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IN VITRO PRIMARY CULTURE OF *EISENIA FETIDA* COELOMOCYTES
AS A TOOL FOR SOIL HEALTH ASSESSMENT USING NEUTRAL RED
RETENTION ASSAY

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Project Presentation

The present work was developed as an integrated part of the PhD project of Amaia Irizar in the Laboratory of Cell Biology and Histology of the Department of Zoology and Animal Cell Biology at the University of Basque Country, which was initiated in September of 2009. The mentioned PhD project embraces multidisciplinary approaches for the study of the earthworm *Eisenia fetida* as a whole-organism and particularly their immune coelome cells (coelomocytes) as potential tools in the development of valid, reliable and cost-effective methodologies for soil health assessment.

Publications

The present work has been partially presented as a poster in the Europe 20th Annual Meeting of the Society of Environmental Toxicology and Chemistry held in Sevilla (Spain) from 23-24 of May in 2010: Amaia Irizar, Daniel Duarte, Ionan Marigómez, Manu Soto. In vitro culture of *Eisenia fetida* coelomocytes (immune cells) as a reliable and cost-effective tool for soil health assessment.

Abbreviations

A	ampere
A. caliginosa	Aporrectodea caliginosa
Cd	cadmium
CO₂	carbon dioxide
Cu	copper
DNA	deoxyribonucleic acid
E. andrei	Eisenia andrei
E. fetida	Eisenia fetida
FBS	fetal bovine serum
HBSS	Hank's balanced salt solution
hr	hour
Hz	hertz
L. rubellus	Lumbricus rubellus
L. terrestris	Lumbricus terrestris
L-15	Leibovitz-15 medium
NCS	newborn calf serum
Ni	niquel
NR	neutral red
NRH⁺	protonated neutral red
NRR	neutral red retention
NRRT	neutral red retention time
NRSS	neutral red stock solution
°C	degrees celsius
Pb	lead
RCF	relative centrifugal force
RN	rouge neutre
RVN	retenção de vermelho neutro
RRN	rétenção du rouge neutre
SD	standard deviation
V	volt
VN	vermelho neutro
W	watt

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Resumo

Os metais são elementos ubíquos nos ecossistemas podendo causar efeitos deletérios diversos em diferentes níveis de organização biológica. Durante décadas as minhocas de solo como a *Eisenia fetida* foram conhecidas como sendo importantes bioindicadores e sentinelas de poluição de solos. Os celomócitos, células imunitárias livres da cavidade do celoma, tem recebido especial atenção em estudos sobre efeitos ecotoxicológicos devido à sua sensibilidade a compostos tóxicos, nomeadamente metais. A maioria das experiências de avaliação sobre o efeito de poluentes é limitada. O desenvolvimento de um protocolo *in vitro* pode fornecer um método rápido na análise de poluentes evitando a exposição de organismos. A medição da estabilidade membranar dos lisossomas através do teste de retenção de vermelho neutro (RVN) é um dos biomarcadores de minhocas de solo mais usados. O colorante vermelho neutro (VN) é uma base acidotrópica fraca que se acumula em compartimentos intracelulares ácidos como os lisossomas através do mecanismo “ion-trapping”. A integridade da membrana lisossômica é avaliada através da quantificação de VN retido pelo organelo. Este método tem sido usado sobretudo em celomócitos derivados da exposição *in vivo* de minhocas de solo. Mas a optimização do RVN para exposições *in vitro* de celomócitos pode permitir a avaliação de solos de campo ou de laboratório contaminados para identificar os seus contaminantes e perfil toxicológico. Este último pode vir a ser integrado na Regulação da Comissão Europeia REACH. Assim o presente trabalho consistiu na tentativa de optimizar e aplicar o RVN no estudo dos efeitos *in vitro* de poluentes metálicos de solos (cádmio, cobre, níquel e chumbo) na estabilidade de membrana dos lisossomas em culturas primárias de celomócitos de *E. fetida*.

