
**THE EFFECTS OF LONG-TERM EXPOSURE TO CADMIUM ON
THE EARTHWORM *EISENIA FETIDA* (OLIGOCHAETA): AN
INVESTIGATION OF THE DEVELOPMENT OF GENETIC METAL
RESISTANCE**

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DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:

Date.....

ABSTRACT

The development of potential genetically based resistance to cadmium (Cd) after long-term exposure to this metal at a sublethal concentration, was investigated in earthworm specimens belonging to the genus *Eisenia*. Adult (clitellate) earthworms from a long-term laboratory Cd-exposed population (> 78 generations) and from other populations having no previous history of metal exposure were exposed to increasing concentrations (0; 2.5; 5; 10 and 20 mg/l) of Cd in the form of CdSO₄. Different biomarkers and molecular markers were used to determine whether the specimens from the long-term Cd-exposed population had acclimatized or adapted to the metal contaminated environment.

Acclimation was investigated at different physiological and biochemical levels using the following three biomarkers: the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT assay) measuring mitochondrial activity and cell viability; the single cell gel electrophoresis assay (comet assay) measuring DNA strand breaks and the biosynthesis of metallothioneins (MTs) that have the function of lowering metal toxicity. Earthworms from the long-term exposed substrate as well as specimens from populations not exposed to Cd were used.

Adaptation was investigated by looking at both allozyme polymorphism at seven enzyme encoding loci and DNA polymorphism using chosen neutral and selectable genetic markers. The markers used were cytochrome c oxydase subunit I (COI) and metallothionein-2 (*mt-2*) respectively. This was done amongst the same pool of populations. Additionally, the DNA polymorphism study also aimed at genetically identifying the species utilized in this study thereby determining whether these earthworms belong to the species *Eisenia fetida* or *Eisenia andrei*.

MTs biosynthesis did not generate the expected data mainly due to the fact that a suitable antibody could not be obtained (discussed in Chapter 5 section 5.3.3.). Similarly, the assessment of DNA polymorphism at the chosen selectable genetic marker *mt-2* did provide information relevant to understanding the potential development of resistance to Cd in the

long-term metal contaminated group. COI sequences generated in this study were compared to *E. fetida* and *E. andrei* COI sequences available on Genbank. Consequently, specimens used in this study were identified as possibly belonging to the species *E. andrei*.

Allozyme polymorphism revealed no fixed genetic differences between the long-term Cd exposed laboratory culture and the rest of the populations. All the populations departed from Hardy-Weinberg equilibrium ($\chi^2 > 5.9$; $p < 0.05$), and showed a low mean heterozygosity per locus ($H_o \leq 0.21$), probably due to inbreeding.

Cell viability and proliferation as tested by the MTT assay revealed that coelomocytes, isolated from the long-term Cd-exposed group showed the highest viability (98.42%) compared to those from other groups (+/- 80%). Kruskal-Wallis ANOVA ($H_{2, 225}=109.7165$ $p < 0.001$) revealed that the long-term Cd-exposed laboratory culture showed a better response to acute exposure to Cd, thus demonstrating that these worms have developed some kind of tolerance to Cd.

Similarly, the comet assay showed that in the long-term Cd-exposed specimens, less DNA breaks occurred after Cd exposure than in the unexposed groups. Of all the comet parameters assessed in this study (comet tail length, tail moment and tail DNA percentage), tail DNA percentage seemed to be more sensitive although all three parameters indicated that long-term Cd-exposed specimens were more resistant than unexposed specimens as shown by the number of single strand DNA breaks induced by exposure to higher concentrations of Cd ($p < 0.001$).

The comet and the MTT assays indicated that the earthworms with a previous history of Cd exposure have developed increased fitness towards higher doses of Cd, compared to previously unexposed groups. These findings mainly proved that several mechanisms could come into play at the physiological and biochemical level to allow the Cd exposed population to acclimatize to its chemically stressful environment. Clear genetic support for the differences found between the tested populations was not obtained, but needs to be investigated further using Cd selectable markers such as the *mt-2* gene, in order to come to a more conclusive deduction.

OPSOMMING

Die moontlike ontwikkeling van geneties gebaseerde weerstand teen kadmium (Cd) by erdwurms van die genus *Eisenia*, na langtermyn blootstelling aan die metaal by 'n subletale konsentrasie, is ondersoek. Volwasse (klitellate) erdwurms van 'n langtermyn laboratorium Cd-blootgestelde bevolking (< 78 generasies) asook van ander bevolkings met geen voorgeskiedenis van metaal blootstelling nie, is blootgestel aan 'n reeks van Cd konsentrasies (0; 2.5; 5; 10; en 20 mg/l) in die vorm van CdSO₄. Verskillende biomerkers en molekulêre merkers is gebruik om vas te stel of die erdwurms geakklimeer of aangepas het by die metaal in die gekontamineerde omgewing.

Akklimasie is op verskillende fisiologiese en biochemiese vlakke getoets deur die volgende drie biomerkers te gebruik: Die 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromied toets (MTT toets), wat mitochondriale aktiwiteit en sel lewensvatbaarheid meet; die enkel sel elektroforese toets (Komeettoets) wat DNS string breuke meet; die biosintese van metallotioniene (MT's) wat metaalvergiftiging verlaag. Erdwurms van die langtermyn blootstellingsubstraat asook eksemplare van bevolkings wat nie aan kadmium blootgestel was nie, is gebruik.

Aanpassing is ondersoek deur gebruik te maak van allosiem polimorfisme, waar 7 ensiem koderende lokusse gebruik is asook van DNS polimorfisme deur neutrale genetiese merkers te selekteer. Die merkers wat gebruik is, was sitochroom c oksidase, subeenheid I (COI) en metallotionien-2 (*mt-2*) respektiwelik. Hierdie toetse is met dieselfde groepe bevolkings uitgevoer. 'n Bykomende van die studie aspek waar ook van DNS polimorfisme gebruik gemaak is, was daarop gemik om die spesie wat vir die huidige studie gebruik is, geneties te identifiseer en om vas te stel of die erdwurms aan die spesie *Eisenia fetida* of *Eisenia andrei* behoort.

Die MT biosintese het nie die verwagte data gegenereer nie, hoofsaaklik weens die feit dat geskikte teenligaampies nie beskikbaar was nie (bespreek in Hoofstuk 5 afdeling 5.3.3). Soortgelyk, het die waardebevestiging van DNS polimorfisme by die geselekteerde genetiese merker *mt-2* nie genoegsame informasie verskaf om die potensiaal van die ontwikkeling van weerstand teen Cd in die langtermyn blootgestelde groep te verstaan nie. COI geenvolgordes van *E. fetida* en *E. andrei* wat op Genbank beskikbaar was, is gebruik om met die resultate van die huidige studie te vergelyk. Die spesie wat tydens die huidige studie gebruik is, is op hierdie wyse geïdentifiseer as *E. andrei*.

Alloisim polimorfisme het geen vaste genetiese verskille tussen die langtermyn blootgestelde laboratoriumkulture en die ander bevolkings getoon nie. Al die bevolkings het verskil van die Hardy-Weinberg ewewigstoestand ($\chi^2 > 5.9$; $p < 0.05$) en het 'n lae heterosigositeit per lokus getoon ($H_o \leq 0.21$), moontlik as gevolg van inteling.

Sellewensvatbaarheid en proliferasie soos getoets met die MTT toets, het getoon dat selomosiete, geïsoleer vanuit die langtermyn Cd blootgestelde groep, die hoogste lewensvatbaarheid (98.42%) gehad het in vergelyking met die ander groepe (+/- 80%). Kruksal-Wallis ANOVA ($H_{2, 225} = 109.7165$ $p < 0.001$) het getoon dat die langtermyn Cd blootgestelde laboratoriumkultuur 'n beter respons vir akute blootstelling aan Cd gehad het. Hierdeur is gedemonstreer dat hierdie wurms 'n soort toleransie teenoor kadmium ontwikkel het.

Soortgelyk het die komeettoets aangetoon dat daar in die langtermyn blootgestelde eksemplare minder DNS breuke voorgekom het na verdere blootstelling aan Cd as in die ander groepe. Van al die komeet parameters wat tydens die studie gemeet is (komeet stertlengte, Olive stert moment en stert DNS persentasie), het die stert DNS persentasie geblyk om die mees sensitiefste te wees, alhoewel al drie parameters aangetoon het dat die langtermyn Cd blootgestelde eksemplare meer weerstandbiedend was teen induksie van DNS enkelstring breuke weens Cd blootstelling ($p < 0.001$), as die wat nie voorheen blootgestel was nie.

Die komeet en MTT toetse het aangetoon dat erdwurms met 'n voorgeskiedenis van Cd blootstelling 'n toenemende fiksheid teen hoër dosisse van Cd ontwikkel het as die wat nie voorheen aan kadmium blootgestel was nie. My bevindings kon hoofsaaklik toon dat verskeie meganismes op die fisiologiese en biochemiese vlak 'n rol sou kon speel om Cd blootgestelde bevolkings by 'n chemies stresvolle omgewing te laat akklimeer. Duidelike ondersteuning vir die verskille tussen die getoetsde bevolkings deur van Cd geselekteerde merkers soos die *mt-2* geen gebruik te maak om 'n meer finale afleiding te kan maak is nie verkry nie en behoort verder ondersoek te word.

RÉSUMÉ

Le développement potentiel d'une résistance génétique au cadmium (Cd), après une exposition à long terme à une concentration sub-létale de ce métal en laboratoire, fut l'objet d'une étude sur des vers de terres appartenant au genre *Eisenia*. Des vers de terre adultes provenant d'une population longtemps exposée au Cd (> 78 générations) et d'autres populations n'ayant pas eu de contact antérieur avec des métaux, furent exposés à des concentrations croissantes (0; 2.5; 5; 10 et 20 mg/l) de Cd sous forme de CdSO₄. Différents bio-marqueurs et marqueurs moléculaires furent utilisés pour déterminer si les spécimens provenant de la population longtemps exposée au Cd s'étaient acclimatés ou plutôt adaptés au milieu contaminé par le métal.

Des évidences d'acclimatation furent recherchées à différents niveaux physiologiques et biochimiques en utilisant les trois bio-marqueurs suivants: Le 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide essai (essai de MTT) qui mesure l'activité des mitochondries et la viabilité cellulaire; l'essai d'électrophorèse de cellules isolées (l'essai des comètes) qui mesure la rupture des maillons d'ADN; la biosynthèse des metallothioneins (MTs) qui ont pour fonction de réduire la toxicité des métaux. Des spécimens provenant du milieu longtemps contaminé au Cd et ceux provenant de milieux neutres furent utilisés.

Des évidences d'adaptation furent recherchées en sondant à la fois le polymorphisme des allozymes à 7 loci codants et celui de l'ADN à deux marqueurs, l'un spécifique et l'autre non spécifique. Ces marqueurs furent respectivement, le cytochrome c oxydase sous unité I (COI) et le gène metallothionein-2 (*mt-2*). Ceci fut réalisé en utilisant le même groupe de populations. Parallèlement, l'étude du polymorphisme de l'ADN avait pour but d'aider à identifier génétiquement l'espèce de vers de terre utilisée dans cette étude déterminant ainsi si elle est de l'espèce *Eisenia fetida* ou *Eisenia andrei*.

La biosynthèse des MTs ne généra pas les données attendues essentiellement dû au fait qu'un anticorps approprié ne put être acquis (fait développé au Chapitre 5 section 5.3.3.). Similairement, l'estimation du polymorphisme de l'ADN au marqueur spécifique *mt-2*, ne fournit pas d'information permettant de comprendre le potentiel développement de la

résistance au Cd dans le groupe exposée à long terme. Les séquences de COI générées durant cette étude furent comparées à celles d'*E. fetida* et *E. andrei* disponibles sur Genbank. En conséquence, les spécimens utilisés dans cette étude furent identifiés comme pouvant appartenir à l'espèce *E. andrei*.

Le polymorphisme des allozymes ne révéla aucune différence génétique entre la population exposée à long terme au Cd et le reste des populations. Toutes les populations furent hors de l'équilibre de Hardy-Weinberg ($\chi^2 > 5.9$; $p < 0.05$), et démontrèrent des très basses moyennes d'hétérozygotie par locus ($H_o \leq 0.21$).

La viabilité et prolifération cellulaire testées par l'essai de MTT, montra que les cellules isolées du group longtemps exposé au Cd étaient plus viables (98.42%) que celles provenant des autres groupes (+/- 80%). L'ANOVA de Kruskal-Wallis ($H_{2, 225}=109.7165$ $p < 0.001$) révéla que la population de laboratoire longtemps exposée au Cd déploya la meilleure réponse face à une exposition aigue au Cd, prouvant ainsi que ces vers de terre doivent avoir développé une sorte de tolérance vis-à-vis du Cd.

Similairement l'essai des comètes révéla moins de ruptures d'ADN dans la population longtemps exposée au Cd, comparée aux populations jamais exposées avant. De tous les paramètres mesurés dans cette étude (longueur de la queue des comètes, moment de la queue et pourcentage d'ADN dans la queue), le pourcentage d'ADN dans la queue sembla plus sensible même si tous les trois paramètres indiquèrent, au nombre des ruptures d'ADN, que les spécimens provenant de la population longtemps exposée au Cd, étaient plus résistant que ceux des autres groups ($p < 0.001$).

L'essai des comètes et l'essai de MTT indiquèrent que les vers de terre ayant antérieurement été exposés au Cd ont développé une robustesse vis-à-vis des doses plus élevées de Cd. Ces résultats prouvent principalement que plusieurs mécanismes pourraient rentrer en jeu sur le plan physiologique et biochimique afin de permettre à la population longtemps exposée au Cd de s'acclimater à son environnement chimiquement éprouvant. Les différences enregistrées entre ces populations testées n'ont néanmoins eu aucun support génétique net. Il est par

conséquent recommandé que plus d'amples recherches soient menées en utilisant des marqueurs spécifiques tel que le *mt-2* afin d'arriver à des déductions plus conclusives.

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DEDICATION

Les prénoms des anges

Mère, j'ai écouté ce que disent les anges
Dans leur somptueux palais avant de s'endormir.
De biens doux murmures mais tout aussi étranges
Qu'on croirait entendre ton nom dans leurs soupirs.
Je sais qu'ils prient à Dieu de te garder la place
Chérie si convoitée près du trône, à Son ombre.
Ils t'ont écrit un chant qui égaie leur palace
Car déjà ils savent que tu es de leur nombre.
Dieu en te faisant te mise en cette terre.
Il désira longtemps que tu fus nommée mère
Avant de demeurer logée au firmament.
Car il y'a de beau dans les prénoms des anges
Le fait qu'ils les prennent aux refrains des louanges
Lorsque deux ou trois mots sonnent comme "maman".

Patrick Voua Otomo
19/12/2002 Stellenbosch

A toi mère, mon inspiration!
Avec amour.

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1. GENERAL INTRODUCTION

1.1. General pollution threats

It is estimated that the world human population will reach 8.2 billion by the year 2025 (Depledge 1992). This population growth will occur at the cost of an even bigger amount of chemical waste released into the environment. It is feared that the increase of pollution levels will have a detrimental impact on all components of the environment. According to Kale (2004), the repeated use of chemicals in tropical countries is negatively impacting on soil properties, productivity and fertility. Forbes & Forbes (1994) warned that pesticides and related organic compounds were the greatest and most pressing danger to marine life. Freshwater ecosystems are also threatened as a result of pollution due to industrialisation and urbanisation (Young 1997; Younger 2001 and Munafo *et al.* 2005). The imminent threat of high levels of environmental pollution has compelled scientists to endeavour to derive safe environmental concentrations for the most likely toxicants to be released into the environment (Posthuma *et al.* 2002). However, despite the increasing public awareness, it will take some time before the implementation of efficient waste-recycling policies becomes effective in many countries, especially in sub-Saharan Africa, Latin America and Asia, where 90% of the human population growth is expected to take place within the next two decades (Depledge 1992).

Several consequences can be predicted from the increasing levels of pollutants in the environment. Since different species are expected to respond differently to a substance at a given concentration (Posthuma *et al.* 2002), higher levels of toxicants in the environment might wipe out more sensitive species and promote the survival of more robust ones. This concern is being addressed in the species sensitivity distribution (SSD) concept, in which differences in sensitivity to a compound among test organisms can be used to infer a safe environmental concentration that can subsequently help to set an environmental quality criterion (Posthuma *et al.* 2002). However, more resistant natural populations might not react as sensitively as those not previously exposed and results of studies which include these populations might therefore be biased. This might in some instances trigger the outpouring of chemicals at levels which are higher than what can be handled by the environment. For instance in the agricultural industry more pesticides would be needed if pest control programmes become less efficient overtime because of more resistant agricultural pests. The possible extinction of certain species would inexorably cause the rise of localised or

generalised ecological disequilibria. Such a phenomenon occurred in Malaysia when the thatched roofs of some villages started collapsing after the spraying of DDT (Dichlorodiphenyltrichloroethane) as a means to control malaria (Connell *et al.* 1999). Non-target wasps that fed upon selected moth larvae were wiped out by the pollutant. Moth larvae that were more resistant to DDT, eventually swarmed and as they fed upon the thatch, the roofs collapsed. Moreover, as more sensitive individuals or species disappear, current ecotoxicological data might sooner or later become obsolete in predicting or assessing environmental problems in the field.

The genetic structure of impacted populations could also be affected by pollution through increasing mutation rates, causing directional selection on tolerant genotypes, bottleneck events, and by altering migration patterns (Van Straalen & Timmermans 2002). One detrimental consequence, after exposure to one or more pollutants, would be the loss of genetic diversity that could jeopardize the survival of affected populations or species during later exposure to more harmful compounds. Scientists are currently interested in finding out to which degree pollution could directly or indirectly play a role in these aspects and thus influence *evolution* (Medina 2002).

1.2. Heavy metals

The main pollution threats to marine, freshwater and terrestrial ecosystems are pesticides, litter, oil, domestic and industrial waste, and heavy metals (Forbes & Forbes 1994). Heavy metal pollution has become a great source of concern. Forbes & Forbes (1994) listed mercury (Hg), cadmium (Cd) and lead (Pb) as the most hazardous heavy metals to humans and ecosystems and emphasized the significant dangers that copper (Cu), zinc (Zn), silver (Ag) and chromium (Cr) can also pose. Heavy metals are byproducts of industrial activities and enter ecosystems through air, rivers and dumping (Forbes & Forbes, 1994). Nriagu & Pacyna (1988) estimated the portion of the total production per metal that is annually discarded as waste and eventually loaded into soils. For manganese (Mn), molybdenum (Mo), nickel (Ni), antimony (Sb) and vanadium (V), they assumed a 1-5% of wastage rate. For Cd, Cu, Pb, Cr and Zn, the rate was 5-10% and for Hg and selenium (Se), they estimated the wastage rate to be 10-15% of the total annual production. If these rates were still actual, for Cd which in 2003 a worldwide production of 16 900 metric tons was recorded (Plachy 2003), 845 to 1690 metric tons of Cd would have been dumped into the environment during that year. However, it seems that these rates have increased. Plachy (1997) states that approximately 2 600 tons of Cd are released into the ground each year through the use of fertilizers alone.

1.3. Cadmium

1.3.1. Production

Cadmium is mainly recovered as a byproduct of Zn smelting and refining (Plachy 1997). Natural Cd is bound to zinc in a concentrate of sphalerite (ZnS) and other sulphide ore minerals. During the purification of zinc, together with other metal residues, Cd precipitates at the bottom of the leaching tank. This impure Cd is subsequently purified to more than 99.9% purity. The four largest producers of Cd in 1997 (that accounted for 37% of the world production) were Belgium, Canada, China, and Japan (Plachy 2003). China and Japan are also among the biggest consumers of Cd. In the 1900s, together, they consumed more than half the total world Cd production (Plachy 2003). The United States Geological Survey has estimated from identified zinc resources (containing about 0.3% of Cd), reserves of up to 6 million metric tons of Cd (Plachy 1997).

1.3.2. Consumption

During the last 40 years, the human population has used eight times more Cd than ever before (Willuhn *et al.* 1996). Worldwide consumption of Cd for various uses in 2003 was as follows: batteries, 79%; pigments, 12%; coatings and plating, 7.5%; stabilizers for plastics, 1%; others, 0.5% (Plachy 2003). The use of Cd in rechargeable batteries has been increasing steadily in the last two decades. In the early 1990s approximately 55% of the Cd produced was used in the manufacture of Ni-Cd batteries. In 2003 the portion of Cd used in Ni-Cd batteries was near to 80% of the total consumption (Plachy 2003). Consumption rates, however, vary from one country to the other because of differences in environmental regulations. In Europe, OECD (Organisation for Economic Co-Operation and Development) country members are recording a decline in Cd consumption due to more stringent environmental regulations (OECD 1994; Plachy 1997).

1.3.3. Environmental issues

As described by the Environmental Protection Agency in the United States (EPA), Cd is a persistent, bioaccumulative, and toxic (PBT) pollutant (Plachy 2003). Plachy (1997) and Plachy (2003) listed four main environmental and human concerns regarding Cd. As close to 80% of the Cd used goes into the manufacturing of Ni-Cd batteries, concerns are raised around occupational exposure, manufacturing emission and wastes, product use, and product disposal. Of these concerns, product disposal gets the most attention as all the other issues are

fairly manageable (Plachy 1997; Plachy 2003). Four disposal options are available. They are composting, incineration, landfilling, and recycling. Of these options, recycling is highly promoted because Ni-Cd batteries are 100% recyclable (Plachy 1997). However, 75% of the Cd used in Ni-Cd batteries ends up in the hands of small consumers, which clearly limits recycling rates. When discarded into the environment, Cd is readily absorbed and accumulated in plants and animals (Siekierska & Urbanska-Jasik 2002).

