28d-30ppm-3	28	72.33
G3-28d-30ppm-csoil-1	28	52.81
G3-28d-30ppm-csoil-2	28	48.02
G3-28d-30ppm-csoil-3	28	42.61
G3-56d-Cont-1	56	1.74
G3-56d-Cont-2	56	2.20
G3-56d-Cont-3	56	2.09
G3-56d-30ppm-1	56	97.82
G3-56d-30ppm-2	56	104.09
G3-56d-30ppm-3	56	109.74
G3-56d-30ppm-csoil-1	56	27.11
G3-56d-30ppm-csoil-2	56	33.25
G3-56d-30ppm-csoil-3	56	28.72

Identities of named genes from the Generation 2 recovery experiment

Generation 2 recovery: Differentially expressed genes (minimum 15-fold different) after 56 days of recovery experiment.

Gene	Protein name	Gene ontology	Level of expression		
			Control	30-CS	90-CS
TRI56	E3 ubiquitin-protein ligase TRIM56	E3 ubiquitin-protein ligase that plays a key role in innate antiviral immunity, defence responsive to virus	297	0	0.2
BRICHOS	BRICD5	multi-pass membrane protein	652	0	0.8
YHU2	Uncharacterized threonine-rich GPI-anchored glycoprotein PJ4664.02	cell adhesion mediator activity	1018	0	0.3
RSPH1	Radial spoke head 1 homolog	axoneme assembly, meiotic cell cycle, spermatid development	357	0	0.6
PRAM	PML-RARA-regulated adapter molecule 1	lipid binding, protein kinase binding	439	45.4	0
TRP	Transient receptor potential protein		465	0	0.1
G3P	Glyceraldehyde-3-phosphate dehydrogenase	glucose metabolic process	283	0	0.7

The protein expressed by *TRI56* is responsible for play a key role in an antiviral immunity (Wang et al., 2011). The molecular function of YHU2 is cell adhesion mediator activity. This protein has ability to mediate adhesion of cell to the external substrate or another cell (UniProt, 2019e). The expression of gene *RSPH1* produces a protein called Radical spoke head 1 homolog which plays a major role in male meiosis. The sperm with more Radical spoke head 1 homolog protein are considered as greater quality (Li et al., 2019). The function of gene *PRAM* is not

identified properly yet. The gene might be involves in myeloid differentiation and integrin signalling in neutrophils. The molecular function of gene TRP is calcium channel activity. The protein produced by *G3P*is Glyceraldehyde-3-phosphate dehydrogenase which is involved with glucose metabolic process.

Out of 3103 10-fold differentially expressed genes there were 3 named genes which are related to reproduction and 18 genes related to growth. These are listed below.

Identified and named ≥10-fold differentially expressed genes after Generation 2 recovery period that were related to growth and reproduction

Reproduction	**Growth**	
MMP12	*XDH*	*XDH*
ACE	*ERAP1*	*PGM1*
MMP3	*MMP12*	*LMNA*
	FAT1	*MMP3*
	FBP1	*MUSK*
	LDHB	*BAG1*
	ACE	*COX17*
	DCLK1	*RAN*
	CHIA	
	HGF	

Identities of named genes from the Generation 3 exposure and recovery experiments

Generation 3 worm genes showing ≥ 15 fold differential gene expression after 56 days Cd exposure (30 mgCd/kg) and 26 d recovery

Gene symbol	Protein name	Putative function	level of expression Control	30 mgCd/kg	30-CS
CATC	Dipeptidyl peptidase 1	Dipeptidylpeptidase activity an act as both an exopeptidase and endopeptidase	515	0	293
ENT1	Epsin-1	Actin cortical patch assembly, actin filament organization and endocytosis	424	0	0
RSPH1	Radial spoke head 1 homolog Protein	Play an important role in male meiosis (axoneme assembly, meiotic cell cycle and spermatid development)	339	0	221
LRR2	PFF0380w	Defense response	2935	0	634
MMP12	Macrophage metalloelastase	Tissue injury and remodeling and metal(Zn and Ca) binding	392	0	21.1
TMPS9	Transmembrane protease serine 9	Serine-type endopeptidase activity	145.476	539.21	0

Among 6 genes there is one gene which is related to reproduction (*RSPH1*), which effects the ability of making viable eggs.The gene (*TMPS9*) which has serine-type endopeptidase activity.

Out of 3772 ≥10-fold differentially expressed genes, 290 had names. Of these 21 are related to growth and 3 to reproduction. These are listed below.