Abstract

Metals are ubiquitous elements in ecosystems that may cause a great variety of deleterious effects at different levels of biological organization. For decades earthworms like *Eisenia fetida* are known as an important bioindicator and sentinel of soil pollution. Coelomocytes, free flowing immune cells of the coelomic cavity, have been given special attention for ecotoxicological effects studies due to their sensibility to toxicants exposure, namely metals. Most of experiments carried out to assess pollutant effects are barely scarce. Development of an *in vitro* protocol could provide a faster method for testing pollutants and would avoid the exposure of whole organisms. The measurement of the lysosomal membrane stability using the neutral red retention assay (NRR assay) is one of the most used earthworm biomarkers. The neutral red (NR) is an acidotropic weak base dye that accumulates in intracellular acid compartments like lysosomes through the ion-trapping mechanism. So the lysosomal membrane integrity is evaluated by the quantity of NR retained. This method has been applied mainly in coelomocytes derived from *in vivo* exposures of earthworms. But the optimization of the NRR assay for *in vitro* exposures of coelomocytes can permit the evaluation of field or artificially contaminated soils to identify soil contaminants and establish their toxic profile. The latter could be integrated in the European Commission Regulation REACH. Taking this into account in this work it was attempted to optimize and use the NRR assay to study *in vitro* the effects of described soil metal pollutants (cadmium, copper, nickel and lead) on the lysosomal membrane stability on coelomocyte primary cell cultures from *E. fetida*.

Résumé

Les métaux sont des éléments omniprésents dans les écosystèmes peut avoir des effets délétères sur les différents niveaux d'organisation biologique. Pendant des décennies, les vers de terre *Eisenia fetida* sols qui étaient connus pour être des bio-indicateurs importants de sentinelles et de la pollution des sols. Le celomocyte, les cellules immunitaires coelome cavité libre, a reçu une attention particulière dans les études des effets écotoxicologiques en raison de leur sensibilité à des composés toxiques, en particulier des métaux. La plupart des expériences sur l'évaluation des effets des polluants est limitée. Développement d'un protocole *in vitro* peuvent fournir une méthode rapide dans l'analyse des polluants en évitant l'exposition des corps. La mesure de la stabilité de la membrane lysosomale par essai de rétention du rouge neutre (RRN) est l'un des biomarqueurs utilisés vers de terre pour le sol plus. Le colorant rouge neutre (RN) est une base faible acidetropique qui s'accumule dans les compartiments intracellulaires acides tels que les lysosomes par le mécanisme de "ion-trapping". L'intégrité de la membrane lysosomale est évaluée par la quantification RN retenus par l'organite. Cette méthode a été utilisée principalement pour celomocytes dérivés de l'exposition *in vivo* des vers de terre dans le sol. Mais l'optimisation de la RRN pour les expositions celomocytes *in vitro* peuvent permettre l'évaluation des sols contaminés sur le terrain ou en laboratoire pour identifier les contaminants et profil de toxicité. Celui-ci pourrait être incluse dans le règlement REACH de la Commission Européenne. Le présent travail était de tenter d'optimiser et de mettre en œuvre la RRN étude *in vitro* des effets des polluants métalliques dans les sols (cadmium, cuivre, nickel et plomb) dans la stabilité de la membrane des lysosomes dans des cultures primaires de celomocytes *E. fetida*.

Chapter 1 - Introduction

1.1. State of the art

In the last decades it has been evident the effects arising from human and industrial activities in different habitats. Anthropogenic or natural compounds with potential to provoke adverse effects in biological systems have raised concerns. Among them are several specially relevant organic compounds (e.g. polybrominated diphenyl ether), pesticides (e.g. polychlorinated biphenyl) and metals (e.g. cadmium, copper, nickel, lead) (Keith and Telliard 1979).

1.2. Metals, ecosystems and toxicity

Metals are a category of pollutants that are consistently delivered to the environment and retained in various biological matrices from different sources (Shaw and Haddad 2004, Chen *et al.* 1997).

In natural environment, cadmium (Cd) content in igneous rocks is generally low (Pan *et al.* 2010). More than 90% of Cd in the surface environment is from anthropogenic sources including rock phosphate fertilizer, the ash from fossil-fuel combustion, waste from cement manufacture and metallurgical works, municipal refuse and sewage sludge, and atmospheric deposition. Actually in industrialized countries, the former and later alone account for over 90% of the anthropogenic sources of cadmium (Pan *et al.* 2010). Chronic exposure to Cd at low levels may result in completely different toxic effects than the high levels that are sufficient to cause diseases like 'itai-itai' (osteoporosis-like bone disease) (Pan *et al.* 2010). Cd has recently been shown to be an endocrine-disrupting chemical with estrogenic properties and a potential prostate carcinogen. In addition to being persistent and toxic, Cd is bioaccumulated at high concentrations particularly in the kidney of mammals as also in the chloragogenous tissue of earthworms (Pan *et al.* 2010).