1.3.4. Effects on organisms

In humans, Cd mainly affects the renal and respiratory systems and increases risks of bone fractures and of cancer developing (Nawrot *et al.* 2006). Cadmium and many of its derived compounds are listed as carcinogens by The International Agency for Research on Cancer (Plachy 1997). Cadmium has also been shown to be harmful to several organisms among which: plants (Carpena *et al.* 2003; Nouairi *et al.* 2006); insects (Cervera *et al.* 2004), amphibians (Loumbourdis *et al.* 1999) and rats (Lafuente & Esquifino 2002; Kim *et al.* 1998)

Oligochaetes show the ability to accumulate Cd and a number of studies have been done to investigate the effects of Cd on these organisms (Klerks & Bartholomew 1991; Morgan & Morgan 1999). Earthworms especially, feed on the organic fraction of the soil to which Cd preferably binds (Li & Shuman 1996). Several studies have been conducted on the physiological effects of Cd on various oligochaete species such as *Eisenia fetida*, *Eisenia andrei*, and *Dendrobaena veneta* (Bengtsson & Rundgren 1992; Spurgeon *et al.* 1994; Reinecke & Reinecke 1996; Reinecke *et al.* 1999). These studies have reported a spectrum of physiological and morphological changes that range from disturbance in water and homeostatic balance (Reinecke *et al.* 1999); changes in the ovarian structure (Siekierska & Urbanska-Jasik 2002), nephridial degeneration (Prinsloo 1999) to reduction in cocoon production and hatching (Spurgeon *et al.* 1994; Bengtsson & Rundgren 1992).

1.4. Experimental earthworms

1.4.1. The species used

Earthworms of the genus *Eisenia* occur naturally in northern Europe in places rich in organic matter (Lokke & Van Gestel 1998). However, because of their resilience and fairly wide temperature and moisture tolerance range, they have become ubiquitous with a worldwide distribution (Dominguez *et al.* 2005). The worms used for the present study were obtained from Europe as the species *E. fetida*.

According to Sims & Gerard (1985) the classification of *E. fetida* is as follows:

Phylum: Annelida

Subphylum: Clitellata

Class: Oligochaeta

Order: Haplotaxida

Suborder: Lumbricina

Superfamily: Lumbricoidea

Family: Lumbricidae

Subfamily: Lumbricinae

Genus: *Eisenia*

Species: *E. fetida* (Savigny 1826)

Various authors (Bundy *et al.* 2002; Dominguez *et al.* 2005 and Pérez-Losada *et al.* 2005) have suggested that the species complex *E. fetida/andrei* were unresolved. *E. fetida* Savigny 1826 and *E. andrei* Bouché 1972 were historically described as different species on the basis of their pigmentation (André 1963). Subsequently, Bouché (1972) designated them as subspecies, renaming them *E. foetida foetida* (for the current *E. fetida*) and *E. foetida unicolour* (for the current *E. andrei*). This was based on the fact that *E. fetida* has no pigmentation on the area around the intersegmental groove while *E. andrei* appears uniformly red (Reinecke & Viljoen 1991; Dominguez *et al.* 2005). Most recently, Pérez-Losada *et al.* (2005) employed two molecular markers cytochrome *c* oxydase subunits I (COI) and 28S subunits of the nuclear ribosomal RNA (28S rRNA) to resolve the taxonomy of this species complex. Based on their results, in combination with the morphological differences mentioned above, they proposed species status for *E. fetida* and *E. andrei*.

In the light of the recent taxonomic reclassification of the *E. fetida/andrei* group, it was not quite clear whether the earthworms in the laboratory cultures housed at the Ecotoxicology group at Stellenbosch University (hence those used for the long-term Cd exposure) were in fact of the species *E. fetida*. Although both species are quite often referred to as *E. fetida* in

the current literature (Dominguez *et al.* 2005 and references therein), it is important to have a clear understanding of the taxonomy of the species under investigation. Albani *et al.* (2003) and Dominguez *et al.* (2005) argued that because of metabolic differences between both *E. fetida* and *E. andrei*, these two species might respond differently to ecotoxicological testing and consequently lead to biased interpretations and recommendations. In the light of this, one of the aims of this project was to determine the correct classification for the species used in this study. To avoid confusion and for ease of presentation, the species employed in this study were initially referred to by the generic name *E. fetida* until the taxonomy was resolved (see Chapter 6, section 6.2.4.)

1.4.2. Background information on *E. fetida*

E. fetida is hermaphroditic and reproduces sexually. Within four days of mating, cocoon production begins (Venter & Reinecke 1988). After reaching sexual maturity, each worm may produce 2 to 5 cocoons weekly (Edwards & Bohlen 1992) which incubate for \pm 23 days before hatching (Venter & Reinecke 1988). Each cocoon produces \pm 3 hatchlings. The species takes 7 to 8 weeks to complete its life cycle (Venter & Reinecke 1988; Edwards & Bohlen 1992) and individuals may live up to five years (Reynolds 1977). Earthworms are important in soils, where they aid in the decomposition of organic matter, soil aeration, water transport and soil structure (Reinecke & Reinecke 2004).

E. fetida especially has gained international status for being one of the test organisms recommended by the Organisation for Economic Co-Operation and Development (OECD 1984; OECD 2000) for ecotoxicological testing. For the aquatic as well as the terrestrial environments, some oligochaetes seem to fulfil most criteria required for excellent test organisms (Landis & Yu 1995). Among many other invertebrates earthworms can also be used as bioindicators of pollution in the environment (Reinecke & Reinecke 2004). Because they are soft bodied and live in direct contact with their environment, earthworms are expected to respond faster than most organisms to relatively low concentrations of environmental pollutants in soil. These criteria make *E. fetida* a suitable organism to investigate the possible genetic effects of heavy metal pollution in general and Cd in particular. In addition, the species is easy to culture and handle.

In order to fulfil their role as test organisms, earthworms have to be relatively sensitive to the chemicals being tested (Reinecke *et al.* 1999; Spurgeon & Hopkin 2000). Localised variations in resistance among different populations may well cause some discrepancies in comparative

studies. The rise of field resistance due to pollution would mislead predictions and recommendations inferred from laboratory testing. Similarly, the use of a resistant laboratory culture for testing would also not reflect the field situation. Should field populations or laboratory cultures of *E. fetida* be able to develop genetically based resistance to certain toxicants over time, the value of laboratory tests results would be compromised.

1.5. Resistance

1.5.1. Definition

Resistance, according to Moriarty (1999), is a genetically based decrease in response of a population to a polluting agent as a result of previous exposure to that agent. According to Weis & Weis (1989) and Forbes & Forbes (1994), *resistance* also called *tolerance*, is an organism's ability to function successfully during exposure to an environmental stress such as a toxicant. The latter definition, however, does not take any genetic implications into account. Likewise, Landis & Yu (1995) defined resistance as the increased capacity of an organism that has been pre-exposed to an agent to resist the effect of later exposure to ordinarily lethal doses of the same agent. This response they termed *adaptation*. However, according to Klerks & Weis (1987), *adaptation* is a result of natural selection on genetically based individual variation in resistance whereas *acclimation* is an acquired degree of tolerance during exposure to sublethal concentrations of a pollutant. From these definitions it is apparent that terms like *resistance*, *tolerance* and *acclimation* have been used interchangeably in the literature. However, *genetic adaptation* or simply *adaptation* has become synonymous with changes in gene or genotype frequency in a population following exposure to a toxicant to increase the survivorship of the affected population (Klerks & Weis 1987; Donker 1991; Posthuma & Van Straalen 1993; Martinez & Levinton 1996; Belfiore & Anderson 2001).

Resistance to heavy metals, has been reported in several microorganisms, invertebrate and vertebrate species (Klerks & Lentz 1998; Shirley & Sibly 1999; Bruins *et al.* 2000; Kolok *et al.* 2002). In oligochaeta, metal resistance has been shown in species such as the benthic oligochaete *Limnodrilus hoffmeisteri* (Klerks & Levinton 1989) and in earthworms, e.g. *Dendrodrilus rubidus* (Langdon *et al.* 2001), *Lumbricus rubellus* (Langdon *et al.* 2003) and *E. fetida* (Reinecke *et al.* 1999). Several studies conducted in oligochaetes have suggested metal-binding proteins (Metallothioneins, MTs) to be at the basis of metal resistance in those species (Morgan *et al.* 1989; Klerks & Bartholomew 1991, Deeds & Klerks 1999; Gruber *et al.* 2000).

1.5.2. Development of resistance in *E. fetida*

To investigate the development of resistance in the earthworm *E. fetida*, Reinecke *et al.* (1999) used a biometric approach. They compared growth rates, cocoon production, hatchling success and survival between a long-term Cd exposed culture (after 3 years of exposure to 0.01% of CdSO₄) of *E. fetida* and a control group never exposed to Cd.

With regard to survival, results indicated that at a certain concentration (4000 µg g⁻¹ CdSO₄), 100% of control worms died whereas at the same concentration only 50% of the long-term exposed worms succumbed (an LC₅₀ of 4000µg g⁻¹). Biomass variations indicated that long-term exposed earthworms, maintained a higher body mass than earthworms from the control group, when exposed to the selected concentration range. However, with respect to cocoon production and hatchling success, the control group performed better than the long-term exposed group, denoting the negative effect of long-term exposure to Cd in the reproduction of earthworms (Reinecke & Reinecke 1996; Reinecke *et al.* 1999 and Siekierska & Urbanska-Jasik 2002).

These results indicated that earthworms exposed to sublethal concentrations of Cd in the laboratory for a number of years, showed a higher degree of tolerance (with respect to growth rate and survivorship) when exposed to higher concentrations of Cd. Reinecke *et al.* (1999) suggested that worms with a long-term history of Cd exposure might have developed resistance to Cd but stated that more research was needed to determine whether this “resistance” had a genetic basis.

Mechanisms involved in metal resistance in oligochaete species are not well understood. The question whether resistance to metal in oligochaetes is genetically based or not is being addressed by many authors (Martinez & Levington 1996; Spurgeon & Hopkin 2000; Langdon *et al.* 2003). Physiological means, such as metal binding proteins, have been suggested to be at the basis of metal resistance in oligochaetes (Morgan *et al.* 1989; Klerks & Bartholomew 1991, Deeds & Klerks 1999; Gruber *et al.* 2000). However, further investigations on diagnosed metal resistant populations or laboratory cultures are required to shed light on this matter.

1.6. Aims

The main aim of the present study was to investigate the effects of long-term exposure to Cd on the earthworm *E. fetida* with regard to the development of genetically based metal resistance. I formulated the null hypothesis that long-term exposure to Cd (12 years; \pm 78 generations) had not induced any genetic variation in a laboratory culture of *E. fetida*. A secondary aim was to use relevant DNA techniques to be able to establish whether either *E. fetida* or *E. andrei* was the species reared in our laboratory for more than a decade.

The specific objectives were fivefold:

1. To carry out biomarker studies in order to assess differences in biomarker responses between a long-term Cd-exposed laboratory culture and selected unexposed populations of *E. fetida*. The selected biomarkers were the MTT Assay and the Comet Assay
2. To monitor the synthesis of metallothionein proteins between a long-term Cd-exposed laboratory culture and selected unexposed populations of *E. fetida*, using the ELISA technique.
3. To determine if there was a difference in the genetic variation as measured by allozyme electrophoresis between a long-term Cd-exposed laboratory culture and selected unexposed populations of *E. fetida*.
4. To determine, using the *mt-2* gene whether differences in resistance to Cd among laboratory and control populations of *E. fetida* were the result of genetically based tolerance to Cd.
5. To determine, using DNA polymorphism, whether the taxonomic identity of the species that was obtained from Europe and reared and tested for more than a decade in our laboratory as *E. fetida* is correct.

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2 ASSESSING CELL VIABILITY AND MITOCHONDRIAL ACTIVITY USING THE MTT ASSAY

2.1. INTRODUCTION

Biomarkers are tests measuring the effects of stress factors on the sub-individual level in organisms (Svendsen & Weeks 1997). Different species or individuals show different levels of response at cellular level to stress factors such as the exposure to toxicants (Posthuma *et al.* 2002). It might therefore be possible, to differentiate between metal resistant and metal sensitive individuals by using certain biomarkers as there is a possibility that chronically contaminated individuals would respond differently to the same amount of chemical threat than newly exposed individuals at a sub-individual level. Van Gestel & Van Brummelen (1996) defined a biomarker as a biological response following exposure to a certain toxicant that is expressed below the individual level i.e. at the level of biochemical and physiological processes. Dallinger *et al.* (2000) gave the definition for a biomarker as “any molecular, biochemical, histological and physiological parameter at the sub-individual level that varies in response to an environmental pollutant”. It is a specific dose related response that provides early warning of any dysfunction subsequent to a chemical exposure.

Early physiological changes would first take place in the cell, then affect different tissues and organs and later the whole organism (Segner & Braunbeck 1997). Segner & Braunbeck (1997) argued that changes at the cellular level can eventually evolve into ecological changes. Cell viability is therefore important in the health and survival of organisms. As cell viability could be affected by toxicant exposure, the measurement of this parameter could be used as a biomarker (Segner & Braunbeck 1997). An important factor in cell survival is the energy provided by mitochondrial activity (Lodish *et al.* 1999). An unusually low mitochondrial activity may be an indication of cytotoxicity and might have an effect on cell viability (Lodish *et al.* 1999). A suitable method to measure this effect could be the methyl tetrazolium (MTT) as say that measures the survival and proliferation of cells by measuring their mitochondrial activity (Mosmann 1983)

The MTT assay was developed by Mosmann (1983) and can be used for measuring cytotoxicity, cell proliferation or activation by measuring mitochondrial activity. It is a colorimetric assay in which isolated living cells, in contact with the tetrazolium salt MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) cause the tetrazolium ring to

cleave. The coloured product that is produced (blue formazan) is contained within the cell membrane and therefore it accumulates in healthy cells (Fotakis & Timbrell 2006). The concentration of that product gives an estimation of cell survival and proliferation when measured spectrophotometrically. Because this process takes place when active, healthy mitochondria are present, it will only occur in living cells (Mosmann 1983), and could therefore be an indication of cell survival and (thus cytotoxicity) after treatment with a toxicant. The notion that mitochondria are the sites of MTT reduction came from Slater *et al.* (1963). They showed, working on rat liver homogenates, that MTT was reduced at two sites in the mitochondrial electron transport chain, in the presence of succinate as an electron donor. Berridge & Tan (1993), however working on bone marrow-derived cell line, 32D, investigated the sub-cellular localisation of MTT reduction using succinate, NADH, and NADPH as substrates. They concluded that most cellular reduction of MTT occurred extramitochondrially and perhaps involved NADH and NADPH. There is therefore evidence that MTT is reduced both intra and extramitochondrially. Nevertheless, because mitochondria are involved in the process, the MTT assay could be used to some extent as an indication of mitochondrial activity (Slater *et al.* 1963).

This technique has been used recently to assess the effect of the heavy metals copper, zinc and silver on digestive enzyme activities of the cuttlefish *Sepia officinalis* (Le Bihan *et al.* 2004). Of all the metals tested, zinc was found to be the most cytotoxic as it altered enzymatic functions of the cuttlefish digestive cells. Similarly, Seth *et al.* (2004) assessed Cu induced toxicity in the human hepatoma line, HepG2 using the MTT assay together with other biomarker tests and found the method to indicate effects. Fotakis & Timbrell (2006) also recently found the MTT to be a particularly sensitive assay in cases of heavy metal exposure in the hepatoma cell lines HepG2, giving better results than the lactate dehydrogenase leakage assay (LDH) and the neutral red (NRR) assay.

For oligochaetes, the MTT assay has been used to assess the cellular integrity of isolated chloragocytes of the earthworm *Lumbricus terrestris* before analysing enzymatic patterns of the same cells (Affar *et al.* 1998) and to monitor mitochondrial activity in isolated coelomocytes of the earthworm *E. fetida* in simulated micro gravity (Kauschke *et al.* 1997).

The aim of this part of the present study was to compare mitochondrial activity and cell viability between coelomocytes from a long-term Cd-exposed *E. fetida* culture and coelomocytes from other cultures and populations of the same species which had not been

pre-exposed to Cd, when exposed to a selected range of Cd concentrations using the MTT assay.

2.2. MATERIAL & METHODS

2.2.1. Experimental animals

Earthworms of the species *E. fetida* (Family Lumbricidae; Oligochaeta) were used as experimental animals. The original stock culture of *E. fetida* had been maintained in the ecotoxicology laboratory (University of Stellenbosch) since 1992. From this stock culture 200 clitellate (adult) worms were selected and divided (in July 1994) into two laboratory cultures. One culture was exposed to Cd (in the form of CdSO₄) at a concentration of 0.01%, mixed with fresh cattle manure and fed to the earthworms on a weekly basis. The other culture was kept as a control and fed weekly with clean fresh cattle manure. These two cultures were maintained in a climate control room with an ambient temperature of 20°C and a relative humidity (Rh) of 60%. With a life cycle of 7 to 8 weeks (Venter & Reinecke 1988), it has been estimated that the worms in both cultures will have undergone at least 78 generations of change by July 2006.

Additionally, a field population of *E. fetida* was collected from Middelvlei farm; a Cd free site (near the town of Stellenbosch, Western Cape, South Africa), This population was maintained, for the duration of the study, on uncontaminated cattle manure and kept under the same controlled laboratory conditions as the two long-term laboratory cultures.

2.2.2. The MTT Assay

Adult earthworms from the long-term exposed culture, the control laboratory culture and from Middelvlei farm (outdoor population), were exposed in artificial soil water (Kiewiet & Ma 1991) to a range of Cd concentrations (0, 2.5, 5, 10, and 20 mg/L) for 2 days as suggested by Maleri (2006). Five worms were exposed per treatment. Three replicates were performed for each exposure concentration. After exposure, coelomic cells were extracted from the worms by means of an extrusion solution (0,2 g EDTA in 76 ml PBS; 80 mg Guaiacol Gliserol Ether; 4 ml EtOH abs). Each animal was immersed in 1ml of the extrusion solution for 3 min in an Eppendorf tube. Thereafter, the animals were removed and the Eppendorf tubes were centrifuged at 2000 g for four minutes. After centrifugation, most of the supernatant, in each tube, was discarded and the pellet was suspended in PBS up to the 0.5 ml mark. Cell density and viability was assessed in cell suspensions using the trypan blue exclusion method. Cells

($2,5 - 3,5 \times 10^6$ cells/ml) were then transferred to microtiter (96 well) plates and the tetrazolium salt (MTT) was added to the medium. 50 μ L of the cell suspension and 50 μ L of the MTT colouring solution (2.5mg MTT in 5ml PBS) were added to the wells. The plates were incubated at room temperature in the dark for 2 hours before the addition of the MTT extraction buffer (20 μ L 70% HCl in 18ml Isopropanol, 10% Triton X, pH 4.7). After another 2 hour incubation period, absorbance was measured at 570nm using a multiwell scanning spectrophotometer (Multiskan[®] Ex, Thermo Electron Corporation). The reduction of the MTT into the blue formazan product measured, reflected the mitochondrial activity, thus cell viability.

Cell viability was estimated for the different populations using the following formula (Kim *et al.* 2003):

$$\text{Viability (\%)} = 100 \times \frac{\text{absorbance of treated samples}}{\text{absorbance of untreated (control) samples}}$$

2.3. RESULTS

In Fig. 1, it can be seen that there was no clear dose response relationship within each population group. Kruskal–Wallis ANOVA tests ($H_{14, 225}=128.54$, $p < 0.001$) revealed that most of the significant differences occurred between populations rather than within populations ($p \leq 0.049$).

At the 0 mg/L treatments (untreated samples), both the outdoor population (M) and the control culture (C) showed no difference between them. Both groups however differ statistically from the long-term Cd-exposed culture (E) at the same treatment ($p < 0.001$ and $p = 0.02$ respectively; Fig. 1).

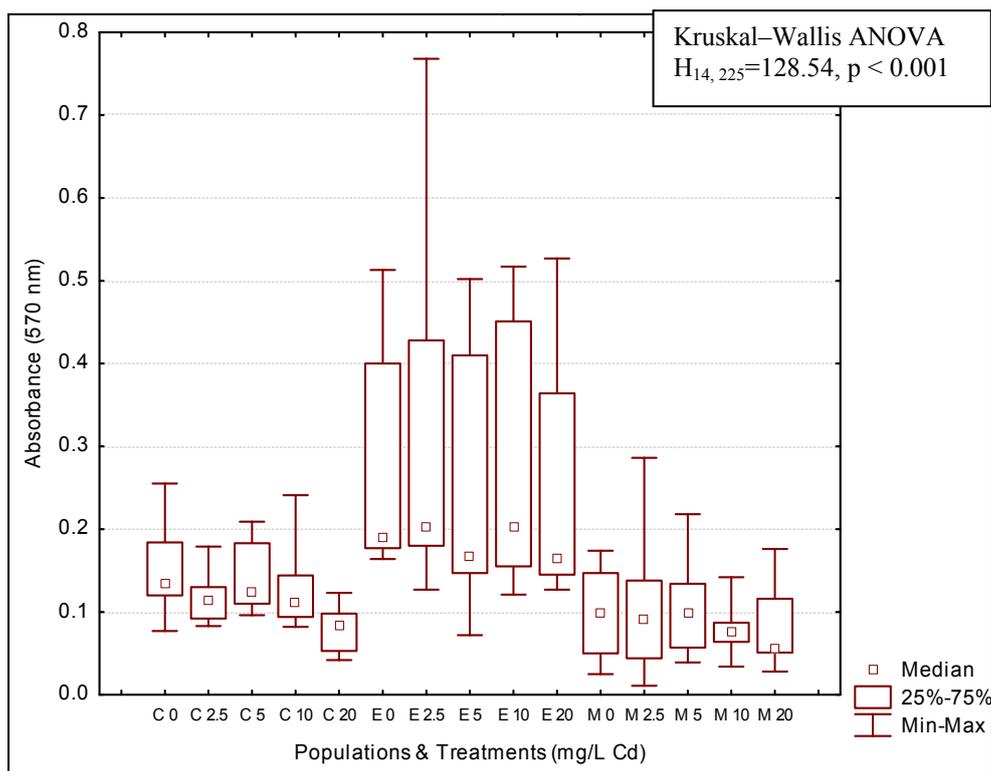


Fig. 1. Absorbance values indicating mitochondrial activity per treatment for all three groups of *E. fetida* (n = 225) after 48h exposure to CdSO₄. C = long-term Control culture, E = long-term Cd Exposed group, M = outdoor population (Middelvlei)

When absorbance values from all treatments within a population were added together, it was found that the three populations differed significantly from one another (Table 1). These absorbance values were the lowest in the outdoor population and the highest in the long-term Cd-exposed laboratory culture (Fig. 2).