≥10-fold differentially expressed genes related to growth and reproduction in Generation 3 worms

Reproduction	Growth	
ACE	*ABCA2*	*KLHL6*
MMP3	*ACE*	*KLKB1*
MMP12	*Adk2*	*LDHB*
	BAG1	*LRP5*
	COX17	*MMP12*
	DCLK1	*MMP3*
	Egr1	*MUSK*
	FBP1	*PGM1*
	FKBP4	*PRDM1*
	HRH2	*RAN*
		TRPM3

Appendix 3: Appendices for Chapter 5

3.1 Generation 1

Initial weights of worms and weights of worms in generation 1 (Control, 10 mgAs/kg and 20mgAs/kg) after 28 days and 56 days. All weights are in grams.

	Initial			Day 28			Day 56		
	Control	**10 mgAs/kg**	**20 mgAs/kg**	**Control**	**10 mgAs/kg**	**20 mgAs/kg**	**Control**	**10 mgAs/kg**	**20 mgAs/kg**
1	0.342	0.402	0.3	0.42	0.212	0.315	0.352	0.54	0.438
2	0.358	0.392	0.219	0.461	0.369	0.419	0.452	0.396	0.39
3	0.256	0.372	0.356	0.455	0.291	0.509	0.422	0.589	0.475
4	0.384	0.343	0.328	0.514	0.313	0.4	0.475	0.336	0.551
5	0.459	0.263	0.296	0.422	0.417	0.346	0.306	0.338	0.736
6	0.364	0.332	0.211	0.333	0.42	0.289	0.396	0.387	0.445
7	0.225	0.33	0.382	0.358	0.177	0.392	0.431	0.136	0.478
8	0.267	0.362	0.27	0.392	0.122	0.345	0.326	0.505	0.294
9	0.356	0.333	0.258	0.433	0.355	0.557	0.412	0.453	0.557
10	0.398	0.263	0.393	0.189	0.074	0.236	0.382	0.508	0.449
11	0.325	0.328	0.413	0.457	0.542	0.2	0.407	0.35	0.412
12	0.386	0.31	0.383	0.313	0.43	0.48	0.422	0.444	0.59
13	0.214	0.352	0.284	0.294	0.361	0.515	0.375	0.759	0.402
14	0.265	0.443	0.275	0.4	0.311	0.192	0.388	0.482	0.297
15	0.225	0.365	0.211	0.494	0.397	0.301	0.298	0.35	0.447
16	0.426	0.389	0.234	0.33	0.385	0.437	0.457	0.644	0.479
17	0.248	0.276	0.315	0.437	0.345	0.427	0.342	0.513	0.617

18	0.456	0.317	0.178	0.347		0.318	0.453	0.385	0.573	0.423
19	0.238	0.314	0.31	0.534		0.428	0.437	0.297	0.511	0.625
20	0.367	0.17	0.306	0.142		0.479	0.193	0.388	0.595	0.453
21	0.325	0.264	0.371	0.383		0.453	0.355	0.267	0.31	0.565
22	0.235	0.24	0.238	0.345		0.423	0.557	0.438	0.411	0.41
23	0.382	0.419	0.487	0.489		0.431	0.35	0.489	0.451	0.553
24	0.286	0.348	0.259	0.33		0.385	0.359	0.324	0.483	0.426
25	0.452	0.266	0.139	0.263		0.321	0.675	0.346	0.352	0.523
26	0.258	0.324	0.228	0.344		0.289	0.349	0.305	0.644	0.385
27	0.375	0.281	0.336	0.301		0.327	0.442	0.356	0.334	0.467
28	0.372	0.39	0.245	0.232		0.411	0.193	0.366	0.35	0.424
29	0.364	0.424	0.232	0.331		0.266	0.413	-	Dead	0.313
30	0.286	0.353	0.234	0.343		0.202	0.204	-	-	0.399
31	0.328	0.277	0.407	0.267		0.304	0.463	-	-	0.443
32	0.245	0.37	0.314	0.362		0.387	0.473	-	-	-
33	0.357	0.324	0.333	0.439		0.298	0.434	-	-	-
34	0.428	0.227	0.406	0.248		0.374	0.435	-	-	-
35	0.328	0.211	0.337	0.402		0.453	0.527	-	-	-
36	0.372	0.183	0.269	0.248		0.329	0.412	-	-	-
37	0.354	0.228	0.289	0.317		0.291	0.338	-	-	-
38	0.254	0.339	0.24	0.34		0.277	0.252	-	-	-
39	0.324	0.273	0.213	0.499	Dead		0.33	-	-	-
40	0.341	0.154	0.242	0.368	Dead		0.408	-	-	-