Copper (Cu) is used as a metal or alloy in machinery, construction, transportation, and military weapons and is an important component of white gold and other alloys used for imitation jewellery. Cu is also used in dental products, intrauterine devices, and in cosmetics (Gaetke and Chow 2003). One of the best-known consequences of Cu toxicity is peroxidative damage to membrane

lipids (Gaetke and Chow 2003). Reactive oxygen species production, DNA damage, liver cirrhosis, hemolysis and damage to renal tubules, brain, and gastrointestinal tract have been also reported (Linder and HazeghAzam 1996).

Due to unique physical and chemical properties, nickel (Ni) and its compounds are widely used in modern industry (electroplating, electroforming, nickel-cadmium batteries, electronic equipment, nickel alloys, coins casting, jewellery and medical prostheses). The high consumption of nickel-containing products inevitably leads to environmental pollution by nickel and its by-products at all stages of production, recycling and disposal. Nickel can affect several organs including cardiovascular and respiratory systems, skin and kidneys. It has also shown teratogenic and carcinogenic potential (Denkhaus and Salnikow 2002).

Lead (Pb) is a metal with neuropsychological and cardiovascular effects, nephrotoxic, carcinogenic and can directly affect reproduction (Goyer 1993). One of the main targets for lead toxicity is the bones.

Depending on the source of pollution and the process from which it derives its emission metals may reach different compartments of ecosystems: atmosphere, hydrosphere or lithosphere (Schroeder and Munthe 1998, Babel and Kurniawan 2003, Basta and Gradwohl 2000).

1.3. Metals in soils and fauna

Metal compounds can enter the soil from different sources. Fertilizers, pesticides, organic and inorganic amendants, wastes and sludge residues can contain variable amounts of these metals (Paoletti 1999). It is worrying that in common soil conditions metals mostly exist as mixtures of several contaminants depending on the source of pollution (Spurgeon, Hopkin and Jones 1994).

The soil is not only a means to role agriculture or a container to dump unwanted materials, but also an important transmitter of various pollutants into all its contiguous systems (Chen *et al.* 1997). The soil pollution represents a menace to the quality of human and other living beings health since ultimately all end up consuming environmental components (Ruby *et al.* 1999).

Animals tend to uptake contaminants as a function of space, time, and characteristics of the surroundings and eventually display a wide variety of biological responses to exposure (Talmage and Walton 1991). There are various

ways in which organisms deal with metals depending on metal dependency, pre-exposure or tolerance induction, species dependency, relevance to trophic transfer in invertebrate food chains and link to uptake routes (Vijver *et al.* 2004). Although some metals are necessary (e.g. Zn, Fe, Cu) in a certain range of concentrations for all living organisms, most of them present toxicity hazard at high concentrations (AbdulRida and Bouche 1997).

Among the most representative animals of soil fauna and one of the most interesting indicator of metals in soil environments are the earthworms (AbdulRida and Bouche 1997). Sentinels serve to map the bioavailable fraction in an ecosystem by retaining the pollutant in their tissues (Beeby 2001). In this scope, the earthworm represent a relevant sentinel species to determined the amount of toxicity linked to soil contaminants or to test the efficacy of remediation protocols (Brousseau *et al.* 1997).

1.4. Soil pollution assessment

The main objective in ecotoxicological work is to provide information to estimate ecological risk (Xiao *et al.* 2006). The complexity of soil ecosystem and its variability in space and time is such that it is very difficult to assume that soil pollution analyses can be used as a tool to predict biohazards (AbdulRida and Bouche 1997). Thus, analyses of biological material assume an important role in ecotoxicology. Earthworms are one of the organisms that have had a notable contribution in terrestrial ecotoxicology (Sanchez-Hernandez, 2006). They have been broadly used to assess environmental impact from metal pollution, and they are typical test organisms in standardized toxicity tests (Marino and Morgan 1999).