Table 1. Multiple comparisons of p values after Kruskal-Wallis test ($H_{2, 225} = 109.7165, p < 0.001$) on all three populations of *E. fetida* after 48h exposure to CdSO₄, with all treatments added together

Populations	Laboratory control	Long-term Cd	Middelvlei
Laboratory control	-	< 0.001	0.002724
Long-term Cd		-	< 0.001
Middelvlei			-

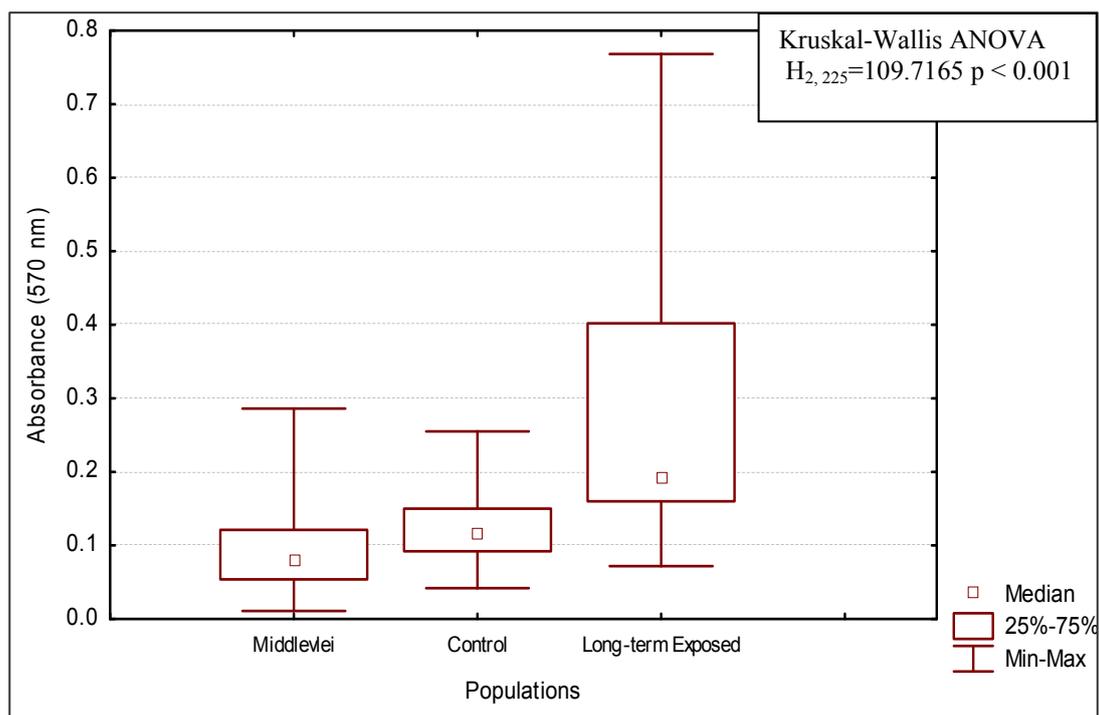


Fig. 2. Total absorbance per population of *E. fetida* after 48h exposure to CdSO₄. “Middelvlei” = outdoor population, “Control” = laboratory control, “Long-term Exposed” = pre-exposed to Cd. Total absorbance was calculated by adding together absorbance values for all treatments within each population.

In table 2, the median absorbance values of treated and untreated samples (used to estimate cell viability) are given.

Table 2. Median absorbance values of treated ($n_1 = 180$) and untreated ($n_2 = 45$) samples for each population of *E. fetida* after 48h exposure to CdSO₄. The untreated samples are the control treatments (0 mg/l) and the treated samples are all the other treatments (2.5, 5, 10, and 20 mg/L).

Populations	Median absorbance values	
	Untreated samples (0 mg/l)	Treated samples
Laboratory control	0.135	0.11
Long-term Cd	0.191	0.188
Middelvlei	0.098	0.079

Fig. 3 shows that the long-term Cd-exposed group had the highest cell viability (98.42%) while the outdoor population showed the lowest percentage of cell viability (80.61%). When comparing absorbance values at the untreated samples (0 mg/L) between the three populations, no statistical differences in absorbance were found between the outdoor population and the laboratory control ($p = 0.7$). Thus, the laboratory control’s cell viability (81.48%) was very close to the value found for the outdoor population (80.61%).

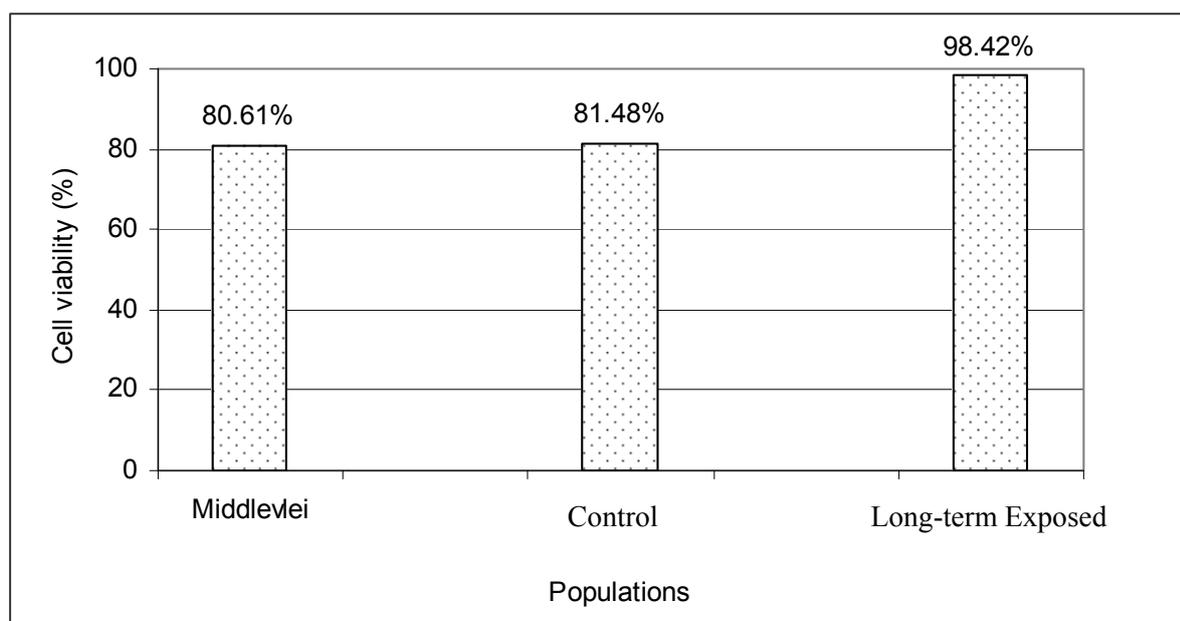


Fig. 3. Percentage cell viability per population of *E. fetida* after 48h exposure to CdSO₄. “Middelvlei” = outdoor population, “Control” = laboratory control, “Long-term exposed” = pre-exposed to Cd. Cell viability was estimated using the formula of Kim *et al.* (2003).

2.4. DISCUSSION

The outdoor population (Middelvlei) and the laboratory reared control culture showed an overall significant difference between them although both these populations did not have a history of previous exposure to Cd. Mitochondrial functions (in the outdoor population) could have been affected by factors other than Cd, such as temperature and food availability. Mitochondrial functions are known to vary for instance with temperature fluctuations (Brooks *et al.* 1971). Some of these factors might have differed amongst these two groups. This was seemingly the case because for more than a decade, the laboratory control culture was fed regularly and maintained in a climate control room with constant ambient temperature and relative humidity as mentioned above. The outdoor population might not have lived under such conditions.

At the control treatment (0 mg/L), both the outdoor population and the laboratory control culture showed no significant differences between them. This fact was reflected by the similarity in cell viability estimations found between these two groups (Fig. 3). Although these groups showed no difference when the control samples alone were compared, differences in absorbance did appear when the groups were exposed to Cd. This fact was reflected by the overall significant difference found between these two groups (Table 1). This might indicate that, without the effects of Cd, the long-term laboratory culture and the outdoor population might have responded similarly to the MTT. It can therefore be deduced that

exposure to Cd together with differences in factors such as food availability and weather conditions may have caused the significant difference found between the Middelvlei farm population and the long-term laboratory control culture.

The long-term Cd-exposed culture and the outdoor population differed significantly in terms of absorbance values at population level ($p < 0.001$) even though all three populations showed significantly different responses from one another (Table 1). Looking at the comparison between both their untreated samples (0 mg/L), the long-term Cd-exposed culture and the outdoor population already showed significant differences (Fig. 1). It might therefore be that a history of pre-exposure to Cd in the long-term Cd-exposed culture (to a greater extent than differences in factors such as food availability and weather conditions) may have caused the significant difference found between the Middelvlei farm population and the long-term Cd-exposed culture.

Because the long-term laboratory control culture and the long-term Cd-exposed culture were reared under the same controlled conditions (see section 2.2.1), the difference found between both these cultures (Table 1) could have been induced directly by Cd.

If we define resistance as “*the increased capacity of an organism that has been pre-exposed to an agent to resist the effect of later exposure to ordinarily lethal doses of the same agent*” (Landis & Yu 1995), then perhaps the long-term Cd exposed group is showing some attributes of that feature. In this present case, doses applied were not lethal. However the fact that the earthworms from long-term Cd exposed group had been pre-exposed to Cd before may have contributed to their increased ability to cope with a subsequent Cd exposure.

2.5. CONCLUSION

In the light of these findings, it seems possible that Cd could have caused physiological changes which could play a role in the development of resistance in the long-term Cd-exposed group. That group performed better than the other two groups, when exposed to the same concentration range of Cd, demonstrating a greater tolerance to Cd and even a greater chance of survival in case of future exposure to that heavy metal.

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3 INVESTIGATION OF CADMIUM GENOTOXICITY USING THE COMET ASSAY

3.1. INTRODUCTION

Deoxyribonucleic acid (DNA) is the cellular storehouse containing all the information necessary to build cells, tissues and organs in an organism (Lodish *et al.* 1999). It consists of two polynucleotide strands held together through hydrogen bonds to form a three-dimensional double helix structure. DNA finds stability not only through numerous hydrogen bonds but also through hydrophobic and Van der Waals interactions between adjacent nucleotides. Despite these bonds and interactions, the double helix is flexible about its long axis. The double helix can thus twist on itself, creating what is called supercoils. To ensure their survival organisms need to pass on their DNA between generations. For that reason DNA can be perfectly copied through a process called replication and passed on during reproduction.

DNA replication occurs in a semiconservative manner whereby each old polynucleotide strand from the original DNA is paired with a new strand “copied” from it following a complementary precise and regular base-pairing scheme controlled by DNA polymerases. However, during replication copying errors are introduced by DNA polymerases. Mutagenic chemicals such as ethyl methane sulfonate (EMS) and certain types of radiation such as UV and X-rays are also known to generate similar errors (Lodish *et al.* 1999; Shugart 2000). These errors are called mutations and if left uncorrected, can accumulate in cells to the point of causing detrimental dysfunctions (Shugart 2000). DNA mutations are often associated to cancer for all carcinogenic agents are known to be mutagenic i.e. they can cause changes in the DNA sequence (Lindahl 1993; Lodish *et al.* 1999). Fortunately DNA possesses several repair mechanisms that come into play depending on the type of mutation (Lindahl 1993). Proofreading by DNA polymerases corrects copying errors that occurs during replication. It is estimated that *E. coli* DNA polymerase III for instance introduces about 1 incorrect base in 10^4 inter nucleotide linkages during in vitro replication (Lodish *et al.* 1999). The mismatch repair-system repairs for instance, single base mispairs that could arise from errors during DNA replication. The end-joining repair of nonhomologous DNA mechanism repairs double strand breaks that are caused by ionizing radiation and even anticancer drugs such as bleomycin (Lodish *et al.* 1999).

Östling & Johanson (1984) developed an electrophoretic technique for the visualization and quantification of DNA damage in individual cells. The technique was called the single cell gel assay (SCG) or microgel electrophoresis (MGE). In their study, murine lymphoma cell line L5178Y-S and Chinese hamster fibroblast cells (CI-1) were irradiated, suspended in melted agarose, casted on microscope slides, lysed in a neutral detergent solution and electrophoresed.

Slides stained later with the fluorescent dye acridine orange revealed a green fluorescence in a microscope photometer indicating the presence of double-stranded DNA (Östling and Johanson 1984). The loose pieces of DNA, negatively charged, had migrated towards the anode of the electrophoretic field, further than the bulks of the DNA from the nuclei. The damaged cells hence had a comet-like appearance with their tail sections (formed by DNA fragments) giving an estimation of DNA damage. Östling & Johanson (1984) were themselves inspired by the work of Rydberg & Johanson (1978) that first quantified DNA damage in individual cells embedded in agarose and cast on microscope slides. However, while Rydberg & Johanson (1978) work under mild alkalic conditions, Östling & Johanson (1984) preferred working under neutral conditions. These choices directly affected the types of DNA damages (DNA breaks) that could be visualized. Neutral conditions for lysis and electrophoresis allowed the detection of double stranded DNA breaks but they left out single stranded ones (Singh *et al.* 1988). Consequently, Singh *et al.* (1988) improved these methods by mainly working under stronger alkaline conditions thereby allowing the visualization of both DNA break types after staining with ethidium bromide. Under these conditions, cellular RNA was degraded creating less interference in the quantification of actual DNA damage and therefore increasing method sensitivity (Singh *et al.* 1988). Besides its usefulness in the detection of DNA strand breaks following ionizing radiation, the Single Cell Gel Electrophoresis Assay (also known as the comet assay; because of the appearance of damaged cell under the microscope) has found relevance in the assessment of (1) excisable DNA damage (e.g. strand breaks produced by DNA repair mechanisms); (2) DNA interstrand crosslinks (lesions mainly caused by chemotherapeutic agents); (3) oxidative stress damage (from reactive oxygen species such as hydrogen peroxide); (4) cellular death by apoptosis and (5) genetic toxicology (Fairbairn *et al.* 1995; Collins 2004).

The comet assay is also a useful tool in ecotoxicology because various hazards encountered in the environment could cause DNA strand breaks. The level of genotoxicity caused by toxic substances can for example be assessed using this assay. Moreover, Bickhan & Smolen (1994) suggested that traditional approaches of environmental toxicology could benefit from

molecular genetics to help understanding the effects of pollutants on population genetics. The resulting merge would lead to the emergence of “evolutionary toxicology” that would deal with the effects of environmental toxins at DNA level (Bickhan & Smolen 1994).

According to Eastman & Barry (1992), few agents directly instigate DNA breaks but studies investigating the genotoxicity of various compounds are on the increase (Fairbairn *et al.* 1995; Belfiore & Anderson 2001) because most substances rather act as indirect genotoxic agents. Genotoxicity, according to Belfiore & Anderson (2001) is the alteration in genetic patterns such as genotype frequencies and genetic variability in a population, subsequent to the exposure to any contaminant. Shugart (2000) defines a genotoxicant as an environmental chemical and/or physical agent that is capable of interacting with and modifying DNA structure.

In terms of genotoxicity, Cd is regarded as an indirect genotoxic metal because it interferes with antioxidant defence mechanisms by promoting the production of reactive oxygen species (ROS) which subsequently alter gene expression and induce apoptosis (Risso-de Faverney *et al.* 2001 and Waisberg *et al.* 2003). Pruski & Dixon (2002) for instance found that Cd (0.2mg/l CdCl₂) alone is not genotoxic to *Mytilus edulis* (mussel) gill cells under acute or chronic exposure conditions. Cadmium nevertheless was found to enhance the genotoxicity of hydrogen peroxide (H₂O₂) which is a ROS, in the same mussel species by hindering DNA repair mechanisms (Pruski & Dixon 2002).

Halliwell & Aruoma (1991) listed two ways through which ROS can damage DNA and subsequently alter gene expression. The first way follows Fenton chemistry principles where hydroxyl radical (·OH) is formed after interaction between hydrogen peroxide (H₂O₂; from activated neutrophils and macrophages for instance) and metal ions chelates (such as Fe²⁺ or Cu⁺) already bound to DNA. The free radical (·OH) thereafter interacts with DNA to cause deoxyribose fragmentation, DNA base modification and DNA strand breakage (Halliwell & Aruoma 1991). In the second way, oxidative stress (caused by poor removal of H₂O₂ or O₂⁻ after phagocytosis for instance) causes the rise of intracellular free Ca²⁺ that subsequently activates endonuclease, leading to DNA fragmentation (Halliwell & Aruoma 1991).

Cadmium therefore might interact with either or both of these mechanisms since it can inhibit DNA repair processes (Waisberg *et al.* 2003 and Fatur *et al.* 2003; Hook and Lee, 2004). Other authors have nevertheless reported that Cd could directly be involved into DNA strand

breaks, which they found to happen in rat Leydig cells (Yang *et al.* 2003) and human lung fibroblasts cells (Mourón *et al.* 2001).

In oligochaetes, a number of studies have looked at the assessment of genotoxic pollutants using the comet assay (Verschaeve & Gilles 1995; Salagovic *et al.* 1996; Zang *et al.* 2000 and Reinecke & Reinecke 2004). With regard to heavy metal genotoxicity, Reinecke & Reinecke (2004) assessed the effect of nickel (Ni) in the earthworm *E. fetida* and reported the genotoxic potential of this metal. Their results also suggested that earthworms may be useful organisms for the assessment of heavy metal genotoxicity using the comet assay. The present study aimed at comparing the genotoxic capability of Cd between a long-term Cd-exposed culture and a long-term control culture of *E. fetida*, using the comet assay. We formulated the null hypothesis that Cd would not cause significant DNA damages in the individuals from the long-term Cd-exposed, as they have been proven to be physiologically more tolerant to Cd (Reinecke *et al.* 1999).

In order to assess the genotoxicity of Cd on the earthworm *E. fetida*, and seeking to find out whether the metal would act differently on two populations with different histories of Cd exposure, the comet assay was performed on individuals from both a long-term Cd-contaminated population and an uncontaminated control population.

3.2. MATERIAL & METHODS

Earthworms used during this study came from the set of cultures and populations of *E. fetida* described in chapter 2, section 2.2. For the purpose of this study however, only the long-term control and the long-term Cd exposed cultures were used.

To assess the genotoxicity of Cd, adult earthworms were exposed to increasing concentrations of Cd in artificial soil water (Kiewiet & Ma 1991). One litre of artificial soil water is made up to resemble groundwater by mixing 100mg NaHCO₃; 20mg KHCO₃; 200mg CaCl₂·2H₂O and 180mg MgSO₄ in a litre of distilled water (pH ± 8.2). Because of container size constrain and in order to provide enough oxygen for the exposed specimens, groups of five earthworms were exposed in that medium for 48 hours (OECD 1984) to 0, 2.5, 5, 10, and 20 mg/l of Cd in the form of CdSO₄ (Maleri 2006). Two replicates of the experiment were conducted. Additionally, 10 earthworms from both the long-term Cd-exposed culture and the long-term control culture were exposed (in groups of five) to a nickel treatment (20mg/l Ni in the form of NiSO₄) to serve as a positive control. Ni has been reported to show genotoxic capabilities (Reinecke & Reinecke 2004). After the 48 hour exposure period, coelomic cells were

extracted from the earthworms by means of an extrusion solution (0,2 g EDTA in 76 ml PBS; 80 mg Guaiacol Gliserol Ether; 4 ml EtOH abs) (Eyambe *et al.* 1991). Each animal was immersed in 1ml of the extrusion solution for 3 min in an Eppendorf tube. Thereafter, the animals were removed and the Eppendorf tubes were centrifuged at 2000 g for four minutes. After centrifugation, most of the supernatant, in each tube, was discarded and the pellet was suspended in PBS up to the 0.5ml mark. Cell density and viability was assessed in cell suspensions using the trypan blue exclusion method. Cells ($2,5 - 3,5 \times 10^6$ cells/ml) were then transferred to agarose coated microscope slides. The slides were first coated with a layer of 1% normal melting point agarose and they were dried in an oven at 60°C. Then 10µl of the prepared cell suspensions mixed with 0.5% low melting point agarose (70µl) were transferred onto the slides. The slides were covered with cover slips and kept on ice until that second layer of agarose had hardened. The cover slips were removed and another layer of low melting point agarose (75µl) was put on top. The slides were covered again and kept on ice until that last layer of agarose had hardened and the cover slips removed. For each animal, two slides were prepared. The uncovered slides were immersed in cold lysing solution (37.2g EDTA, 146.1g NaCl, 1.2g Tris and 8g NaOH in 890ml H₂O, completed just before use by adding 10ml of Triton X-100 and 100ml DMSO, pH 10) and left overnight at 4°C. Thereafter the slides were washed with distilled water and immersed in electrophoresis buffer (3% NaOH, 05% of EDTA in distilled water, pH >13) and left for 20 minutes for unwinding of the DNA strands to take place. Then, electrophoresis was carried out for 10 min at 25V (300mA). After electrophoresis, the slides were washed with distilled water and with a neutralization buffer (48.5g of 0.4M Tris in 1000ml of H₂O, pH 7.5). The slides were then stored away from light and dust, in slide boxes until staining and scoring were carried out. Slides were stained with $\pm 100\mu\text{L}$ of ethidium bromide (20µg/ml) and covered with a cover slip before visualisation. Visualisation of DNA damage was done under a Leitz Diaplan fluorescent microscope (Ploemopak 2.3, excitation filter 515-650nm, barrier filter 580nm). 50 cells were scored per slide, therefore a total of 100 cells per animal. The comets were assessed using two separate software packages which were IM50 V1.20 (Leica Microsystems AG, Heerbrugg, Switzerland) and CASP (Konca *et al.* 2003). The first, helped to take pictures of the cells on microscope slides and the latter was used to measure various comet parameters such as comet tail length (µm), tail DNA percentage and tail moment (which is a product of the first two parameters). Generated data were tested for normality using the Lillifors test and the homogeneity of variances was assessed using the Levene test (Statsoft 2004). Statistical analyses were carried out using the software Statistica 7 (Statsoft 2004). The level of significance was $p < 0.05$.

3.3. RESULTS

The three parameters measured (tail DNA percentage, tail length and tail moment) during the present study using the comet assay did not all display increasing damage as Cd concentrations increased. Within population analyses revealed one dose response relationship within the control population when the parameter comet tail length was considered (Fig. 4a). Within the same group, tail DNA percentage and tail moment reached their maximum values in the 5 mg/l treatment and thereafter decreased as the concentration of Cd increased (Fig. 4b & c). In this control population, nickel (Ni) genotoxicity varied depending on the parameter under investigation. Looking at tail length, Ni caused the highest effect and differed significantly from all the other treatments ($p \approx 0$; Fig. 4a). For the other two parameters, Ni, did not cause the highest genotoxic effect (Fig. 4b & c). For all the parameters, the control treatment (0 mg/l) showed significantly less damage ($p < 0.001$) compared to all the other treatments.