Cocoon production of worms after 28 days and 56 days of As exposure

Sample	Time		
	Day 28	**Day 56**	**Equivalent estimate cocoons for 10 worms – 56 days**
control 1	6	13	16
control 2	8	17	21
control 3	10	11	14
control 4	5	14	18
10 (1)	0	22	24
10 (2)	0	19	31
10 (3)	2	34	56
10 (4)	6	30	42
20 (1)	0	41	59
20 (2)	0	42	60
20 (3)	0	54	67
20 (4)	0	44	49

Concentration of As(mg/kg) in E. fetida at the beginning of the experiment and after 28 and 56 days period of As exposure experiments

Sample	Time (days)	[As] ng/ml	Weights of worms	[Cd] mgAs/kg
G1 / Initial 1	0	4.73	0.178	1.328
G1 / Initial 2	0	3.48	0.211	0.825
G1 / Initial 3	0	3.64	0.257	0.708
As /1/28/C1	28	4.37	0.214	1.021
As /1/28/C2	28	2.79	0.247	0.564
As /1/28/C3	28	5.57	0.308	0.904
As /1/28/10 (1)	28	23.37	0.192	6.085
As /1/28/10(2)	28	24.19	0.303	3.991
As /1/28/10(3)	28	25.48	0.385	3.309
As /1/28/20(1)	28	18.74	0.175	5.355
As /1/28/20(2)	28	124.81	0.318	19.625
As /1/28/22(3)	28	43.23	0.184	11.747
As/1/56/C(1)	56	6.97	0.44	0.792
As/1/56/(C2)	56	4.98	0.146	1.705
As/1/56/C(3)	56	5.34	0.288	0.926
As/1/56/10(1)	56	93.25	0.353	13.209
As/1/56/10(2)	56	20.29	0.097	10.457
As/1/56/10(3)	56	29.04	0.108	13.443
As/1/56/20(1)	56	313.29	0.481	32.567
As/1/56/20(2)	56	2.86	0.365	outlier
As/1/56/20(3)	56	220.34	0.289	38.122

Initial weight of E. fetida and weight after 28 days of As recovery experiment. All weights are in grams

	Initial					Day 28				
	Control	**10 mgAs/kg**	**20 mgAs/kg**	**10-CS**	**20-CS**	**Control**	**10 mgAs/kg**	**20 mgAs/kg**	**10-CS**	**20-CS**
1	0.4	0.426	0.465	0.54	0.438	0.522	0.624	0.418	0.261	0.374
2	0.414	0.562	0.424	0.396	0.39	0.442	0.598	0.624	0.637	0.486
3	0.339	0.586	0.45	0.589	0.405	0.355	0.712	0.512	0.554	0.425
4	0.409	0.521	0.521	0.336	0.451	0.422	0.604	0.422	0.835	0.476
5	0.411	0.498	0.428	0.338	0.736	0.396	0.556	0.512	0.364	0.58
6	0.418	0.573	0.478	0.136	0.378	0.252	0.562	0.617	0.548	0.423
7	0.492	0.475	0.562	0.483	0.417	0.587	0.453	0.506	0.201	0.496
8	0.2628	0.486	0.511	0.644	0.424	0.319	0.621	0.361	0.173	0.672
9	0.573	0.644	0.447	0.508	0.294	0.468	0.711	0.393	0.342	0.502
10	0.367	0.513	0.479	0.35	0.557	0.46	0.604	0.306	0.377	0.516
11	0.3424	0.573	0.617	0.444	0.449	0.311	0.532	0.443	0.494	0.522
12	0.338	0.511	0.423	0.759	0.412	0.572	0.58	0.732	0.387	0.431
13	0.262	0.595	0.625	0.482	0.59	0.298	0.439	0.772	0.485	0.386
14	0.427	0.31	0.453	0.35	0.402	0.347	0.839	0.518	0.466	0.621
15	0.463	0.411	0.253	0.352	0.523	0.447	0.639	-	0.716	0.529
16	0.285	0.451	0.426	0.334	0.385	0.306	0.346	-	0.377	0.34

Cocoon production by E. fetida after 28 days in the As recovery experiment

Sample	**Number of cocoons**	
	Day 28	**Equivalent estimate cocoons for 10 worms**
control (1)	5	6.25
control (2)	4	5
10(1)	81	101.25
10(2)	78	97.5
20(1)	60	75
20(2)	65	81.25
10-CS (1)	31	38.75
10-CS (2)	22	27.5
20-CS (1)	25	31.25
20-CS (2)	20	25

As concentration in the metal extracts and concentration of As in the worm tissue