1.4.1. Earthworms – a pivotal model organism

The terrestrial invertebrate fauna has a pivotal role in the services provided by soil ecosystems (Lavelle *et al.* 2006). A clear proof of that are the earthworms who have the status of “ecosystem engineers” (Jones, Lawton and Shachak 1997).

In many temperate and tropical ecosystems earthworms are the most important member of soil fauna being involved in the regulation of

decomposition and nutrient cycling processes, and modifying their physical properties (Lavelle and Spain 2002). In fact, earthworms are a special case of the importance of invertebrate fauna in ecosystems by exerting effects on the microbial number, biomass, dispersion, decomposition activity, and on the structure, fertility and soil productivity. The earthworm populations, soil volume, microbial and invertebrate populations influenced directly or indirectly by earthworm activities are termed drilosphere (Brown and Doube 2004). By their activity of ingestion of various biological soil fractions (dead plants, microorganisms, humus, possibly fine living roots and most mineral fractions) an assimilable fraction is taken up and partially excreted, so their body content reflects the bioavailability of soil contaminants (AbdulRida and Bouche 1997).

Terrestrial invertebrates are known to be efficient accumulators of metals and to respond to pollution in a sensitive and measurable manner therefore widely used as sentinels of environmental pollution in soils. Several authors suggested that earthworms can be used in soil health assessment in the manner that mussels and other molluscs are used in marine pollution monitoring programs. A great deal of studies have established the ability of *Eisenia fetida* (Neuhauser *et al.*, 1985; Spurgeon and Hopkin, 1995; Lock and Janssen, 2001), *Aporrectodea caliginosa* (Khalil *et al.*, 1996) and *Lumbricus rubellus* (Svendsen and Weeks, 1997b; Langdon *et al.*, 2001) to accurately reflect environmental metal levels in soils. Thus, earthworms are sentinels whom biomarkers of exposure and effect recorded can indicate soil health.

The specie *E. fetida* (Fig. 1) is easily maintained in laboratory conditions and is the normally chosen earthworm because of the standardization of acute and chronic ecotoxicological assays (Sauve *et al.* 2002, Xiao *et al.* 2006). It is considered a suitable model species and is prescribed as test organism (OECD 1984).

1.5. Hierarchical responses to metal stress

Ecotoxicology is based on a central paradigm: the existence of a differentiated cascade of biological responses, i.e. a hierarchical cascade that relates the severity of exposure to chemicals with the various levels of biological

organization in which the effects can be measured (Fig. 2A) (Spurgeon *et al.* 2005).



Figure 1. *Eisenia fetida*, showing the classic coloured ring pattern of the more common species living in manure and compost. Adapted from Paoletti (1999).

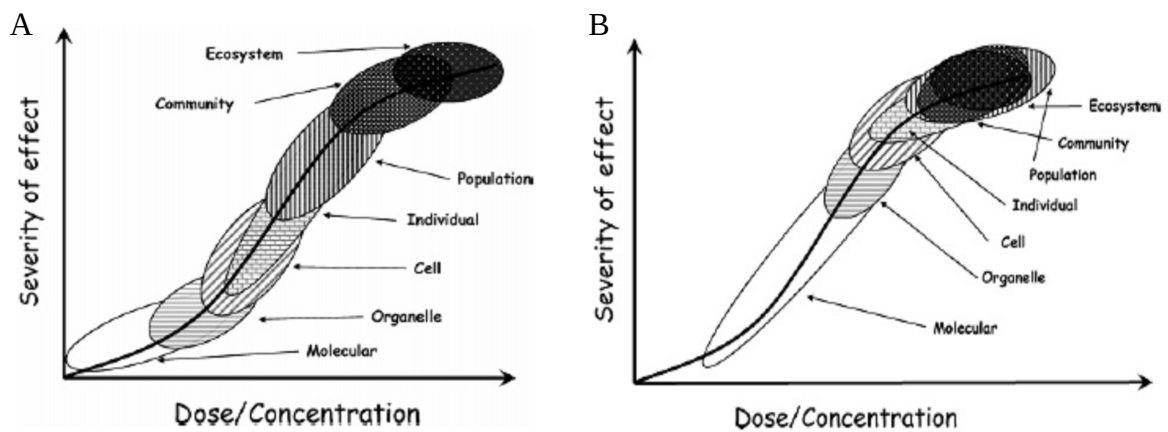


Figure 2. Schematic diagram of the hierarchical relationship between ecotoxicological responses measured at different levels of biological organization (A) and the sensitivity relationship of responses at different levels of biological organization based on comparative analysis (B). Adapted from Spurgeon *et al.* (2005).