Within the long-term Cd-exposed culture, for all three parameters assessed, Cd genotoxicity reached its peak in the 2.5 mg/l treatment and thereafter remained constant (Fig. 5). However, none of the Cd treatments showed statistically significant differences amongst themselves. The control treatment (0 mg/l) differed significantly ($p \approx 0$) from all the other treatments and showed less damage. In contrast, the positive control treatment (exposed to nickel) displayed the highest effect for all the parameters assessed and differed significantly from all other treatments for all three parameters ($p \approx 0$; Fig. 5).

A comparison of the different treatments, using all three parameters, amongst the two populations revealed that individuals from the control culture generally suffered more DNA damage than their counterpart from the long-term Cd-exposed (Fig. 6). In the negative control sample (0 mg/l), tail length did not show statistically significant differences between the two populations whereas tail DNA percentage and tail moment revealed that background DNA damage were already significantly higher at 0 mg/l in the control population as compared to 0 mg/l in the long-term exposed group ($p < 0.001$ and $p < 0.02$ respectively; Fig. 6).

The response to Ni between the two populations and at all parameters also revealed that individuals from the control culture statistically endured more Ni damage than their counterpart from the long-term Cd-exposed group ($p < 0.001$; Fig. 7).

When whole population responses were considered, i.e. when responses from all treatments were pooled with respect to the parameters under investigation, the control culture

consistently showed statistically higher DNA damage than the long-term Cd-exposed culture ($p \approx 0$; Fig. 8).

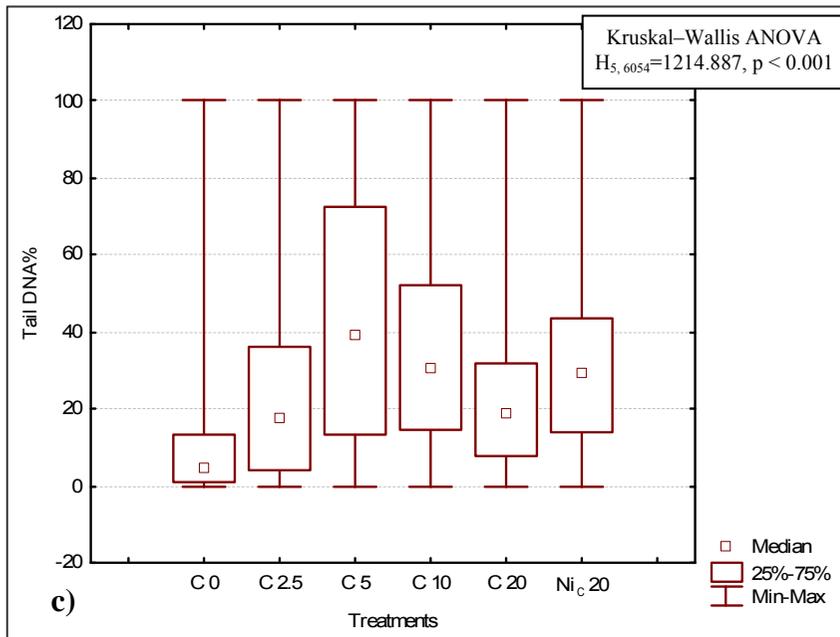
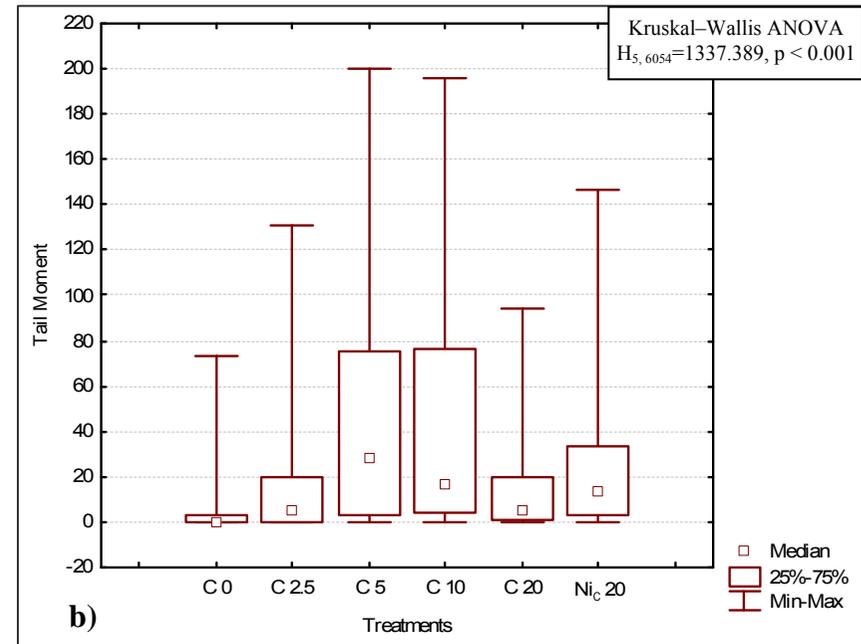
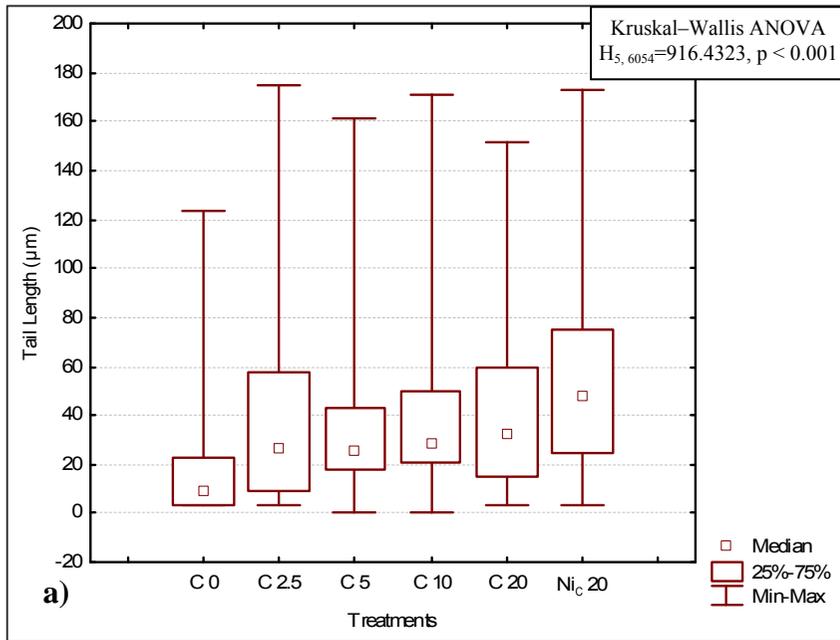


Fig. 4. Comet assay analyses within the long-term control culture of *E. fetida* (number of nuclei scored = 6054) after 48h exposure to CdSO₄, using three different parameters: a) tail length (µm); b) tail moment; and c) tail DNA percentage. On X-axis C = long-term Control culture and numbers indicate respective Cd concentrations (mg/l). Ni_c 20 = Nickel positive control treatment (20 mg/l).

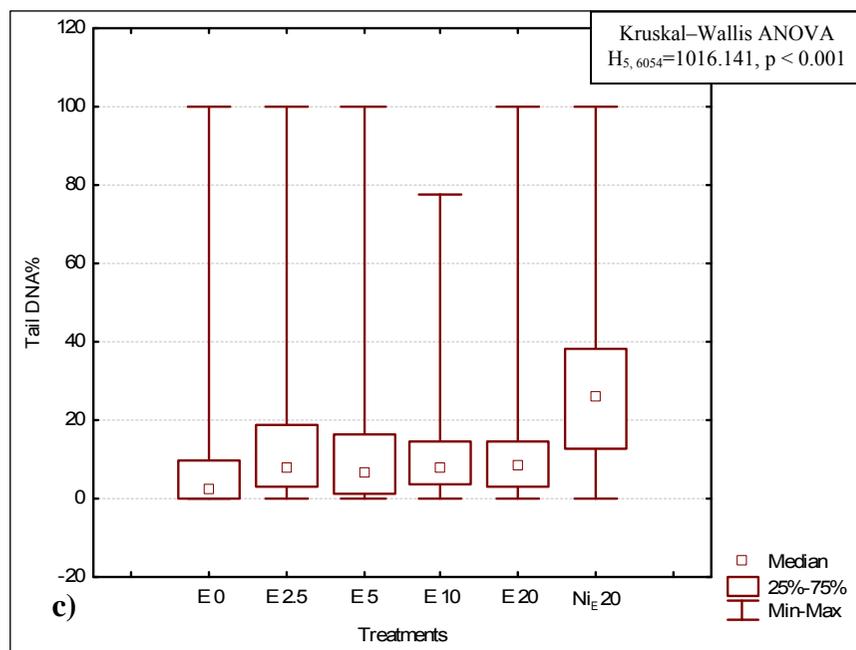
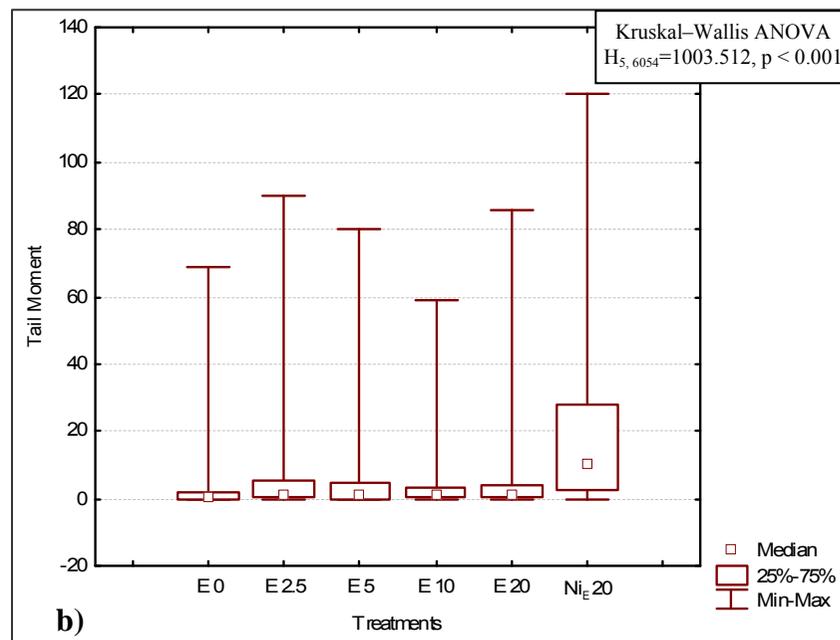
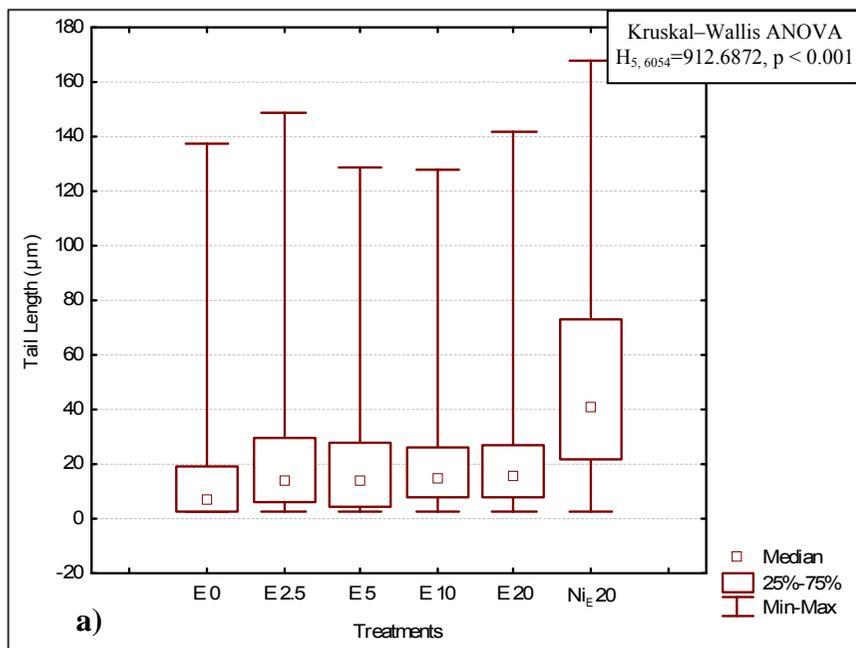


Fig. 5. Comet assay analyses within the long-term Cd-exposed culture of *E. fetida* (number of nuclei scored = 6054) after 48h exposure to CdSO₄, using three different parameters: a) tail length (µm); b) tail moment; and c) tail DNA percentage. On X-axis E = long-term Cd Exposed group and numbers indicate respective Cd concentrations (mg/l). Ni_E20 = Nickel positive control treatment (20 mg/l).

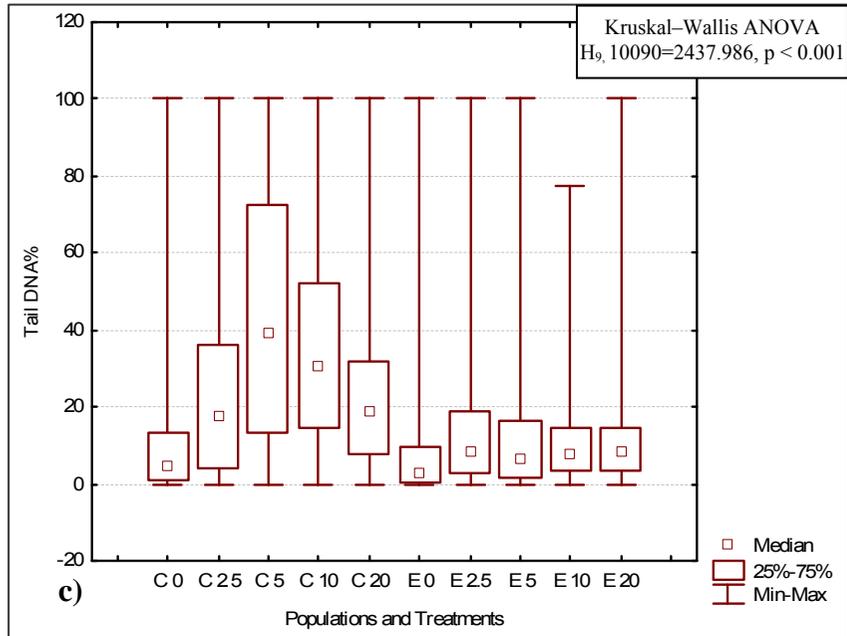
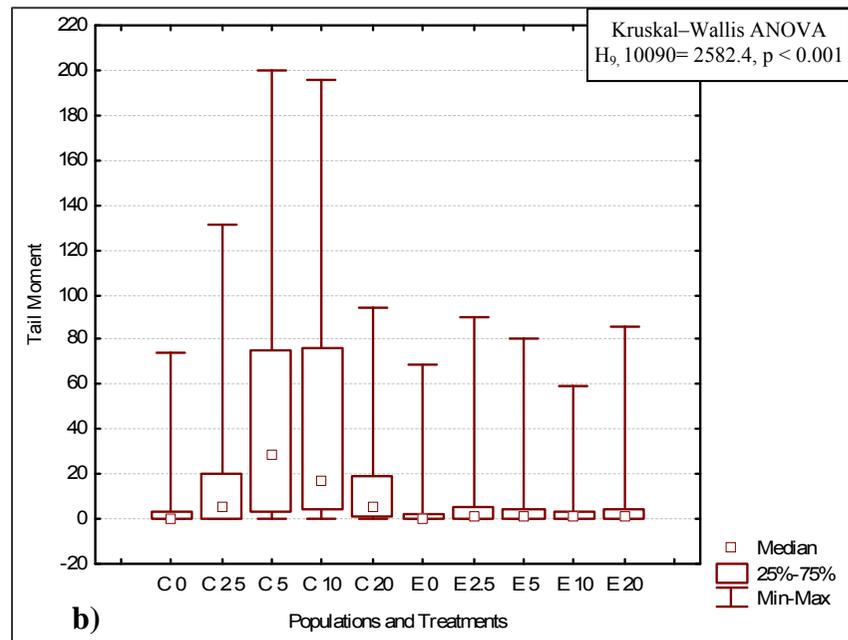
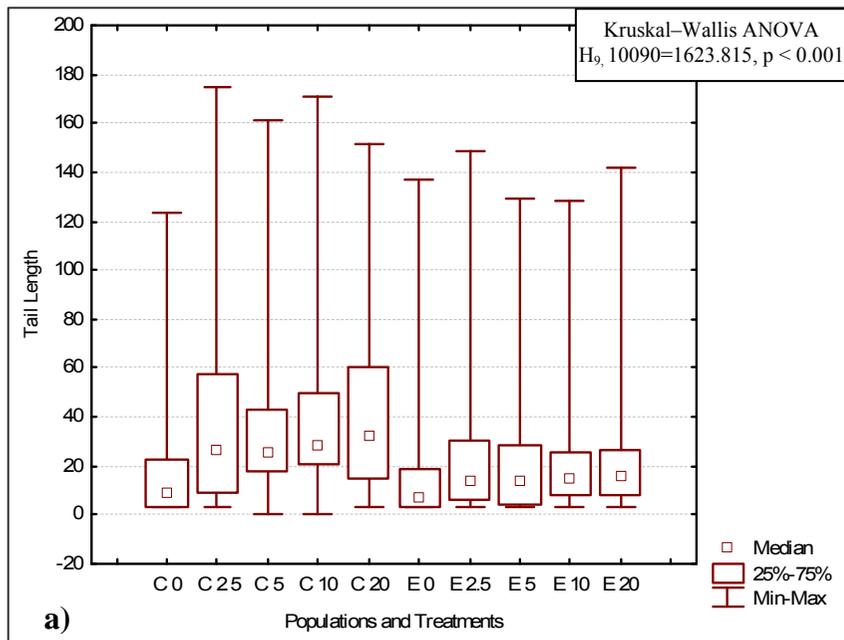


Fig. 6. Comet assay analyses amongst the long-term control culture and the long-term Cd-exposed culture of *E. fetida* (number of nuclei scored = 10090) after 48h exposure to CdSO₄, using three different parameters: a) tail length (μm); b) tail moment; and c) tail DNA percentage. On X-axis E = long-term Cd Exposed group, C = long-term Control culture and numbers indicate respective Cd concentrations (mg/l).