Sample	Time (days)	[As] ng/ml	Weights of worms	[As] in worm tissues mgAS/kg
As/1/84/C(1)	84	28.29	0.215	6.58
As/1/84/C(2)	84	25.39	0.135	9.40
As/1/84/C(3)	84	21.43	0.631	1.70
As/1/84/10(1)	84	303.10	0.47	32.24
As/1/84/10(2)	84	793.13	0.973	40.76
As/1/84/10(3)	84	404.65	0.569	35.56
As/1/84/20(1)	84	479.35	0.445	53.86
As/1/84/20(2)	84	407.56	0.457	44.59
As/1/84/20(3)	84	641.87	0.592	54.21
As/1/84/10-C(1)	84	337.18	0.546	30.88
As/1/84/10-C(2)	84	189.19	0.793	11.93
As/1/84/10-C(3)	84	192.26	0.385	24.97
As/1/84/20-C(1)	84	271.50	0.434	31.28
As/1/84/20-C(2)	84	128.96	0.453	14.23
As/1/84/20-C(3)	84	179.09	0.447	20.03

3.2 Generation 2

Initial Weights of E. fetida and weight after 28 and 56 days of As exposure. All weight are in grams

		Initial			Day 28			Day 56	
	Control	**10 mgAs/kg**	**20 mgAs/kg**	**Control**	**10 mgAs/kg**	**20 mgAs/kg**	**Control**	**10 mgAs/kg**	**20 mgAs/kg**
1	0.342	0.415	0.076	0.386	0.426	0.082	0.412	0.388	0.243
2	0.421	0.36	0.095	0.421	0.496	0.134	0.384	0.382	0.296
3	0.311	0.489	0.174	0.412	0.45	0.243	0.398	0.435	0.148
4	0.301	0.435	0.14	0.384	0.361	0.238	0.443	0.37	
5	0.359	0.265	0.191	0.356	0.52	0.127	0.418	0.359	
6	0.364	0.481	0.096	0.411	0.54	0.064	0.367	0.41	
7	0.352	0.368	0.081	0.298	0.391	Dead	0.347	0.315	
8	0.278	0.467	0.102	0.356	0.573	Dead	0.421	0.329	
9	0.325	0.305	0.241	0.478	0.422	Dead	0.478	0.336	
10	0.278	0.46	0.051	0.344	0.498	Dead	0.385	0.387	
11	0.277	0.32		0.365	0.296		0.358	0.346	
12	0.315	0.621		0.491	0.504		0.347	0.221	
13	0.292	0.541		0.345	0.423		0.388	0.469	
14	0.351	0.325		0.382	0.653			0.475	
15	0.362	0.422		0.312	0.486			0.579	
16	0.351	0.377		0.384	0.617				
17	0.345	0.316		0.401	0.326				
18	0.325	0.304		0.322	0.306				
19	0.381	0.466		0.301	0.328				
20	0.214	0.411		0.412	0.388				

Cocoon production by E. fetida in generation two after 28 and 56 days of As exposure

	Time		
Sample	**Day 28**	**Day 56**	**Equivalent estimate cocoons for 10 worms (56 days)**
control (1)	5	24	30
control (2)	7	21	26.3
10(1)	44	74	92.5
10(2)	32	59	84.3
20(1)	0	8	26.7

Concentration of As (mg/kg) in the extracted samples and in the worm tissues of E. fetida

Sample	Time (days)	[As] in the sample	weight of worm	[As] in worm tissue
As/2/Ini/C(1)	0	3.35	0.189	0.886
As/2/Ini/C(2)	0	20.87	0.287	3.636
As/2/Ini/C(3)	0	10.14	0.268	1.892
As/2/Ini/10(1)	0	45.56	0.25	9.112
As/2/Ini/10(2)	0	44.98	0.211	10.658
As/2/Ini/10(3)	0	65.83	0.297	11.083
As/2/Ini/20(1)	0	278.13	0.467	29.778
As/2/Ini/20(2)	0	46.65	0.108	21.595
As/2/28/C(1)	28	4.00	0.243	0.823
As/2/28/C(2)	28	7.11	0.315	1.129
As/2/28/C(3)	28	7.23	0.336	1.076
As/2/28/10(1)	28	317.65	0.317	50.103
As/2/28/10(2)	28	116.07	0.207	28.037
As/2/28/10(3)	28	317.42	0.445	35.665
As/2/28/20(1)	28	307.16	0.21	73.133
As/2/56/C(1)	56	4.00	0.269	0.743
As/2/56/C(2)	56	4.00	0.619	0.323
As/2/56/C(3)	56	4.00	0.363	0.551
As/2/56/10(1)	56	145.41	0.207	35.123
As/2/56/10(2)	56	481.84	0.395	60.993
As/2/56/10(3)	56	448.60	0.387	57.959
As/2/56/20(1)	56	116.23	0.163	35.654
As/2/56/20(2)	56	264.94	0.29	45.679