Normally a severity of an effect is associated with greater levels of biological hierarchy but in an interesting study Spurgeon and colleagues (2005) concluded that from the retrospective risk assessment and monitoring point of view the low organization-level effects offer a potential advantage of inherent sensitivity over other measuring “higher” endpoints (e.g. population, reproduction, diversity). The processes and mechanisms present at a given level ultimately can assume considerable consequences at higher levels of biological organization. But what appears to be commonly accepted is the significant value of the lower levels of biological states when one considers the effects and influences on higher levels of organization (Decaens *et al.* 2006). Accordingly, acute toxicological tests on earthworms are useful to establish contamination levels, but for a proper protection of the environment, it is necessary to develop more sensitive markers of toxicity (Sauve *et al.* 2002). That is why several reviews and international workshops have stressed the need for increasing understanding and applicability of earthworms’ biomarkers in the ecological risk assessment process (Sanchez-Hernandez, 2006).

1.5.1. Biomarkers

To assess toxic responses in the heterogeneous, complex, and often unique conditions found in the field, a number of biomarkers have been established in an effort to close the gap between results obtained in field and laboratory studies (Maleri *et al.* 2008).

The use of biological markers or biomarkers measured at the molecular or cellular level have been proposed as sensitive “early warning” tools for biological effect measurement in environmental quality assessment (McCarthy and Shugart 1990). The selected biomarkers should indicate that the organism has been exposed to pollutants (exposure biomarkers) and/or the magnitude of the organism’s response to the pollutant (effect biomarkers or biomarkers of stress). Biomarkers, which address sub-cellular alterations, have been evaluated previously in several studies and correlated well with endpoints investigated at higher physiological levels demonstrating its anticipative features (Maleri *et al.* 2008). Then they are defined as short-term indicators of long-term biological effects. Although the quantitative nature of homeostasis relationships is uncertain, this concept underlies the relevance of biomarker analysis as they offer

the possibility to determine where the organism is situated in this continuum and can indicate early deviations of the “normal” functioning of biota (De Coen, Janssen and Giesy 2000).

Immunotoxic effects of environmental exposure to chemical contaminants can be evaluated by monitoring cellular and functional features of immune system of sentinel species (Brousseau *et al.* 1997).

1.5.2. Coelomocytes

Earthworm coelomic fluid, a part of the hydrostatic skeleton, acts as communicator between the inner and outer milieu, plays an important role in homoeostasis maintenance and contains an abundant population of immunocompetent cells, coelomocytes (Kurek *et al.* 2007). Oligochaeta coelomocytes are characterized by pronounced variability; their quantitative and qualitative composition changes depending on environmental factors, the age of the worm, and their physiological condition (Di Marzio *et al.* 2005). It has been shown that different pollutants (organic and inorganic residues) can perturb integrity and functions of earthworm coelomocytes, and that these responses can be used as biomarkers of sub-lethal, chemical-induced stress (Sample *et al.* 1999, Diogene *et al.* 1997).

1.5.2.1. Classification

The classification of earthworm coelomocytes is largely based on differential staining, ultrastructure and granule composition, as well as behavioural traits such as adherent and chemotaxis. The origin and relationships of the main populations of coelomocytes, namely amoebocytes and eleocytes are not yet completely known (Fig. 3). Perhaps amoebocytes derive from the mesenchymal lining of the coelom while eleocytes originate by the detachment of chloragogen cells covering the intestinal tract. All these coelomic cells are involved in various aspects of cellular and humoral immunity: the former by phagocytosis, encapsulation, and cytotoxicity, and the latter by secretion of antimicrobial substances (Cholewa *et al.* 2006).