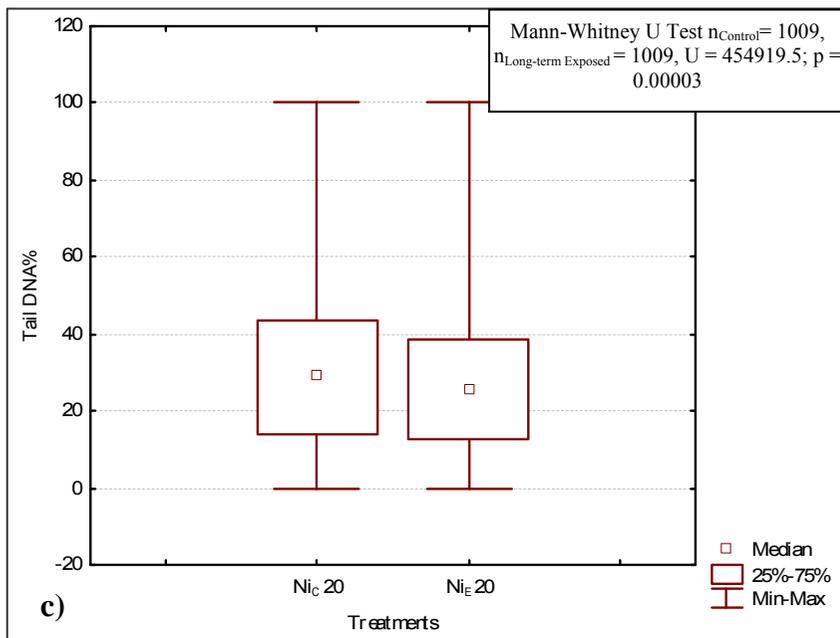
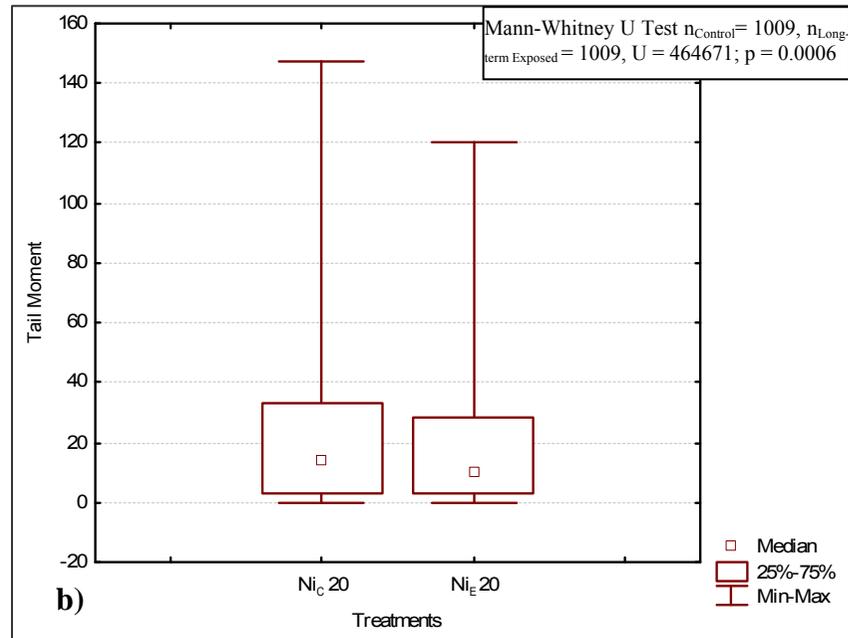
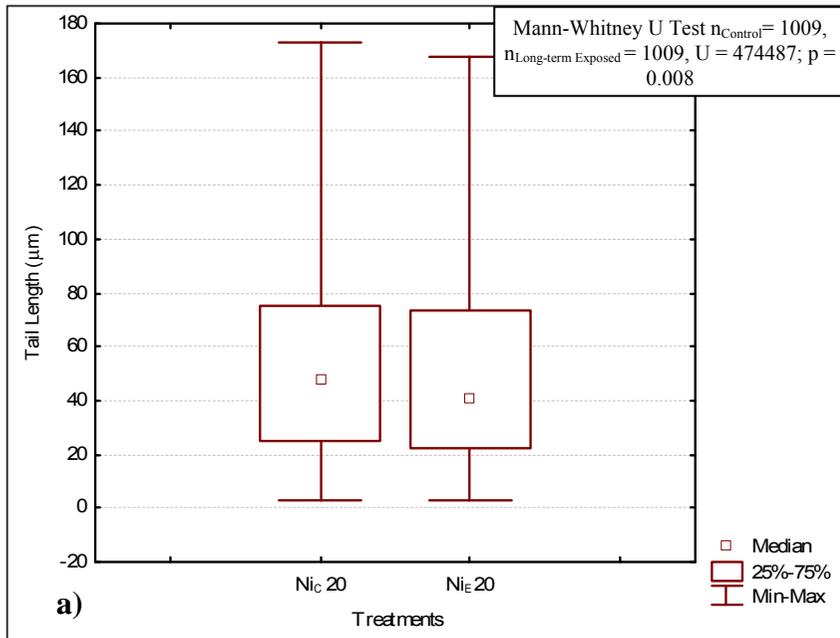
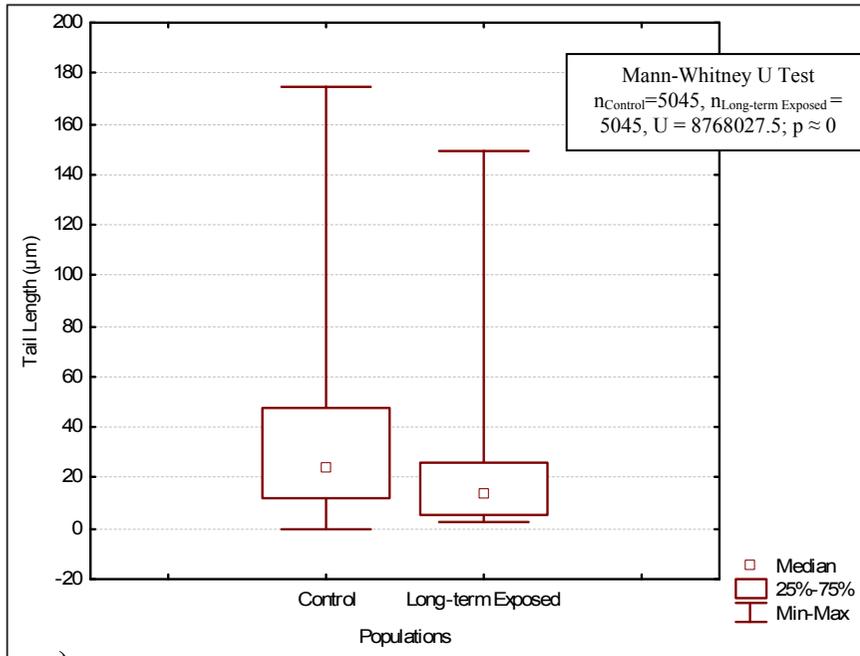
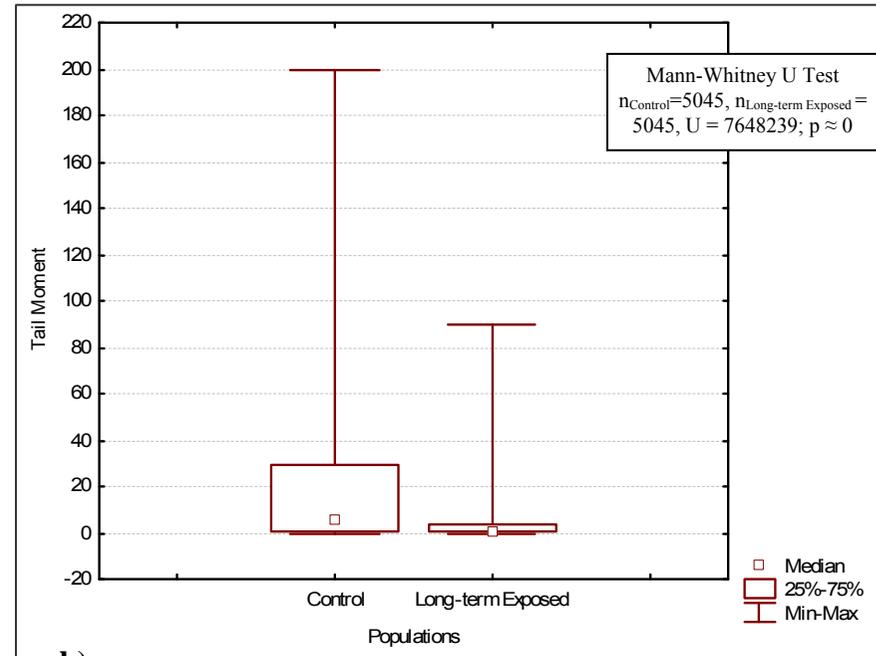


Fig. 7. Comet assay analyses between positive controls from both the long-term control culture and the long-term Cd-exposed culture of *E. fetida* (number of nuclei scored = 1009) after 48h exposure to CdSO₄, using three different parameters: a) tail length (μm); b) tail moment; and c) tail DNA percentage. On X-axis Ni_E 20 = Nickel positive control treatment from (20 mg/l) the long-term Cd-exposed culture and Ni_C 20 = Nickel positive control treatment (20 mg/l) from the long-term control culture.



a)



b)

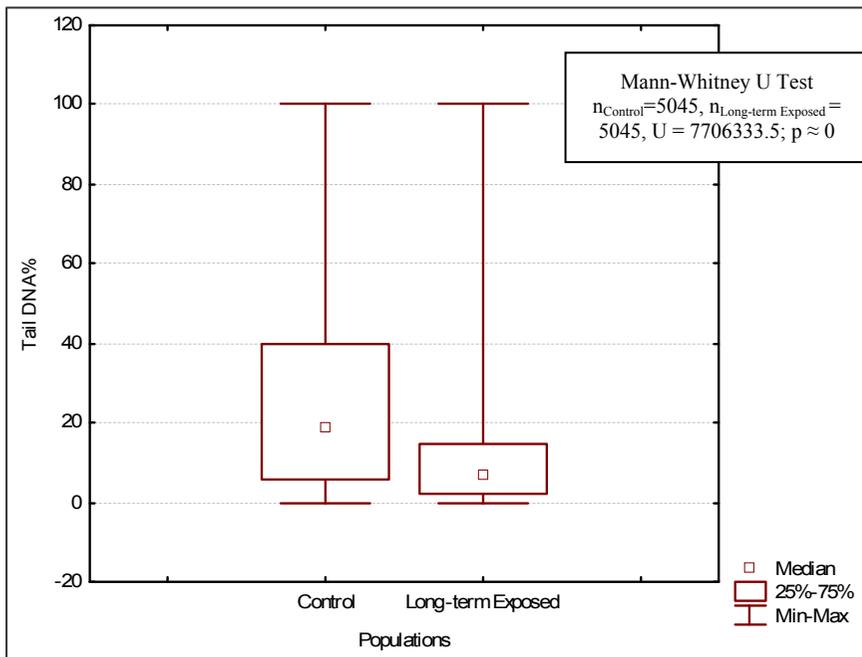


Fig. 8. Comet assay response at population level between the long-term control and the long-term Cd-exposed cultures of *E. fetida* (number of nuclei scored = 5045) after 48h exposure to CdSO₄, using three different parameters: a) tail length (μm); b) tail moment; and c) tail DNA percentage.

3.4. DISCUSSION

The comet assay carried out between two populations of *E. fetida* with different histories of metal exposure revealed that chronic exposure to Cd seems to cause less DNA damage in pre-exposed than in non pre-exposed earthworms. All three parameters assessed in this study demonstrated that Cd exposure for more than 12 years (\pm 78 generations) has probably caused the long-term Cd-exposed laboratory culture of *E. fetida* to display less genotoxic sensitivity when further exposed to Cd. Despite some discrepancies amongst the selected parameters with regard to the positive and negative controls (Fig. 4 and 5), results highlighted significant differences in Cd generated genotoxicity between the long-term Cd contaminated group and the control group. When whole population responses were considered, all parameters pointed towards an increased tolerance to Cd induced genotoxicity in the long-term Cd-exposed laboratory culture (Fig. 8). This could be potentially advantageous in case of future acute exposure to the same and perhaps even to a different metal. Our data revealed that the long-term Cd-exposed culture was also less sensitive to Ni (Fig. 7).

Many mechanisms might nevertheless come into play in order to keep the whole earthworm organism alive under continuous metal stress. Most biomarkers help both to generate snapshots of the early development of a specific toxic stress and endeavour to provide acceptable explanation behind the recorded response. In the present case, the alkaline comet assay helped to measure the occurrence of single strand DNA breaks in the two populations under investigation. However the mechanisms behind the recorded increased tolerance to Cd induced genotoxicity in the long-term metal contaminated population still needs to be elucidated.

A plausible explanation calls for the involvement of differences in DNA repair mechanisms. Boiteux *et al.* (1978) discussed the eventuality that some unrepaired DNA lesions could contribute to increasing population fitness by promoting genetic variability. Such DNA lesions would not be strand breaks but rather modifications of DNA bases or the phosphate backbone. Eastman & Barry (1992) reported that most DNA damage indeed occur in that form. The comet assay however was designed to detect DNA lesions

in the form of either single strand and/or double strand breaks (Rydberg & Johanson 1978; Östling & Johanson 1984; Singh *et al.* 1988). It could therefore perhaps be possible that DNA lesions other than strand breaks have accumulated in the long-term metal exposed population over time, to the point of contributing to increased population fitness in a Cd environment. Investigating DNA repair processes and assessing the presence of other types of DNA lesions other than strand breaks in the long-term Cd-exposed population could perhaps help to answer this question.

Van Goethem *et al.* (1997) looking at the genotoxic effects of cobalt powder and tungsten carbide on human lymphocytes reported that while the comet assay was sensitive enough to assess DNA breaks, the micronucleus test allowed to detect other types of lesions such as chromosomal aberrations. This might be an ideal assay to further investigate this question. The development of increased tolerance to Cd induced genotoxicity in long-term metal contaminated populations might perhaps not totally operate through the promotion of genetic variability as suggested by Boiteux *et al.* (1978). Allozyme studies have indeed revealed a low mean heterozygosity per locus ($H_o \leq 0.21$) in both populations presently under investigation (see Chapter 4). Cadmium however, is known to inhibit DNA repair processes, to promote chromosome aberrations and the formation of DNA crosslinks (Zasukhina *et al.* 1977; Snow 1992 and Waisberg *et al.* 2003). The observed difference in Cd induced genotoxicity in the two populations investigated in the present study could lie in one or more of these aspects.

The present findings are in accord with those recorded using the MTT assay (see Chapter 2) where it was pointed out that long-term exposure to Cd had conferred to the long-term Cd-exposed culture a potential increased cell viability and better chances of survival in case of future exposure to Cd. Despite the reported increased tolerance to genotoxicity due to Cd in the long-term Cd-exposed group, and because of the scope of the comet assay, the present findings may not reflect the development of genetically based resistance to Cd in the metal contaminated group. The comet assay detects structural damages rather than functional impairments inflicted by genotoxicants (Shugart 2000). The present findings could on the contrary show that increased tolerance to Cd induced

genotoxicity in the metal contaminated group is due to improved or overworking DNA repair mechanisms.

With regard to the many parameters that have been used to assess genotoxicity using the comet assay (head area, tail area, head DNA, tail DNA, head DNA%, tail DNA%, head radius, tail length, comet length, tail moment, olive tail moment), only three were chosen for use during the present study. We obtained fairly congruent results with these parameters but there is a controversy surrounding the choice of parameters for the comet assay in the literature. Collins (2004) argued that tail length and tail moment were not very useful parameters in the assessment of genotoxicity using the comet assay. Tail length, he said, increases at an early stage during exposure to a genotoxic compound but tends to reach a limit as the damage increases. Even though tail intensity might increase with the damage, because of the DNA forming the tail of the comet, tail length will not increase beyond a certain limit. Similarly he argued that tail moment, despite its frequent use in the literature, does not follow a dose response pattern and therefore is not as relevant in quantifying genotoxicity (Collins 2004). He further recommended the use of tail DNA percentage as a more effective parameter as the quantity of DNA in the tail is a reflection of DNA strand breaks (Collins 2004).

In the present study we used three parameters and found that parameter choice is indeed critical in the assessment of genotoxicity using the comet assay. Fig. 7 for instance revealed that Ni had a higher influence on tail length than tail moment. Knowing that tail moment is the product of tail length and tail intensity (which is correlated to the quantity of DNA in comet tail) it is clear that tail length and tail intensity do not always vary in a linear manner.

Few studies have used the comet assay to assess the genotoxicity of various compounds in oligochaetes species. Amongst those studies, the main parameter chosen to assess DNA damage was the comet tail length. Martin *et al.* (2005) used the comet tail length to assess the genotoxicity of the polycyclic aromatic hydrocarbon benzo[*a*]pyrene and the organochlorine lindane on the earthworm *Apporrectodea longa*. Reinecke & Reinecke (2004) also used the same parameter to study the genotoxic potential of Ni on the

earthworm *E. fetida*. Similarly, Verschaeve & Gilles (1995) used it to assess the genotoxicity of polluted environmental soil samples, X-rays, dioxin and mitomycin C on the earthworm species *E. fetida* and *Lumbricus terrestris*.

In terms of sensitivity amongst the selected parameters in the present study, tail length failed to signal statistically significant background DNA damage between the negative control treatments of the two populations under investigation (Fig. 6). Tail DNA percentage and tail moment successfully reported the difference. These latter parameters therefore appeared more sensitive than tail length. Bauer *et al.* (1998) however acknowledged that the comet assay is still in need to be standardized in terms of the parameters used to measure DNA damage.

3.5. CONCLUSION

As accumulating evidences tend to support the acclimation hypothesis over the genetic adaptation hypothesis in the development of metal resistance in the earthworm *E. fetida*, there is in parallel a need to discover the mechanisms that are endorsing these observations. The present study highlights one consequence (i.e. increased genotoxic tolerance) of long-term Cd exposure in *E. fetida* but does not make a connection with the inherent contributing mechanisms. This calls for further investigations preferably using a method such as the micronuclei test to find out whether DNA lesions other than strand breaks are more abundant in long-term metal exposed *E. fetida* individuals.

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4. ASSESSING ALLOZYME POLYMORPHISM TO INVESTIGATE THE DEVELOPMENT OF RESISTANCE TO CADMIUM

4.1. INTRODUCTION

Van Straalen (1999) and Van Straalen & Timmermans (2002) listed three different ways by which genetic variations are usually assessed in toxicant-stressed populations. These are: (i) quantitative analyses (biometric approach), (ii) the measurement of allozyme polymorphism and (iii) assessment of DNA polymorphism.

Using quantitative analyses, some investigations of the genetic basis of metal resistance in some oligochaete species have failed to provide strong supportive evidence (Bengtsson & Rundgren 1992; Aziz *et al.* 1999). Bengtsson & Rundgren (1992) collected earthworms *Dendrobaena octaedra* from two sites contaminated as a result of a brass industry in the area for several decades, and monitored their growth, survival and cocoon production under laboratory conditions. They found that worms from the contaminated sites did not significantly differ from those of the control population in terms of all the parameters assessed. They concluded that metal pollution had not been a significant selective agent on *D. octaedra* at the two sites studied. Aziz *et al.* (1999) examined metal resistance in the earthworm *Lumbricus rubellus* and demonstrated that growth rates between F1 generation offspring from Pb-Zn mines and F1 generation offspring from metal free populations did not reveal any evidence of genetic adaptation.

Other such studies have either reported findings supporting the genetic basis of metal resistance (Martinez & Levington 1996) or a heritable component of resistance to heavy metals (Spurgeon & Hopkin 2000; Langdon *et al.* 2003). Martinez & Levington (1996) found that *Lumbricus hoffmeisteri* from Foundry Cove (New York) had evolved genetic based resistance to heavy metal after estimating the minimum number of genes contributing to the difference in resistance between individuals from that population and a control population. The minimum number of genetic factors was established following the method of Lande (1981), which uses parameters such as phenotypic differences and

genetic variance among populations to deduce the number of genes contributing to a specific character. They found that resistance to heavy metals in *L. hoffmeisteri* from Foundry Cove was controlled by a single gene (Martinez & Levington 1996).

Working on the earthworm *E. fetida*, Spurgeon & Hopkin (2000) found an increased resistance to Zinc for F1 and F2 generations of earthworms reared on a Zn-contaminated medium (contact filter paper) but originally collected from a metal free population. They exposed parent earthworms and a F1 generation of *E. fetida* to a range of concentrations varying from 48.3 to 290 $\mu\text{g Zn ml}^{-1}$. Similarly, F2 earthworms were exposed to increasing concentrations of 48.3 to 363 $\mu\text{g Zn ml}^{-1}$. Results showed that $\text{LC}_{50\text{S}}$, $\text{LC}_{90\text{S}}$ and $\text{LC}_{99\text{S}}$ values found for the F2 generation were higher than those found for the F1 generation and parent earthworms. Toxicokinetic experiments undertaken to verify whether changes in Zn kinetics could be held accountable for the increased resistance to Zn failed to indicate any consistent change amongst the three generations. It was thus suggested that *E. fetida* had developed genetically inherited resistance to Zn under laboratory conditions (Spurgeon & Hopkin 2000). Likewise, Langdon *et al.* (2003) studied cocoon production over two generations for adult *L. rubellus* collected from an abandoned arsenic (As) and copper (Cu) mine and from an abandoned tungsten (W) mine. The earthworms were kept in clean soil long enough to complete their lifecycle during this study and the cocoon production was monitored. Cocoons (F1) from each population were allowed to hatch and to reach sexual maturity on a clean substrate. F1 adults were thereafter exposed to either 2 $\mu\text{g As mg}^{-1}$ as sodium arsenate or 0.3 $\mu\text{g Cu mg}^{-1}$ as copper chloride for 28 days. Results indicated that the F1 generation of *L. rubellus* from the As and Cu mine demonstrated resistance to As but not to Cu. The control population suffered high mortality in both sodium arsenate and copper chloride substrates. The F1 adults and F2 cocoons from different populations did not have considerably higher body burdens of As than the control population implying that resistance to As was probably inherited.

Looking at allozyme polymorphism amongst conspecific population is another way to assess genetic variations. Allozymes are different forms of oligometric enzymes which differ from one another by their thermal stability, kinetic constants and electrophoretic

mobility (Wilson & Walker 2000). Differences in allozyme mobility on a starch gel allow quick and cost effective, though indirect (two steps away from the gene) quantification of genetic variations (Gillespie & Guttman 1993). After electrophoresis, the starch gel is cut into thin slices that are then specifically stained to view migration patterns. This method is used in population studies to describe allele frequency changes, breeding structure, gene flow and species boundaries (Murphy *et al.* 1996).

No fixed differences in allozyme frequency in oligochaete species have yet been correlated with metal contamination. The use of allozymes has helped to establish that Tributyltin (TBT), a biocide found in anti-fouling paints, could impact on the genetic structure of the oyster *Crassostrea gigas* by promoting allozyme differentiation between TBT sensitive and TBT resistant individuals (Tanguy *et al.* 1999). *C. gigas* individuals collected from the Bay of Brest (France), a region relatively contaminated by TBT were exposed to higher concentrations of the substance. Among the 12 loci analyzed, Tanguy *et al.* (1999) found that frequencies at three loci; aspartate-amino-transferase (*Aat-2*), adenylate kinase (*Ak*) and phosphoglucomutase (*Pgm*) significantly varied between TBT-sensitive and TBT-resistant individuals. Moraga & Tanguy (2000) made similar observations while assessing the effect of certain pesticides (atrazine, isoproturon, alachlore, metolachlore and diuron) on the same oyster species (*C. gigas*). They reported that adenylate kinase (*Ak*), phosphoglucose isomerase (*Pgi*) and phosphoglucomutase (*Pgm*) differed in frequency between sensitive and resistant individuals of *C. gigas*. Frati *et al.* (1992) also observed a correlation between metal tolerance and allozyme variation in northwestern European populations of *Orchesella cincta* (Collembola) at the glutamate-oxaloate transaminase (*Got*) locus. However, not as many studies on allozyme variations in terrestrial toxicant stressed populations have been done as for their aquatic counterpart (Gillespie & Guttman 1993; Belfiore & Anderson 2001).

The aims of the present study were twofold. Firstly, to determine if there was a difference in the genetic variation as measured by allozyme electrophoresis between a culture of *E. fetida* that was exposed to Cd for several generations in the laboratory, and a Cd free culture. Secondly, to compare the level of genetic variation between these laboratory cultures and three field populations of *E. fetida* to determine the general level of genetic

variation in this species, occurring locally, but having been introduced from the Northern Hemisphere over many decades.

4.2. MATERIAL & METHODS

Earthworms used during this study came from the set of cultures and populations of *E. fetida* described in chapter 2, section 2.2. To these cultures and populations, two other populations were added. They were collected from Cd free sites in the course of March 2004. These sites are at the Spier, and at Welgevallen farm near the town of Stellenbosch, Western Cape, South Africa

Earthworms' homogenates were prepared as follows: The whole earthworm was washed and placed in 100 μ l of 0.01 M Tris buffer (pH 8) and chemically clean sand was added (to act as a lysis agent). This mixture was then crushed up and homogenized using a glass rod attached to a variable-speed electric motor. The homogenates were centrifuged for 5 min at 12 000 g and stored at -80°C until electrophoresis could be performed. The supernatant that was the source of the enzymes was used for the electrophoresis phase.

Horizontal starch gel electrophoresis was carried out in continuous buffer systems. Enzymes utilized in the studies and their respective buffer systems are listed in Table 3. Depending on the buffer system, the starch gel was prepared as follows: a specified volume, for each specific buffer, was placed in a measuring cylinder and diluted in water until a 330ml solution was reached. From this solution 230 ml were poured into a volumetric flask and brought to boiling point by means of a hot plate. The remaining 100ml were mixed with 42g of hydrolysed potato starch (Sigma Aldrich) in an Erlenmeyer flask. When the content of the volumetric flask started boiling, it was transferred to the Erlenmeyer flask, which was placed on the hot plate until the mixture became gelatinous. By means of a vacuum, created by a system of taps and rubber tubes, air was removed from the gel, which was poured into a mould. After the gel had cooled down, it was stored at 4°C.

To transfer the proteins to the gel, filter paper wicks impregnated with the supernatant were inserted into the starch gel. The gels were covered with plastic and run for 4 hours at 40mA, with ice packs on top. After electrophoresis, the gels were cut into thin horizontal slices and each slice was stained using specific protein staining solutions. The chemical composition of each staining solution depends on the locus to be stained. After each electrophoresis run, the allozymes were numerically scored.