Appendix 4: Appendices for Chapter 6

Initial Weights of E. fetida and weights after 28 and 56 days of Co-exposure experiment

	Initial		28 days		56 days	
	Control	**Test**	**Control**	**Test**	**Control**	**Test**
1	0.224	0.241	0.249	0.249	0.388	0.172
2	0.153	0.241	0.271	0.158	0.3	0.296
3	0.222	0.184	0.299	0.267	0.304	0.241
4	0.213	0.253	0.243	0.158	0.419	0.182
5	0.162	0.214	0.553	0.151	0.384	0.287
6	0.262	0.159	0.344	0.237	0.363	0.289
7	0.346	0.15	0.371	0.24	0.495	0.184
8	0.215	0.222	0.35	0.248	0.348	0.139
9	0.187	0.243	0.4	0.167	0.397	0.255
10	0.176	0.171	0.27	0.212	0.291	0.36
11	0.184	0.206	0.393	0.135	0.25	0.322
12	0.199	0.196	0.27	0.225	0.4	0.265
13	0.16	0.194	0.398	0.163	0.354	0.168
14	0.212	0.156	0.274	0.176	0.441	0.274
15	0.208	0.186	0.351	0.209	0.371	0.336
16	0.222	0.227	0.288	0.246	0.4	0.209
17	0.228	0.247	0.325	0.249	0.45	0.236
18	0.208	0.185	0.328	0.233	0.372	0.321
19	0.24	0.201	0.279	0.226	0.29	0.278
20	0.222	0.176	0.314	0.185	0.302	0.28
21	0.298	0.19	0.288	0.233	0.434	0.117
22	0.151	0.21	0.298	0.147	0.591	0.254
23	0.192	0.192	0.372	0.179		0.156
24	0.276	0.243	0.324	0.262		0.352
25	0.198	0.215	0.266	0.223		0.143
26	0.195	0.168	0.293	0.283		
27	0.245	0.219	0.4	0.249		
28	0.152	0.229	0.381	0.148		
29	0.188	0.154	0.227	0.222		
30		0.183		0.253		

Cocoon production by E. fetida in co-exposure experiment

Sample	**Time**		
	Day 28	**Day 56**	**Equivalent estimate cocoons for 10 worms (56 days)**
Control (1)	4	22	27.5
Control (2)	4	36	45
Control (3)	4	32	35.5
Test (1)	0	0	0
Test (2)	0	0	0
Test (3)	0	0	0

Concentration of As and Cd in the worm tissues of E. fetidain Co –exposure experiment

Sample	Time (days)	As concentration	Cd concentration
G1 / Initial 1	0	1.328	4.214
G1 / Initial 2	0	0.824	4.621
G1 / Initial 3	0	0.708	4.028
Co-ex/28/C1	28	0.696	3.842
Co-ex/28/C2	28	0.980	6.163
Co-ex/28/C3	28	0.784	5.028
Co-ex/28/T1	28	10.18	89.92
Co-ex/28/T2	28	8.497	90.42
Co-ex/28/T3	28	18.30	63.32
Co-ex/56/C1	56	0.439	3.638
Co-ex/56/C2	56	0.467	5.171
Co-ex/56/T1	56	13.6	109.1
Co-ex/56/T2	56	9.39	109.8

Appendix 5: Appendices for methodology

Species identification

Sample number	Species	Matching percentage	Accession number
511	*E. fetida*	100%	AF212166
512	*E. andrei*	100%	KJ912190.1
513	*E. fetida*	100%	EU167736.1
514	*E. fetida*	100%	AF212166.1
516	*E. fetida*	100%	FJ214228.1
517	*E. andrei*	100%	KP422645.1
520	*E. fetida*	100%	JQ739935.1
521	*E. fetida*	100%	JQ739951.1
523	*E. fetida*	93.38	KJ911974.1
525	*E. fetida*	100%	EU167735.1
526	*E. fetida*	100%	Kj912310.1
528	*E. fetida*	99.34%	AF212166.1
529	*E. fetida*	100%	JQ739907.1
530	*E. fetida*	100%	MT126996.1
531	*E. fetida*	100%	KX651309.1
532	*E. andrei*	100%	KP420646.1
533	*E. fetida*	100%	KX651275.1
534	*E. fetida*	100%	FJ214228.1