Statistical analyses were carried out using BIOSYS-1 (Swofford & Selander 1981) and FSTAT (Goudet 2000). The different tests performed included χ^2 -goodness-of-fit, to test if populations were in Hardy-Weinberg equilibrium; calculation of the mean heterozygosity (H) per locus for each population (Nei 1978) ; the mean unbiased genetic identity (I) and distance (D) for each pair-wise comparison of populations (Nei 1978). Furthermore, F statistics (Wright 1965) were used to estimate respectively the mean value of genetic differentiation within subpopulations ($F_{(IS)}$), the mean value of genetic differentiation over the entire population ($F_{(IT)}$) and the genetic differentiation between any two subpopulations ($F_{(ST)}$).

Table 3. Enzymes and buffer systems utilized with starch gel to assess allozyme variations in the laboratory cultures and the field populations.

Enzymes	E.C numbers	Buffer systems
Phosphoglucomutase (<i>PGM</i>)	5.4.2.2	Tris-borate-EDTA- buffer system pH 8.6
Glucose-6-phosphate isomerase (<i>GPI</i>)	5.3.1.9.	Tris-borate-EDTA- buffer system pH 8.6
Peptidase (glycyl-leucine as substrate; <i>GL</i>)	3.4.11.-	Tris-borate-EDTA- buffer system pH 8.6
Malic Enzyme (<i>ME</i>)	1.1.1.40.	Tris-citrate buffer pH 6.9
Isocitrate deshydrogenase (<i>IDH</i>)	1.1.1.42.	Tris-citrate buffer pH 6.9
Peptidase (leucyl-tyrosine as substrate; <i>LT</i>)	3.4.11.-	Tris-borate-EDTA- buffer system pH 8.6
Peptidase (Leucylglycylglycine as substrate; <i>LGG</i>)	3.4.-.-	Tris-borate-EDTA- buffer system pH 8.6

4.3. RESULTS

Although we investigated allozyme polymorphism at 7 enzyme encoding loci (Table 3), one locus, *LGG* consistently displayed two alleles that were called (*LGG-1* and *LGG-2*). Eight enzyme coding loci were therefore consistently scored. Four of these loci were monomorphic (*IDH*, *GL*, *LT* and *GPI*) while the remaining four loci were polymorphic (*ME*, *LGG-1*, *LGG-2* and *PGM*). The allele frequencies at the polymorphic loci are given in Table 4. In the laboratory control culture, two alleles were present at the *LGG-1* locus and three alleles at the *LGG-2* locus. In the Cd exposed population, two alleles were resolved at both the *ME* and at the *PGM* loci. In the Spier population, two alleles were resolved at the *PGM* locus and in both Welgevallen and Middelvlei populations, two alleles were resolved at the *LGG-2* and at the *PGM* loci.

Table 4. Allele frequencies for the two cultures and the three populations of *E. fetida* at each polymorphic locus. With *N* denoting the sample size.

Loci	<u>Populations</u>				
	Lab. control	Cd-exposed	Spier	Welgevallen	Middelvlei
<i>ME</i>					
(<i>N</i>)	30	30	30	30	30
A	1.000	0.933	1.000	1.000	1.000
B	0.000	0.000	0.000	0.000	0.000
C	0.000	0.067	0.000	0.000	0.000
<i>LGG-1</i>					
(<i>N</i>)	30	30	30	30	30
A	0.967	1.000	1.000	1.000	1.000
B	0.033	0.000	0.000	0.000	0.000

Table 4. (Continued)

Loci	Populations				
	Lab. control	Cd-exposed	Spier	Welgevallen	Middelvlei
<i>LGG-2</i>					
(N)	30	30	30	30	30
A	0.933	1.000	1.000	0.750	0.667
B	0.033	0.000	0.000	0.000	0.000
C	0.033	0.000	0.000	0.250	0.333
<i>PGM</i>					
(N)	30	30	30	30	30
A	1.000	0.767	0.817	0.767	0.867
B	0.000	0.233	0.183	0.233	0.133

All the polymorphic loci were out of Hardy-Weinberg equilibrium ($P < 0.05$). These included: *LGG-1* ($\chi^2 = 30$) and *LGG-2* ($\chi^2 = 60$) in the laboratory control; *ME* ($\chi^2 = 30$) and *PGM* ($\chi^2 = 19.86$) in the Cd exposed population; *PGM* ($\chi^2 = 5.9$) in Spier; *LGG-2* ($\chi^2 = 24.9$) and *PGM* ($\chi^2 = 11.8$) in Welgevallen and *LGG-2* ($\chi^2 = 30$) and *PGM* ($\chi^2 = 30$) in Middelvlei.

The mean observed heterozygosity per locus (H_o) was zero in the laboratory control culture and the Middelvlei population. In the remaining culture and populations, $H_o = 0.08$ in the Cd exposed culture, $H_o = 0.21$ in both Spier and Welgevallen (Table 5). Generally low levels of heterozygosity were present. The percentage of polymorphic loci was 12% for the Spier population and 25% for the other two field populations and both laboratory cultures (Table 5).

Table 5. Mean heterozygosity per locus (H_o), mean number of alleles per locus and percentage of polymorphic loci for cultures and populations of *E. fetida*.

Populations	Mean heterozygosity per locus (H_o)	Mean number of alleles per locus	Percentage of polymorphic loci
Laboratory control	0.0	1.4	25%
Cd exposed	0.08	1.3	25%
Spier	0.21	1.1	12%
Welgevallen	0.21	1.3	25%
Middelvlei	0.0	1.3	25%

$F_{(ST)}$ values at all polymorphic loci ranged from 0.027 to 0.17 with a mean of 0.104. $F_{(IT)}$ values ranged from 0.722 to 1 with a mean value of 0.85 and $F_{(IS)}$ ranged from 0.706 to 1 with a mean value of 0.832 (Table 6).

Table 6. Summary of F-statistics at all loci.

Locus	$F_{(IS)}$	$F_{(IT)}$	$F_{(ST)}$
<i>ME-1</i>	1.000	1.000	0.054
<i>LGG-1</i>	1.000	1.000	0.027
<i>LGG-2</i>	0.965	0.971	0.170
<i>PGM-1</i>	0.706	0.722	0.057
Mean	0.832	0.850	0.104

As depicted by Table 7; the estimated pairwise $F_{(ST)}$ among population ranged from -0.0114 to 0.1634.

Table 7. The estimated pairwise $F_{(ST)}$ among cultures and populations of *E. fetida*. With $P < 0.05^*$ and $P < 0.01^{**}$.

	Lab. Control	Cd Exposed	Spier	Welgevallen	Middelvlei
Lab. Control	0.0000	0.1307*	0.1095**	0.1467**	0.1595*
Cd Exposed		0.0000	-0.0114	0.0712*	0.1507**
Spier			0.0000	0.0862*	0.1634*
Welgevallen				0.0000	-0.0082
Middelvlei					0.0000

The dendrogram of genetic similarity (Fig. 9) obtained from the matrix of unbiased genetic identities showed that these conspecific populations were genetically invariant, clearly indicating the absence of genetic structure.

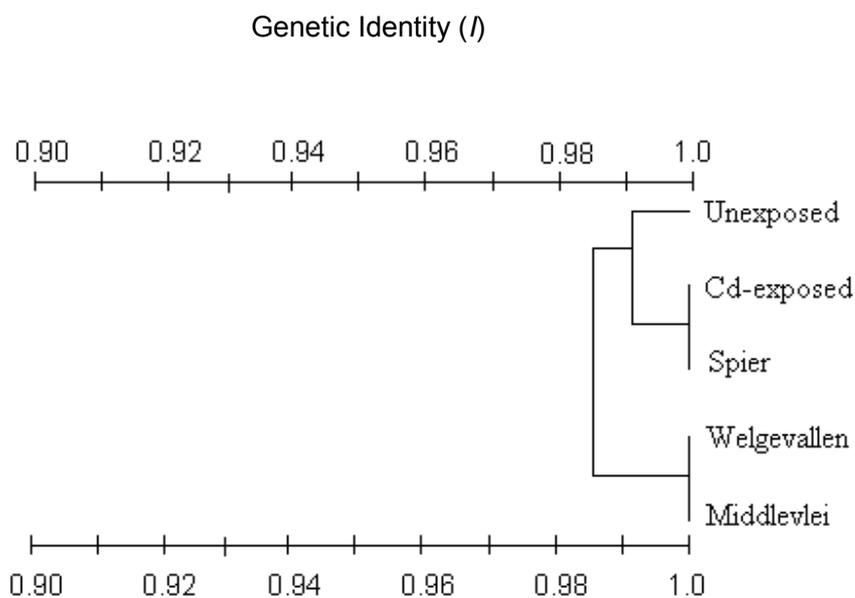


Fig. 9. Dendrogram constructed from the matrix of genetic identities (*I*) from pair-wise comparisons of all the populations.

4.4. DISCUSSION

No fixed genetic differences were observed between the Cd exposed culture and the uncontaminated (Cd free) culture and populations of *E. fetida*. The absence of fixed genetic differences in the present study may be attributed to four factors. Firstly, the molecular marker utilized may be too conservative, secondly the limited number of loci scored, thirdly the number of generations following exposure to Cd may be too limited to induce variation and fourthly, it is possible that Cd does not act as a selective agent.

Our results also suggest a very low level of genetic divergence ($< 2\%$; $D \leq 0.016$; Fig. 9) and a general low level of heterogeneity and polymorphism among populations of *E. fetida*.

The χ^2 -goodness-of-fit test results imply that one or more of the five assumptions of Hardy-Weinberg was not met. These five assumptions are: (i) infinite population size, (ii) random mating, (iii) absence of selection, (iv) absence of migration, (v) absence of mutation.

While allozyme electrophoresis presents the advantage of being relatively inexpensive and fast, its resolution power has, however, been contested (Mueller & Wolfenbager 1999). In addition, roughly only 30% of the genetic variation within a population may be revealed through allozyme analyses (Van Straalen & Timmermans 2002). The limited number of allozymes scored in the present study may be responsible for the poor differentiation between Cd exposed and unexposed populations and cultures. Van Straalen & Timmermans (2002) recommended examining allozymes variations for at least 20 loci in order to obtain an indication of the degree of genetic variation within a population. Depending on the ecotoxicological question being investigated, authors have studied allozymes variation from two to more than 20 enzyme encoding loci. For example, Tranvik *et al.* (1994) examined genetic variation at two loci (*PGI* and *PGM*) in field populations of the Collembola *Orchesella bifasciata* and reported no significant correlation between allozyme variations and the extent of metal contamination. In

contrast, Frati *et al.* (1992) examined genetic variations at 22 enzyme systems in the Collembola *O. cincta* exposed to heavy metals. These authors (Frati *et al.* 1992) found genetically based tolerance in northwestern European populations of *O. cincta*. In the same study, allozyme frequencies were correlated with metal tolerance traits for the *GOT* (glutamate-oxaloacetate transaminase) locus. More recently, Tanguy *et al.* (1999) were able to separate TBT-resistant from TBT-sensitive *C. gigas* by examining genetic variation at eight allozyme loci. Although only a limited number of loci were scored in the present study, the lack of any genetic differentiation between the Cd exposed culture, the control culture and the field populations could indicate a non genetic basis (i.e. occurrence of physiological acclimation) for the regulation of Cd in these earthworms.

The inclusion of a larger number of enzyme loci may have led to a greater understanding of genetic variations between Cd exposed and Cd free populations at the allozyme level in *E. fetida*. Future endeavors should thus examine more enzyme encoding loci or faster evolving molecular markers such as microsatellites or DNA sequences. These latter methods deal directly with variations encoded at the DNA level and consequently may provide more direct information on the possible effect of Cd exposure.

With regard to the number of generations, the estimated 78 generation changes following Cd exposure (by July 2006) may have been insufficient to cause Cd induced adaptation. Furthermore it has been reported that sublethal concentration of metal chlorides (KCl, CaCl₂, MgCl₂, BaCl₂, MnCl₂, SnCl₂, SrCl₂, AlCl₃ and FeCl₃) can significantly delay reproduction in *E. fetida* (Fisher & Molnár 1997). The long-term Cd-exposed laboratory culture might therefore have had less generation changes than estimated according to life cycle studies (Venter & Reinecke 1988). Under CdCl₂ exposure, as in the present study, it may thus take a longer time period for the potential genetic effect of Cd to manifest because this metal delays sexual maturity and reduces reproductive output (Fisher & Molnár 1997).

It is therefore arguable that life cycle parameters might be a determining factor when examining the possible genetic consequences of metal exposure. For instance, in organisms with shorter life spans such as the fruit fly *Drosophila melanogaster* and the

collembola *O. cincta* which complete their life cycles in two and less than four weeks respectively, genetically induced changes following exposure to metals have been detected (Fрати *et al.* 1992; Shirley & Sibly 1999). Most reported cases of heavy metal adaptations have occurred in fast reproducing organisms such as the springtails *Isotoma notabilis*, *Onychiurus armatus* and *O. cincta* (taking 3 to 5 weeks from hatching to maturity), the blowfly *Lucilia cuprina* (which can take 7 days to complete its life cycle in favorable conditions) and the fruit fly *D. melanogaster* (taking two weeks to complete its life cycle) (Posthuma & Van Straalen 1993). Added to the fact that *E. fetida* has a 7 to 8 weeks long life cycle (Venter & Reinecke 1988), Cd is also known to activate physiological processes that might serve as protection against the potential selective influence of Cd (Suzuki *et al.* 1980). These physiological mechanisms involve the development of metallothionein proteins, specialized in compartmentalizing Cd ions. The question whether physiological mechanisms such as the synthesis of Cd-binding proteins could be the cause of Cd-resistance in *E. fetida* merits further examination.

Our results nevertheless suggest that the physiologically induced differences observed between Cd exposed and Cd free cultures of *E. fetida* (Reinecke *et al.* 1999) might be due to physiological acclimation and thus lack a genetic basis. The lack of genetically based adaptation in *E. fetida* is in accordance with a study by Aziz *et al.* (1999) who examined metal resistance in the earthworm *L. rubellus* and successfully demonstrated that growth rates between F1 generation offspring from lead-zinc (Pb/Zn) mines and F1 generation offspring from non-exposed populations did not reveal any evidence of genetic adaptation.

The very low mean heterozygosity per locus ($H_o \leq 0.21$) recorded in all populations and the deviation from Hardy-Weinberg expectations both indicate a lack of heterozygosity at rare alleles. Inbreeding especially can lead to decreased heterozygosity in populations (Newman 2001). In addition, small sized populations are inclined to promote the fixation of certain alleles over others by means of processes such as inbreeding and genetic drift. Genetic drift, bottlenecks and the founder effect can cause the loss of genetic information and therefore result in major changes in genetic compositions of populations (Newman 2001). Such factors are particularly important for an introduced species such as *E. fetida*.

Furthermore, locally, (without taking into account anthropomorphic influences) *E. fetida* does not seem to display great dispersal ability (Prinsloo 1999). Currently the species is only found in compost heaps that are spatially reduced habitats unable to sustain large population sizes. The patchiness that characterizes that type of ecological niche does not always allow gene flow between populations. These factors would explain the low genetic variation observed.

4.5. CONCLUSION

This study on allozyme variations between the long-term Cd exposed laboratory culture and the other Cd free culture and populations failed to support whether resistance to Cd in the earthworm *E. fetida* has a genetic basis. Therefore, the differences in growth rates, cocoon production, hatchling success and survival observed between the long-term Cd exposed and the Cd free culture of *E. fetida* (Reinecke *et al.* 1999) might be due to physiological acclimation and thus lack a genetic basis.

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5. ASSESSING THE BIOSYNTHESIS OF METALLOTHIONEINS TO INVESTIGATE DIFFERENCES IN CADMIUM RESISTANCE

5.1. INTRODUCTION

Metallothioneins (MTs) were first isolated in 1960 from equine renal cortex and the term “*Metallothionein*” was used to designate the Cd-, Zn-, and Cu-containing sulfure-rich proteins, with high metal-binding capacity (Kagi & Vallee 1960; Kojima 1991). These water soluble proteins have a relatively low molecular weight of 2 000 to 15 000 Da (Suzuki *et al.* 1980; Gruber *et al.* 2000). Their characteristic amino acid composition reveals a high cysteine content (with cysteinyl residues such as Cys-X-Cys) and no aromatic or histidine residues (Kojima 1991). MTs biosynthesis has been found throughout the animal kingdom, as well as in plants and fungi (Vallee 1991). Kagi *et al.* (1984) have described three classes of MTs based on their structural characteristics:

Class 1: This class contains polypeptides with the position of cysteine residues similar to those in equine renal MT. The majority of vertebrate MTs are included in this class (Sturzenbaum *et al.* 1998).

Class 2: In this class, the position of cysteine residues in the polypeptides is quite different to those in equine MT. These include the majority of invertebrate and lower organisms MTs from mollusc to yeast (Sturzenbaum *et al.* 1998)

Class 3: In this class, less common, non-translationally synthesised metal-thiolate polypeptides, such as cadystin, phytometallothionein, phytochelatin and, homophytochelatin are included. These are predominantly found in plants (Sturzenbaum *et al.* 1998).

Beside the classes, MTs occurs as two major isoforms referred to as MT-1 and MT-2 (Klaassen & Lehman-McKeeman 1991). MT isoforms have also been described in

oligochaetes (Suzuki *et al.* 1980; Yamamura *et al.* 1981). Gruber *et al.* (2000) isolated and characterized MT-1 (7 000 Da) and MT-2 (15 000 Da) in *Eisenia fetida*.

Hamer (1988) hypothesised that MTs are involved in the detoxification of essential and non-essential metals in a wide range of organisms. Together with their metal binding function, MTs are also believed to be involved in protecting cells and tissues against oxygen free radical stressors (Sato & Bremner, 1993) and in gene regulatory activities (Zeng *et al.* 1991). However, the function of MTs has, to date, not been well established (Bremner 1991; Vallee 1995). The most common hypothesis has been that MTs are detoxification agents of the same metals which initiate their biosynthesis (Bremner 1991). Contradictory evidence has suggested, in the case of Cd-bound MTs, that these proteins also exhibit toxic properties (Cherian & Goyer 1978).

Several studies conducted in oligochaetes have suggested metal-binding proteins to lie at the basis of metal resistance in those species (Morgan *et al.* 1989; Klerks & Bartholomew 1991, Deeds & Klerks 1999; Gruber *et al.* 2000). The biosynthesis of metal-binding proteins has been reported in several oligochaete species: *E. fetida* (Suzuki *et al.* 1980; Yamamura *et al.* 1981), *Lumbricus rubellus* and *Dendrobaena rubida* (Morgan *et al.* 1989); *Tubifex tubifex* (Mosleh *et al.* 2005); *Limnodrilus udekemianus* (Deeds & Klerks 1999); *Allolobophora caliginosa* (Nejmeddine *et al.* 1992); and *Limnodrilus hoffmeisteri* (Klerks & Bartholomew 1991). It has been commonly accepted that, in oligochaetes, the main function of MTs is metal detoxification due to the overwhelming evidence supporting the correlation between metal exposure and the biosynthesis of MTs, on the one hand and MTs biosynthesis and the decrease in metal toxicity on the other.

However, of the two well documented MT-1 and MT-2 isomers in oligochaetes, MT-2 has been shown to be the only one involved in Cd-binding (Suzuki *et al.* 1980; Yamamura *et al.* 1981; Gruber *et al.* 2000). A recently described isomer MT-3, from *Lumbricus rubellus* seems to be abundant and functionally active in earthworm cocoons (Sturzenbaum *et al.* 2004). The function of MT-3 has, to date, not been clarified (Sturzenbaum *et al.* 2004).

Martinez & Levington (1996) hypothesised that the genetic origin of resistance to metal in earthworms lies in an aspect of evolutionary changes in MT genes. Moreover, it has been suggested by some research groups that MTs are at the basis of metal resistance in oligochaetes (Morgan *et al.* 1989; Klerks & Bartholomew 1991, Deeds & Klerks 1999; Gruber *et al.* 2000). Spurgeon *et al.* (2004) demonstrated that Cd causes significant induction of the *mt-2* gene, encoding the MT-2 isomer, involved in metal detoxification in the earthworm *L. rubellus*.

We hypothesize that populations of *E. fetida* with different histories of Cd exposure could show varying patterns of MT biosynthesis. Thus far, mechanisms supporting the reported resistance to Cd in the earthworms *E. fetida* (Reinecke *et al.* 1999), appear to be of physiological origin and not from genetic adaptation (see Chapter 4). Bengtsson & Rundgren (1992) and Aziz *et al.* (1999) argued that metal resistance in oligochaetes was the product of physiological acclimation rather than the outcome of genetic adaptation. If MTs are the basis of metal resistance in oligochaetes (Morgan *et al.* 1989; Klerks & Bartholomew 1991, Deeds & Klerks 1999; Gruber *et al.* 2000), then perhaps patterns of MTs biosynthesis would differ between metal resistant and non-metal resistant populations of oligochaetes. Consequently, comparative studies between the MT biosynthesis profile of metal resistant and non metal resistant populations, could yield relevant information with regard to heavy metal resistance in oligochaetes.

The aim of the present study was therefore to monitor the effect of long-term exposure to Cd on the biosynthesis of MTs in *E. fetida* by assessing both qualitatively and quantitatively, the biosynthesis of MT-2. A population which had been subjected to long-term exposure to Cd (>78 generations) and one which had been kept as a control, were investigated to establish whether metal resistance in this species could influence MTs biosynthesis.

5.2. MATERIAL & METHODS

The presence of MTs in *E. fetida* was investigated by immunoblotting analysis. The antibody, rabbit anti-MT-2 IgG, used in the study was raised against MT-2 isolated from *L. rubellus*. *L. rubellus* and *E. fetida* both belong to the family Lumbricidae and are therefore fairly closely related. It has been reported that antibodies raised against *L. rubellus* MT-2 cross react with *E. fetida* MT-2 (Homa *et al.* 2005). The rabbit anti-MT-2 IgG used in this study was obtained from Dr. S. Stürzenbaum from Cardiff University, Wales, U.K.

5.2.1. Sample collection and preparation

Earthworms used during this study were obtained from the set of cultures and populations of *E. fetida* described in chapter 2, section 2.2. Five *E. fetida* individuals from the long-term Cd exposed culture were exposed for 48 hours to 20 mg Cd/l of Cd in artificial soil water. Similarly, five earthworms from the metal free control culture were exposed to clean artificial soil water. They served as negative control since metallothioneins are only synthesised upon exposure to a metal. The earthworms were pooled with regards to their respective origins and homogenized in (1/3 w/v) Tris/HCl buffer (10mM, pH 7.5) on ice. Homogenates were centrifuged at 20 000 g for 30 minutes at 4°C. The supernatants were collected and the protein concentrations were determined using the Pierce bicinchoninic acid (BCA) protein assay kit (Pierce Biotechnology) according to the manufacturers' instructions.

5.2.2. Pierce BCA Protein Assay

The BCA protein assay exploits the reduction of Cu^{+2} to Cu^{+1} by proteins in an alkaline medium. The cuprous cation (Cu^{+1}) in turn would react with BCA molecules to give a purple-colored product measurable by spectrophotometry at 562 nm (Smith *et al.*, 1985). The protein concentrations of the samples are subsequently determined with reference to a standard curve derived from protein samples of known concentrations.

A bovine serum albumin (BSA) stock solution (2mg/ml) was used in this study to obtain the standard curve. The stock solution was diluted in duplicate in Tris/HCl buffer (10mM, pH 7.5). The BCA working reagent (WR) was prepared by adding 50 parts reagent A with one part reagent B (Pierce BCA protein assay kit). Earthworm homogenate samples were diluted 5, 10, 25 and 50 times in Tris/HCl buffer (10mM, pH 7.5). The diluted earthworm homogenates and standards were added to 200 μ l WR and pipetted, in duplicate, into a microtitre plate. The microtitre plate was incubated at 37°C for 30 minutes and the absorbance was measured at 562 nm using a plate reader (Titertek Multiskan[®] Plus, LabSystems, Finland).

5.2.3. Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

5.2.3.1. Gel preparation

The proteins in the *E. fetida* homogenates were analyzed by electrophoresis on a 12% discontinuous sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The resolving gel was prepared by mixing 7 ml resolving buffer (0.375 M Tris-HCl, pH 8.8, 0.1% SDS) with 3ml 40 % acrylamide stock solution (40% T, 2.7% C). The mixture was stirred and 10 μ l N,N,N',N'-tetramethylene diamine (TEMED), and 100 μ l 20% ammonium persulfate (m/v) were added. The resolving gel was mixed and poured between the glass plates on a casting stand. The gel was covered with a layer of distilled water and allowed to polymerize for 45 minutes. The stacking gel was prepared by mixing 4.5 ml stacking buffer (0.26 M TRIS-Cl, pH 6.8, 0.1% SDS) with 0.5 ml 40% acrylamide stock solution (40% T, 2.7% C). The mixture was stirred and 5 μ l TEMED followed by 50 μ l of 20% ammonium persulfate (m/v) were added. The stacking gel was mixed and after rinsing the interface with distilled water, it was poured on top of the resolving gel. A “comb” was inserted to create wells and the gel was allowed to polymerize, wrapped in wet tissue and stored overnight at 4°C.

5.2.3.2. Electrophoresis

E. fetida Cd-exposed and control samples were mixed with treatment buffer (1:1), incubated for two minutes in a boiling water bath and placed on ice. Samples (25 μ g

protein) were electrophoresed in duplicate using identical gels, at 30 mA until the electrophoresis front was approximately one centimeter from the bottom of the gel. A molecular weight marker (Bio-Rad) consisting of: phosphorylase b (97 400 Da); bovine serum albumin (66 200 Da); ovalbumin (45 000 Da); carbonic anhydrase (31 000 Da); trypsin inhibitor (21 500 Da) and lysozyme (14 400 Da) was used. In addition, lysozyme (4µg) was included as a positive control. After electrophoresis one gel was subjected to immunoblotting, while the other was stained for visualization.

5.2.3.4. *Immunoblotting*

The electrophoresed proteins were transferred from the acrylamide gel to a nitrocellulose membrane by electrophoretic means using a blot apparatus. The transfer was carried out using electrode buffer (0.05M TRIS, pH 8.3, 0.2M Glycine, 20% Methanol (v/v)) at 120 mA ($\pm 10V$) for 90 minutes.

On completion, the membrane was incubated in 20 ml of casein buffer (10mM TRIS, pH 7.6, 0.15 M NaCl, 0.5% Casein, 0.02% Thiomersol) to avoid non specific binding of primary and secondary antibodies. The nitrocellulose membrane was subsequently incubated in casein buffer containing the primary antibody, rabbit anti-MT-2 IgG (1:500), for an hour at 37°C. Thereafter, the antibody solution was decanted and the membrane was washed four times for five minutes in PBS-Tween (PBS buffer containing 0.1% of Tween₂₀). The secondary antibody, goat anti-rabbit HRP-conjugated, was diluted (1:1000) in casein buffer and the membrane was incubated therein for an hour at 37°C. The solution was decanted and the membrane was washed four times for five minutes in PBS-Tween. The membrane was subsequently incubated in the substrate solution (18mg of 4-chloro-1-naphtol in 6ml of cold methanol, 9µl of H₂O₂ and 30 ml of PBS) for 30 minutes.

The portion of membrane onto which the lysozyme had been transferred was subjected to the procedures described above using the primary antibody rabbit anti-lysozyme IgG diluted in casein buffer (1:500).

5.3. RESULTS & DISCUSSION

5.3.1. Sample protein analysis

Prior to electrophoresis protein concentrations of Cd-exposed and control homogenates were determined using the BCA protein assay. The absorbance values of serial dilutions of BSA, used to obtain a standard curve, are reported in Table 8. The standard curve obtained (Fig. 10) was subsequently used to estimate the protein concentration of both the samples.

Table 8. Absorbance values of bovine serum albumin (BSA) serial dilutions.

BSA concentrations (mg/l)	0	0.4	0.8	1.2	1.6	2
Absorbance 1 at 562 nm	0	0.522	0.903	1.201	1.528	1.975
Absorbance 2 at 562 nm	0	0.371	0.866	1.25	1.424	1.685

Results indicated that the five *E. fetida* specimens from the long-term Cd exposed culture, total weight 2.23g, had a protein concentration of 8.65 mg/ml while the five specimens from the control group, total weight 1.68g, had a protein concentration of 10.53 mg/ml. Total protein content (product of sample volume and protein concentration) was 57.87 mg in the Cd-exposed sample and 53.07 mg in the control samples.

Reinecke *et al.* (1999) documented that *E. fetida* specimens with a previous history of Cd exposure, kept for a period of 35 days on Cd contaminated substrates displayed a significantly higher biomass change than the worms kept on uncontaminated substrates. Long-term Cd-exposed individuals are therefore known to be larger than their unexposed counterparts.

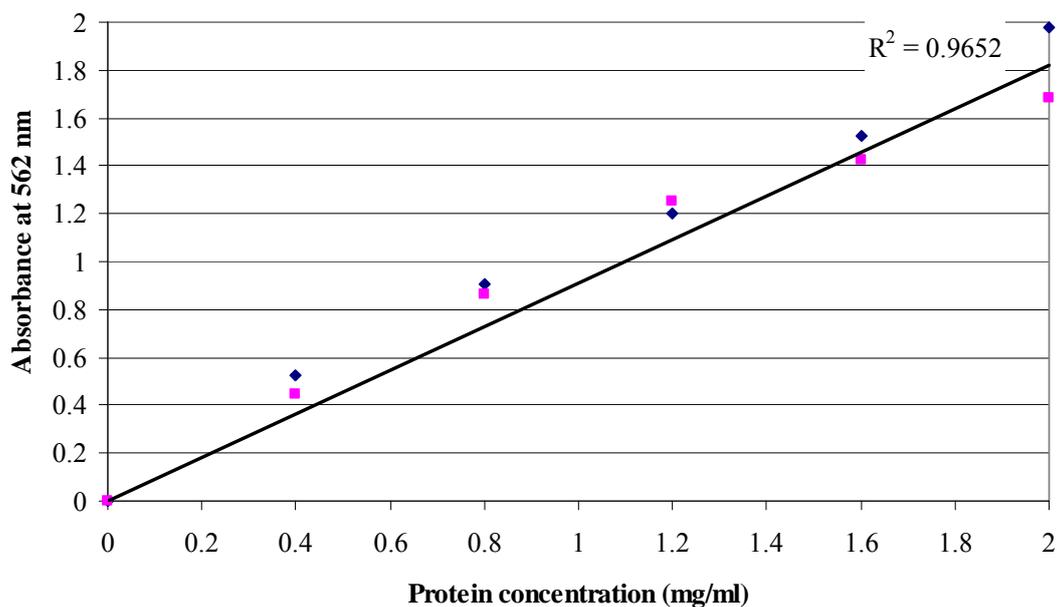


Fig. 10. Standard curve of bovine serum albumin (BSA) serial dilutions (0; 0.4; 0.8; 1.2; 1.6 and 2 mg/l) obtained with the BCA protein assay. Absorbance was measured in duplicate. “◆” indicates first absorbance serie and “■” indicates second absorbane serie.

5.3.2. SDS-PAGE analysis

Analysis of *E. fetida* homogenates, exposed to Cd as well as a control group, showed bands correlating to proteins of interest. Protein bands were clearly visible on the gel indicating that no degradation had occurred during the experiment. Protein visualization of the SDS-polyacrylamide gel however, did not show clear differences between the Cd-exposed (lanes 3 and 4) and the control (lane 1) samples (Fig. 11). There was a protein fraction of similar size (± 15 kDa) to MT-2 in the Cd-exposed sample (lanes 3 and 4) as well as in the control sample (lane 1). A faint protein band indicating the presence of a protein with a possible apparent mass of (7 kDa) is visible in lanes 3 and 4 (indicated by arrow). That protein corresponded to the molecular mass of MT-1 isomer (Fig. 11). MT-1 and MT-2 isomers are both biosynthesized subsequent to metal exposure in *E. fetida* even though MT-2 is the only one that has been shown to bind metal (Suzuki *et al.* 1980; Yamamura *et al.* 1981; Gruber *et al.* 2000).

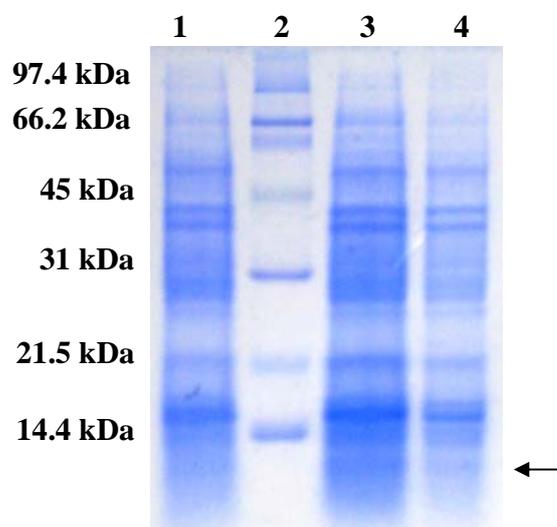


Fig. 11. SDS-polyacrylamide gel of earthworm homogenates from two laboratory cultures of *E. fetida* with different histories of Cd exposure. Lane 1, unexposed earthworms (25 µg protein) kept for 48 hours in clean artificial soil water (control sample); lane 2, molecular weight markers; lane 3, earthworms exposed to 20 mg/l Cd in the form of CdSO₄ in artificial soil water for 48 hours (>25 µg protein); lane 4, earthworms exposed to 20 mg/l Cd in the form of CdSO₄ in artificial soil water for 48 hours (25 µg protein). Arrow shows a faint protein band indicating the presence of a protein with an apparent mass of (7 kDa).

5.3.3. Immunoblotting analysis

In order to assess the presence of MT-2 in a homogenate sample of long-term Cd-exposed specimens of *E. fetida*, immunoblotting experiments were carried out after SDS-PAGE of the relevant sample and a negative control. No specific binding of the rabbit anti-MT-2 IgG on the nitrocellulose membrane was observed. Since no pure aliquot of MT-2 antigen was available as a positive control, lysozyme was used. Lysozyme therefore served as a positive control used to monitor experimental conditions. Positive immunoblotting of lysozyme (not shown) verified the reliability of the experimental procedures.

The lack of a positive signal with the rabbit anti-MT-2 IgG on the nitrocellulose membrane did not necessary indicate the absence of MT-2 in the Cd-exposed sample.

The experimental specimens used in our study were from a long-term Cd-exposed laboratory culture (see Chapter 2, section 2.2.). They had consequently been exposed to Cd and were expected to have a background level of MTs. In addition they were exposed to a sublethal concentration of Cd for 48 hours. As mentioned above, MT-2 is biosynthesized in *E. fetida* under metal toxic stress as a means to diminish the effect of the invading metals on the organism (Suzuki *et al.* 1980; Yamamura *et al.* 1981; Gruber *et al.* 2000). However no specific binding of the rabbit anti-MT-2 IgG on the nitrocellulose membrane was observed.

Homa *et al.* (2005) using a similar approach, detected MT-2 from coelomocytes of *E. fetida* after 3 days of exposure to heavy metals such as Zn, Cu, Pb, and Cd. They used a similar primary antibody in their study, namely rabbit anti-MT-2 IgG raised against the MT-2 isomer of *L. rubellus*. These authors however, reported a cross action between that antibody and *E. fetida* MT-2 isomer. Similar results were expected to be found in the present study but it was not the case.

A band corresponding to a protein with an apparent mass of 15 kDa was present in both samples (seen in lanes 1, 3 and 4). It is possible that other proteins have the same electrophoretic mobility as MT-2 (15kDa). These proteins may have masked MT-2 therefore preventing the binding or the visualization of rabbit anti-MT-2 IgG to MT-2 during immunoblotting. It is therefore possible that MT-2 could have been detected on the nitrocellulose membrane had the protein been purified by subjecting the homogenates to appropriate chromatographic techniques prior to electrophoresis. Chromatographic steps would have purified MT-2 and allowed an increased concentration of the protein to be probed (Wilson & Walker 2000). Even though lysozyme was used to monitor experimental conditions, the lack of a pure MT-2 protein restricted assessing the specificity of the rabbit anti-MT-2 IgG. It is therefore clear that many factors amongst which the lack of purity of our Cd-exposed sample could have contributed towards the MT-2 protein not being detected by rabbit anti-MT-2 IgG.

5.4. CONCLUSION

If the reported resistance to heavy metal in the earthworm *E. fetida* is merely the result of a physiological acclimation, there is reasonable possibility that MTs might be involved, since these proteins are known to be the primary agents against metal toxicity in oligochaetes. This study has revealed the relevance of purification steps such as chromatography techniques prior to immunoblotting experiments. In addition, monitoring the presence of MT-2 should be attempted using a homologous MT-2 protein as a positive control and a specific antibody which will bind the protein being studied. Future endeavours should therefore work both towards the preparation of *E. fetida* specific anti-MT-2 IgG and the production or acquisition of a fairly pure fraction of the same MT-2 isomer.

5.5. References

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6. ASSESSING DNA POLYMORPHISM TO INVESTIGATE THE DEVELOPMENT OF RESISTANCE TO CADMIUM

6.1. INTRODUCTION

Compared to quantitative analyses and the measurement of allozyme polymorphism, the assessment of DNA polymorphism could potentially give better results in terms of resolving subtle differences among data sets (Mueller & Wolfenbarger 1999; Van Straalen & Timmermans 2002). Notwithstanding the usefulness of DNA sequence data, very few studies employ DNA polymorphisms to address ecotoxicological questions in earthworms with the majority of studies focussing on the patterns of gene expression following toxic stress.

Willuhn *et al.* (1994) reported that the accumulation of large amounts of Cd in the enchytraeid worm, *Enchytraeus buchholzi*, coincided with the induction of the *CRP* gene which encodes a cysteine rich non-metallothionein protein was positively associated with Cd exposure (Willuhn *et al.* 1994). Importantly, Willuhn *et al.* (1996a & b) reported that Zn, Cu, Hg as well as Pb could switch on the expression of the *CRP* gene. Other stress conditions such as heat or oxidative stress were not able to induce *CRP* expression (Willuhn *et al.* 1996a). An additional cDNA-clone called *Ebaldh* (*E. buchholzi* aldehyde hydrogenase) was subsequently isolated by Willuhn *et al.* (1996c). This latter gene codes for an aldehyde hydrogenase involved in Cd detoxification in *E. buchholzi*. The expression of *Ebaldh* is more Cd specific compared to the expression of *CRP*. *Ebaldh* expression could not be induced by exposure to Zn, Hg or H₂O₂ (Willuhn *et al.* 1996c).

In metallothionein (MT) producing oligochaetes, gene expression in response to heavy metal exposure has also been investigated. For example, Stürzenbaum *et al.* (1998b) found evidence that certain mRNA transcripts are up-regulated when *Lumbricus rubellus* are exposed to heavy metals. Stürzenbaum *et al.* (1998a) isolated two separate gene sequences encoding two MT isomers (MT-1 and MT-2) in *L. rubellus*. Likewise, Gruber

et al. (2000) successfully linked up-regulation of mRNA genes with the production of MT in the earthworm *Eisenia fetida* by isolating MT cDNA.

Exposure to heavy metals appears to trigger biochemical reactions in oligochaetes, irrespective of whether they produce MT or not, that aim to lower the toxicological effects of these metals on target organs. Exposure of rat and mice cells to high doses of Cd appears to affect the mitochondria specifically with respect to a decrease in the ATP/ADP ratio (Müller, 1986; Dorta *et al.* 2003), loss of plasma membrane potential (Martel *et al.* 1990; Koizumi *et al.* 1994; Dorta *et al.* 2003; Li *et al.* 2003), enhanced lipid peroxidation (Müller, 1986; Dorta *et al.* 2003) as well as the inhibition of respiratory processes (Müller, 1986; Koizumi *et al.* 1994). Long-term exposure of rat cells to Cd causes a 4834-bp DNA deletion in mitochondria of renal epithelial cells (Takaki *et al.* 2004). It is currently unsure whether Cd can cause similar mitochondrial impairments in oligochaetes. Sturmbauer *et al.* (1999) reported different levels of Cd resistance for mitochondrial lineages of the oligochaete *Tubifex tubifex*, however, these variances in resistance were innate and not the result of previous exposure to Cd.

The most accurate way to assess the genetic consequences of exposure to Cd entails targeting a marker that will portray exclusively the selective pressure exerted by Cd alone. An ideal marker will be the *Ebaldh* gene which is entirely dependent on exposure to Cd (Willuhn *et al.* 1996a). However, the mechanism in *E. buchholzi* (from which *Ebald* was isolated) appears unique since Cd detoxification is done exclusively via non-metallothionein proteins; this sets *E. buchholzi* apart from most other terrestrial oligochaetes where Cd detoxification is accomplished through MTs (Morgan *et al.* 1989; Klerks & Bartholomew 1991, Deeds & Klerks 1999; Gruber *et al.* 2000).

If the response of MT genes to exposure has a genetic base, Martinez & Levington (1996) suggested that the origin of resistance could lie in some aspect of evolutionary change in MT genes. Spurgeon *et al.* (2004) demonstrated that Cd causes significant induction of the *mt-2* gene (metallothionein-2; involved in metal detoxification) in the earthworm *Lumbricus rubellus*. Similarly, they reported that Cd seems to lower the expression of the

lgp gene (encoding a lysosome associated-glycoprotein; involved in lysosome function and stability) (Spurgeon *et al.* 2004). Evidence to support a genetic basis for metal resistance comes from the fruit fly *Drosophila melanogaster* (Maroni *et al.* 1987) which is characterized by gene duplications involving MT genes as well as in the oyster *C. gigas* (Tanguy & Moraga 2001)

To our knowledge, no study has employed “neutral” DNA markers (such as mitochondrial DNA) to determine whether there is a genetic basis to heavy metal resistance in oligochaetes. However, the usefulness of these genes (such as the cytochrome *c* oxydase subunits I and II (COI and CO II) as well as the 16S, 18S, and 28S subunits of the nuclear ribosomal RNA (16S rRNA, 18S rRNA and 28S rRNA) in unravelling questions pertaining to population genetics and higher level relationships in oligochaetes has been demonstrated (Apakupakul *et al.* 1999; Beauchamp *et al.* 2001; Heethoff *et al.* 2004; Pérez-Losada *et al.* 2005). An investigation of DNA polymorphism using both specific genetic makers such as the *mt-2* gene and non specific genetic markers such as the COI gene could perhaps be helpful in understanding and explaining the differences in metal tolerance noticed between populations of *E. fetida* with different histories of Cd exposure (Reinecke *et al.* 1999).

A reliable taxonomy is crucial to relevant ecological and ecotoxicological studies. Recent studies by Dominguez *et al.* (2005) and Pérez-Losada *et al.* (2005) highlighted the uncertainty that surrounds the *E. fetida/andrei* taxonomy (see Chapter 1; section 1.4.1.). The most recent classification elevated *E. fetida* and *E. andrei* to species mainly based on sequence differences between them (Pérez-Losada *et al.* 2005). Given this, as well as the fact that there may indeed be physiological differences between these species, one of the objectives of this study was to determine the correct classification for the species currently housed in the Ecotoxicology group at the University of Stellenbosch.

The aims of the present study were therefore twofold:

- 1 To search for evidence that would either support or refute a genetic basis for metal resistance in the earthworm *E. fetida*. For this, sequence data from both neutral (COI) as well as adaptive (*mt-2*) genes were employed.

- 2 To determine the taxonomic status (*E. fetida* or *E. andrei*) of the earthworm cultures housed in the Ecotoxicology laboratory at the University of Stellenbosch.

6.2. MATERIAL & METHODS

6.2.1. Samples

Earthworms included in this study were taken from the set of exposed and control populations of *E. fetida* described in chapter 2, section 2.2. In total, 7 worms of the long-term control group as well as 9 worms of the long-term Cd exposed culture were included.

6.2.2. DNA extraction

Total genomic DNA was extracted using a phenol/chloroform method described by Maniatis *et al.* 1982). Five to ten mg of the tail section of worms were immersed in 250µl of lysis buffer (160mM saccharose, 80mM EDTA, 100mM Tris/HCl, pH 7.8) in the presence of 10µl proteinase K (10mg ml⁻¹). Extractions were incubated overnight at 55°C. Following standard phenol / chloroform extractions, DNA was precipitated in the presence of 100µl of a 7.5M Ammonium Acetate solution and ice-cold ethanol. DNA pellets were dried and resuspended in 250 µl ddH₂O.

6.2.3. PCR amplification and sequencing

6.2.3.1. Nuclear mt-2 amplification

Several attempts, including varying annealing temperatures, DNA concentrations as well as MgCl₂ concentrations, were made to amplify the *mt-2* gene fragment in *E. fetida*; unfortunately, without any success. Forward and reverse primers designed for *Lumbricus rubellus* (Spurgeon *et al.* 2004), were used in our amplification attempts. No other primers for the *mt-2* gene fragment, designed for closely related species, were available for use in this study.

6.2.3.2. Mitochondrial COI amplification

The universal LCO1490 and HCO2198 primer pair (Folmer *et al.* 1994) was used to amplify approximately 650 bp of the mitochondrial COI gene. PCR reactions were performed in a final volume of 30 μ l and contained 10ng of DNA, 3 μ l of PCR buffer, 3 μ l of 25mM MgCl₂, 3 μ l of a 20mM final concentration dNTP mixture, 0.3 units of Taq polymerase (Supertherm) and 30 pmol of each of the required primers. Cycling parameters were as follows: 94°C for 1 minute followed by 35 cycles of 94°C for 30 seconds, 50°C for 30 seconds and 72°C for 45 seconds. A final extension step at 72°C for 5 minutes completed the reactions.

To verify successful amplifications, amplicons were electrophoresed in 1% agarose gels and the correct band excised using a sterile scalpel. PCR products were gel purified with the Wizard SV Gel and PCR clean-up system (Promega) according to the manufacturer's recommendations. Nucleotide sequencing was carried out using the forward primer (i.e. LCO1490) with half-reactions of BigDye[®] Terminator v3.1 mix (Applied Biosystems). Purified sequencing products were run on an ABI 3100 automated sequencer (Applied Biosystems). Electropherograms of the raw data were edited with BioEdit 7.0.5 (Hall 2005). All *E. fetida* sequences were deposited in GenBank (accession numbers DQ914618 to DQ914633).

6.2.4. Sequence analyses

E. fetida sequences were aligned with Clustal X (Thompson *et al.* 1997) using the multiple alignment mode with default parameters and verified by eye. PAUP* (Swofford 2000) was used to assess the nucleotide composition and the number of variable and parsimony-informative sites. Sequence divergences were determined in PAUP*.

6.2.4.1. *Species identification*

To address the uncertainty regarding the taxonomy of *E. fetida* used in this study, several sequences were retrieved from Genbank and aligned to sequences obtained for both the exposed as well as control groups of earthworms housed in the Ecotoxicology laboratory at Stellenbosch University. Genbank sequences included two sequences from *E. fetida* (Pérez-Losada *et al.* 2005; AY874522 & AY874523), two sequences from *E. andrei* (Pérez-Losada *et al.* 2005; AY874511 & AY874512) and two sequences from *E. eiseni* (Pérez-Losada *et al.* 2005; AY874491 & AY874492). *Aporrectodea handlirschi* (Admassu *et al.* 2006; DQ092889) were included to allow resultant topologies to be rooted.

Phylogenetic relationships among taxa were determined using parsimony and neighbour-joining methods carried out by PAUP*. Parsimony analyses were based on heuristic searches with 100 random additions of taxa and TBR branch swapping. For neighbour-joining analyses, trees were constructed using both a simple uncorrected p-distance approach as well as under a more complex evolutionary model that may better explain the data (HKY +G (0.5); (Hasegawa *et al.* 1985) as determined with Modeltest v 3.06 (Posada & Crandall 1998). The confidence of these analyses was assessed using 1000 bootstrap replications.

6.3. RESULTS & DISCUSSION

6.3.1. Metal resistance and population genetics

Several authors (Klerks & Bartholomew 1991; Klerks & Levinton 1991; Reinecke *et al.* 1999; Langdon *et al.* 2001; Langdon *et al.* 2003) have documented resistance in earthworm populations following long-term exposure to heavy metals. Reinecke *et al.* 1999 speculated that changes in genes or genotypes frequency (i.e. genetic adaptation) following long-term exposure of *E. fetida* to Cd may have occurred; but were unable to rule out physiological acclimation resulting in heavy metal resistance. In an attempt to

determine whether there is indeed a genetic base to metal resistance, both neutral (COI) as well as adaptive (*mt-2*) genes were targeted.

Notwithstanding several attempts (see Section 6.2.3.1 above) to amplify the *mt-2* gene in *E. fetida*, amplification efforts always failed. Although both *L. rubellus* and *E. fetida* are segmented worms belonging to the family Lumbricidae, these species are phylogenetically distant and it is possible that the primers designed for *L. rubellus* are too non-specific to allow specific primer binding on the target species.

The mitochondrial COI gene was amplified and sequenced for 7 specimens from the long-term control culture and 9 specimens from the long-term Cd exposed culture. The final data set comprised 524 bp for 16 taxa. In total, 520 characters were constant and the remaining four were variable and informative. Transitions outnumbered transversions 3:1. To confirm the authenticity of our data, sequences were subjected to Blast searches in Genbank with resultant hits always returning *Eisenia sp.* Additionally, the sequence alignment was free of gaps as would be expected for a protein coding gene.

Three haplotypes were identified for the 16 specimens included in the present study. However, specimens could not be separated into groups on the basis of their history of metal exposure or the lack thereof. Two pre-exposed worms (Cd2 and Cd3) shared a unique haplotype, two control worms (KT1 and KT2) were identical with the remainder of the worms from both the control and exposed groups sharing a haplotype. The highest uncorrected sequence divergence between haplotypes was 0.76% (4 mutational steps) between the control (Cd2 and Cd3) and exposed (KT1 and KT2) worms. Both of these haplotypes connected to the main (intermediate) group by 2 steps (0.38% uncorrected p-distance).

These findings of no significant differentiation between control and exposed groups corroborate the outcome of the allozyme study (see Chapter 4; section 4.3). The two groups, despite having been separated for more than a decade (see Chapter 2, section 2.2.) show no or little sign of genetic differentiation. Although these results may be

interpreted as evidence for a lack of a genetic basis to heavy metal resistance, one would have to employ caution. First, mitochondrial DNA genes are neutral genetic markers as far as metal resistance is concerned, and the allozyme loci included in the study (chapter 4) are not directly involved in processes dealing with metal resistance. Therefore, given the data at hand, it is impossible at this stage to rule out the role of selection on genes involved in metal resistance (implying a genetic base to resistance). Secondly, inbreeding and founder effects would have played a significant role in shaping the genetic variation (in this case genetic homogeneity) in the control and exposed groups (Charlesworth & Charlesworth 1987). Both the long-term Cd-exposed and the long-term control cultures were in effect started with more or less 200 individuals. Following on from this point, both *E. fetida* and *E. andrei* have been, until recently, regarded as amphimictic species i.e. with sperm exchange occurring only between two copulating individuals (Gates 1978; Venter & Reinecke 1987). However, Dominguez *et al.* (2003) successfully recorded uniparental reproduction with evidence of self-fertilization in both species. Self-fertilization is the most extreme form of inbreeding and causes a significant reduction in heterozygosity and genetic diversity within populations (Charlesworth & Charlesworth 1987) This self reproductive capability could account for the low mean heterozygosity per locus ($H_o \leq 0.21$) found in these populations (see Chapter 4) and the inability to separate them using both allozyme (see Chapter 4) and mitochondrial DNA data.

6.3.2. The complex *E. fetida/andrei*: laboratory species identification

Although *E. fetida* and *E. andrei* have, until recently, been considered subspecies, several lines of evidence suggest that they are indeed full species. These include differences in pigmentation (Sims & Gerard 1985; Reinecke & Viljoen 1991; Bundy *et al.* 2002), molecular data (McElroy & Diehl 2001; Pérez-Losada *et al.* 2005) and allozyme polymorphism (McElroy & Diehl 2001). Most pertinent to the present study, physiological differences between these species also exist. Bundy *et al.* (2002), using nuclear magnetic resonance (NMR) spectroscopy on tissue extracts and coelomic fluid of both *E. fetida* and *E. andrei*, concluded that these species were noticeably distinct based on their biochemical profiles. Dominguez *et al.* (2005), after rearing cross-couples of *E.*

fetida and *E. andrei* for a period of 15 weeks, documented evidences of reproductive isolation between these forms, suggesting that they are distinct biological species. It is therefore imperative for ecotoxicologists to know the species they work with.

The results of the phylogenetic analyses are shown in Fig. 12. Congruent tree topologies was consistently retrieved irrespective of the method of analyses (parsimony or distance) or evolutionary model employed (uncorrected p-distance or HKY+G in distance analyses).

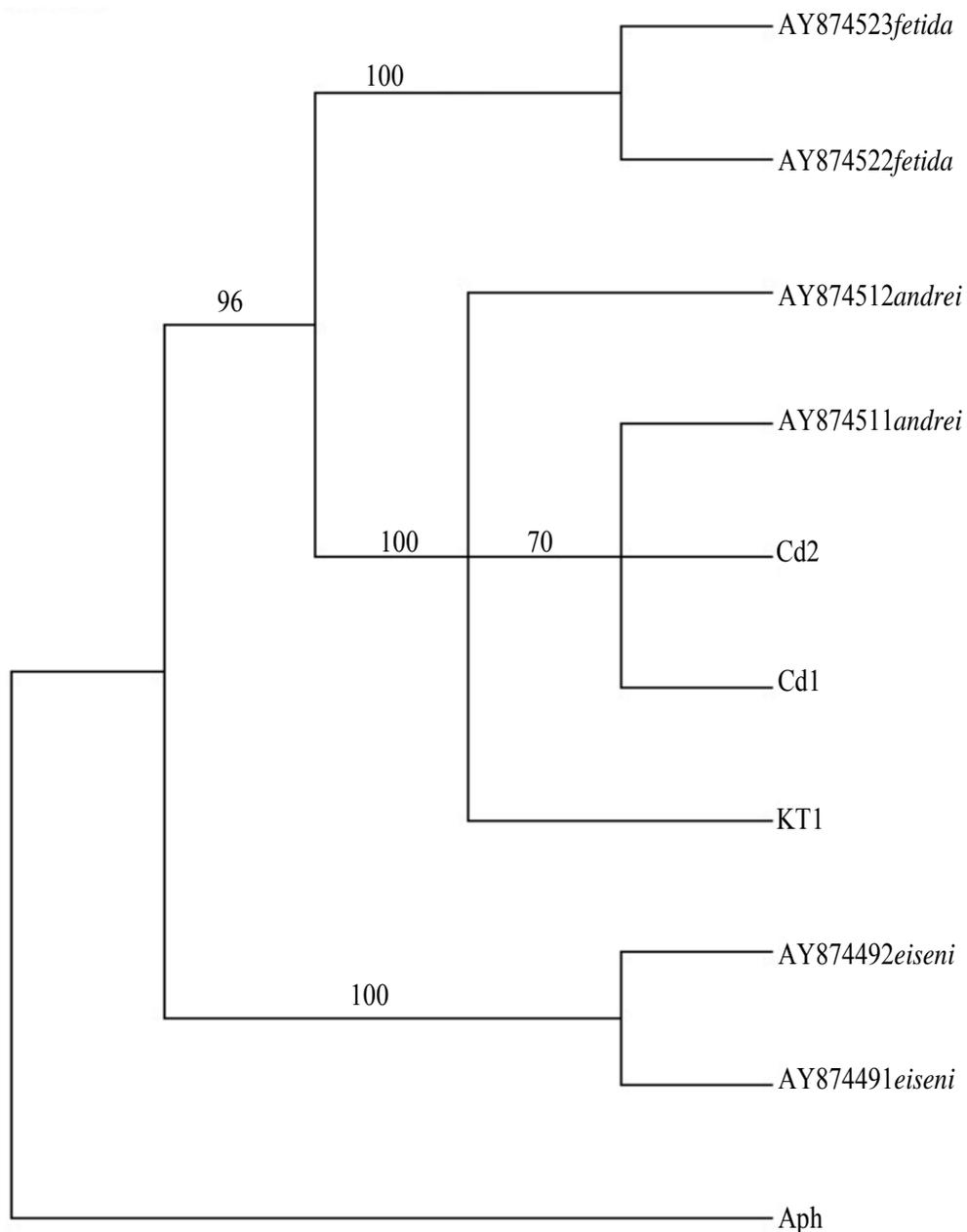


Fig. 12. Strict consensus tree constructed from 3 equally most parsimonious trees (253 steps each). Numbers on branches indicate bootstrap support values. Genbank sequences are identifiable by the access number followed by the *species* designation (*fetida*; *andrei* or *eiseni*). Cd indicates individuals from the long-term Cd exposed culture and KT refers to the long-term control culture. All individuals are from the genus *Eisenia* (n = 9). *Aporrectodea handlirschi* (Aph) was used as an outgroup.

The monophyly of the three species (*E. fetida*, *E. andrei* and *E. eiseni*) were confirmed by 100% bootstrap support. Uncorrected p-distances separating species were consistently greater than 14.2% (see Table 9). These divergences are noticeably higher *cf.* divergence values within species (0.82%). The three specimens representative of the three haplotypes detected in the present study (Cd1, Cd2 and KT1) grouped with 100% support with *E. andrei*. Indeed, two of the haplotypes detected in this study were identical to published *E. andrei* specimens (Cd1 is identical *E. andrei* Genbank accession number AY87451 and KT1 was identical to *E. andrei* Genbank accession number AY874512).

Table 9. Uncorrected p-distance matrix between sequences for the COI gene. Genbank sequences are identifiable by the access number followed by the *species* designation. Cd indicates individuals from the long-term Cd exposed culture and KT refers to the long-term control culture. All individuals are from the genus *Eisenia* (n = 9). Aph is the outgroup.

	1	2	3	4	5	6	7	8	9	10
1 AY874523 <i>fetida</i>	-									
2 AY874522 <i>fetida</i>	0.00000	-								
3 AY874512 <i>andrei</i>	0.14542	0.14542	-							
4 AY874511 <i>andrei</i>	0.14706	0.14706	0.00327	-						
5 AY874492 <i>eiseni</i>	0.18791	0.18791	0.20915	0.20752	-					
6 AY874491 <i>eiseni</i>	0.18464	0.18464	0.21078	0.20915	0.00817	-				
7 Cd2	0.14802	0.14802	0.00779	0.00390	0.20386	0.20574	-			
8 Cd1	0.14405	0.14405	0.00390	0.00000	0.19986	0.20173	0.00382	-		
9 KT1	0.14216	0.14216	0.00000	0.00390	0.20195	0.20382	0.00763	0.00382	-	
10 Aph	0.20823	0.20823	0.19285	0.19472	0.19234	0.19203	0.19847	0.19466	0.19275	-

6.4. CONCLUSION

Future attempts to elucidate potential mechanisms of resistance to heavy metals at the genetic level in oligochaetes should strive to use specific markers such as the *mt-2* gene by designing specific primers for the species under investigation. DNA polymorphism studies in that case, should be preceded by the monitoring of gene expression after sub lethal acute metal exposure, to be able to locate and identify the different genes involved in the reduction of metal toxicity in these organisms.

Regarding species delimitation between (*E. fetida* and *E. andrei*) and the inherent ecotoxicological consequences, it should be recommended that thorough genetic

examination of laboratory stocks be always undertaken in order to avoid discrepancies between comparative studies and misleading recommendations and/or conclusions.

6.5. References

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7. GENERAL DISCUSSION

Belfiore & Anderson (2001) define adaptation as a change in gene or genotype frequency in a population exposed to a specific hazard in order to improve survival despite the continuous presence of the hazard. During the present study it was found that a long-term Cd exposed earthworm population showed signs of potential increased survival as shown by the MTT and the comet assays. However, no support of apparent changes in gene or genotype frequency was found.

Naturally, processes controlling the genetic evolution of a population act in a random manner and are called stochastic processes. According to Newman (2001), these processes depend on factors such as total population size; spatial distribution of individuals within the population; effective population size (potentially mating individuals); mutation and migration rates. Small populations for instance risk enduring random losses of haplotypes, leading to genetic heterogeneity (Van Straalen 1999; Newman 2001). In vertebrates, the estimated mutation rates vary from 1×10^{-10} to 1×10^{-4} mutation per gene per generation (Ayala 1982), which is obviously a very slow process. Estimated mutation rates is not known for earthworms. Stochastic processes could, however, have played a role in the present study as the long-term Cd exposed and control populations were kept in closed containers for more than 10 years and inbreeding could have contributed to the lack of genetic variation in these populations (Charlesworth & Charlesworth 1987).

Medina (2002) introducing the concept of evolution due to pollution, argued that pollution could become the driving force behind evolution mainly by increasing mutation rates within affected population. All forms of pollution unfortunately do not promote the occurrence of mutations. In those case where mutagenic substances are prone to cause mutations, an increase of genetic variation has been recorded. Inversely, some forms of pollution even exert directional selection promoting tolerant genotypes over sensitive ones (Van Straalen & Timmermans 2002). The subsequent consequence is a decrease in genetic variation within affected populations (Van Straalen 1999). This might have been

the case in the present study where low mean of heterozygosity have been recorded using allozyme polymorphism in the long-term metal contaminated population. Van Straalen & Timmermans (2002) and Van Straalen (1999) coined the expression “genetic erosion” to designate the loss of genetic variation in a population due to directional selection, genetic drift or inbreeding. Together with the loss of genetic variation, the population also loses its ability to later adapt to potentially new kinds of stresses. In the present study, we were not able to determine the precise effect of the stressor Cd at the genetic level of these populations either with allozyme or DNA polymorphism. The chosen markers seemed not to be selectable by Cd. This might be why recorded lack of genetic variation was similar in all populations investigated regardless of prior exposure to the metal or the lack thereof. A successful use of a more specific marker such as the *mt-2* gene would have been more informative regarding the development of metal resistance in the metal exposed group.

However, similar values of low mean heterozygosity in these populations do not necessarily imply that they are genetically the same. Allozyme and DNA polymorphism simply revealed that these populations lack genetic diversity but do not give in depth insight as to which genes or haplotypes they have in common. A constraint in this regard was that we only scored 7 enzyme encoding loci (one of them, *LGG* displaying two alleles) in the allozyme polymorphism study and sequenced only one gene in the DNA polymorphism study. There is also a substantial chance that genetic drift acted differently on the long-term metal exposed population and the rest of the populations. Inbreeding and founder effect (subsequent to a genetic bottleneck when these populations were created in the laboratory) could have influenced such genetic drift in the control population while, inbreeding, founder effect and Cd could have been the selecting forces in the long-term metal exposed group. Finding a genetic marker that will portray the selection pressure of Cd alone is critical to further investigating potential changes in genotype frequencies due to this metal.

In terms of genotoxicity, the comet assay indicated increased tolerance to Cd effects in earthworm specimens from the long term metal exposed group as opposed to individuals from the control group. These findings could be explained if Cd sensitive haplotypes

were lost in the population that has been chronically exposed to that metal for over 78 generations. It can therefore perhaps be assumed that the control population has kept its Cd sensitive haplotypes.

These results however do not indicate genetic differences between the two populations i.e. occurrence of genetic adaptation in the Cd stressed population. They can only be a reflection of the ability of Cd to instigate DNA breaks on the genetic material of these populations. Single strand DNA breaks, as assessed by the comet assay in the present study, are repairable with the help of DNA repair mechanisms (Eastman & Barry 1992; Van Goethem *et al.* 1997; Lodish *et al.* 1999). This assay therefore assesses repairable structural DNA damages as opposed to fairly permanent DNA changes such as mutations and chromosomal aberrations that could have a greater influence in the development of metal adaptation mechanisms (Van Goethem *et al.* 1997). A recommended test and a molecular method for investigating such DNA variations are the micronucleus test and the assessment of microsatellite variations (Van Goethem *et al.* 1997; Van Straalen & Timmermans 2002). These methods could be investigated in future.

Increased Cd induced genotoxicity as depicted by the comet assay and cell viability as shown by the MTT assay, reflects increased fitness by the Cd-exposed population. Increased fitness however is believed to correlate with increased heterozygosity (Kopp *et al.* 1992). In the present Cd-exposed population this was probably not the case because of possible directional selection on Cd sensitive haplotypes. Because of its low mean heterozygosity, the Cd-stressed population might perhaps have lost its adaptive potential. Shirley & Sibly (1999) reported cases of *Drosophila melanogaster* that had been reared for 20 generations on Cd contaminated medium and could not subsequently perform well in terms of growth and reproduction on uncontaminated media. The present long-term Cd-exposed earthworm population might be undergoing a similar loss of adaptive potential. The choice or the discovery of exclusive genetic markers will be deterministic in the future of research investigating resistance to metals in oligochaetes (Belfiore & Anderson 2001 and Van Straalen & Timmermans 2002). While neutral markers will give an insight into the operating of stochastic processes between investigated populations, selectable makers will help in determining exclusive stressor effects.

Furthermore, our findings strongly suggest that the *Eisenia* species housed in the Ecotoxicology laboratory at the University of Stellenbosch could be *E. andrei* rather than *E. fetida*. This however needs to be confirmed by comparative genetic studies using clearly identified populations of these two species. The failure to monitor MT-2 biosynthesis between selected populations of *E. andrei* (chapter 5) could even have come from the fact that the acquired antibody was only able to probe *E. fetida* MT-2 isomer and not *E. andrei*'s. Finally, because the OECD guideline for testing of chemicals (OECD 1984) recommends that *E. andrei* be used preferably to *E. fetida* and because of physiological, biochemical and genetic differences reported between the two former subspecies, there is perhaps a need to revise recommendations about the choice of either of the two species for ecotoxicological testing (Bundy *et al.* 2002; Dominguez *et al.* 2005 and Pérez-Losada *et al.* 2005).

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8. APPENDIX

Raw data and additional information

(See attached CD. All files are in PDF format)

Appendix A

- MTT assay raw data
- **Table 1.** Multiple comparisons of p values (after Kruskal-Wallis test) between treatments from both the long-term Cd-exposed population and the control population.

Appendix B

- Comet assay raw data
- **Table 1.** Multiple comparisons of p values (after Kruskal-Wallis test) between treatments from both the long-term Cd-exposed population and the control population (using tail length)
- **Table 2.** Multiple comparisons of p values (after Kruskal-Wallis test) between treatments from both the long-term Cd-exposed population and the control population (using tail moment)
- **Table 3.** Multiple comparisons of p values (after Kruskal-Wallis test) between treatments from both the long-term Cd-exposed population and the control population (using tail DNA percentage)

Appendix C

- Allozyme polymorphism raw data (file name: Allozymes data file)

Appendix D

- DNA polymorphism raw data used to assess Cd effects at the COI gene between the long-term Cd-exposed population and the control population (File name: Nexus file_Cd effects_COI gene)
- DNA polymorphism raw data used for species identification (File name: Nexus file_Species identification_COI gene)