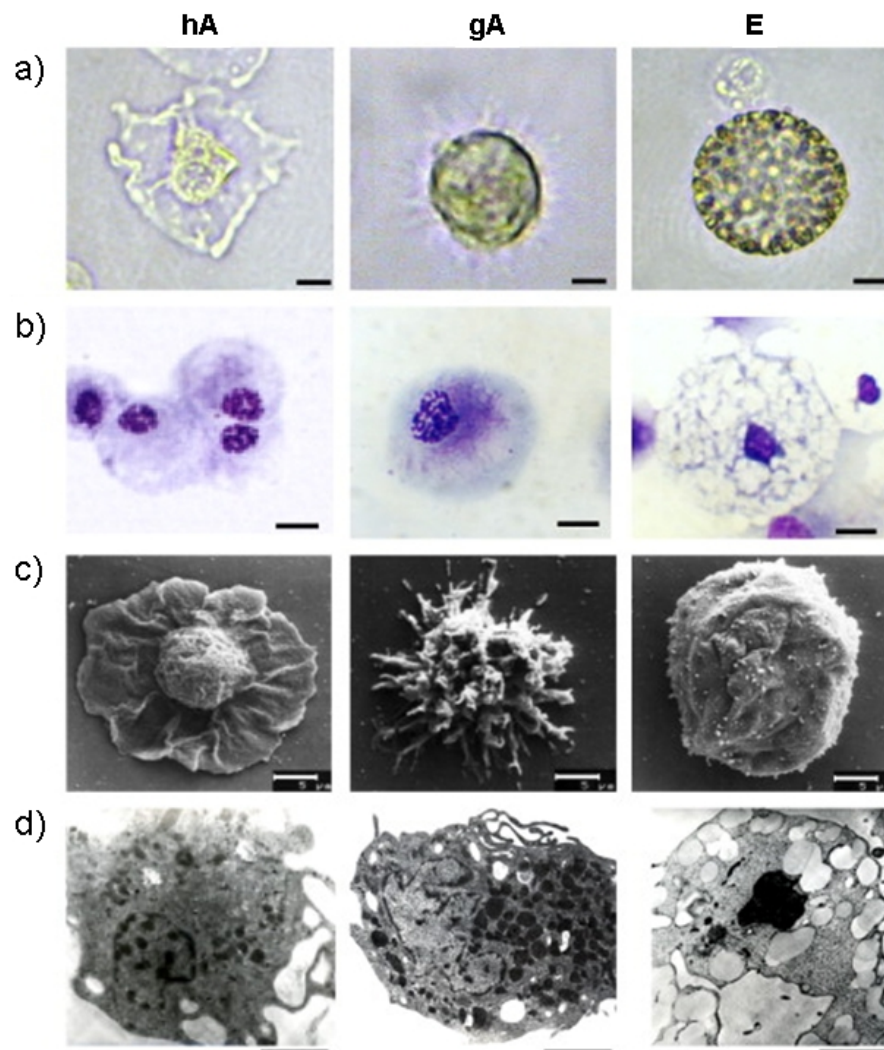


Figure 3. Morphology of coelomocytes of *Allolobophora chlorotica* (E, eleocytes; hA, hyaline amoebocytes; gA, granular amoebocyte; a) vital cells in haemocytometer; b) MGG stained cytopspin preparations; c) SEM preparations; d) TEM preparations; scale bars equal 5 μ m). Adapted from Kurek *et al.* (2007).

1.5.2.2. Usefulness in soil health assessment

Increased metal levels in the environment lead to a depletion in immune function in earthworms. However, the coelomic fluid of earthworms is not aseptic and the earthworms must continually resist to bacterial growth to remain healthy (Sauve *et al.* 2002) even when environmental metal levels are altered. The collection of viable earthworm coelomocytes is easy and clean, making these fluid-suspended cells suitable for developing *in vitro* tests (Eyambe *et al.* 1991).

To assess the environmental damage caused by toxic substances in such a complex system as soil, biomarkers are often applied within a battery of different tests, addressing different ecotoxicological endpoints (Maleri *et al.* 2008). One of the possibilities to reach this purpose is to use the potential of *in vitro* methodologies. And although whole-organism exposure may more closely simulate real-world conditions and subsequent biological responses, there is pressure to develop rapid and highly sensitive cell-based *in vitro* assays (Burch *et al.* 1999). For example recently it has been reported that the coelomocyte populations varies as the proportion of amoebocytes/eleocytes are influenced when the animal is exposed to metals (Homa *et al. in press*).

1.6. *In vitro* in ecotoxicology

Toxicity testing in animals is unpopular with general public, and is therefore unpopular with politicians and with the public relations departments of industrial companies. There are three main reasons for this: many toxicity tests inevitably result in animal suffering, many are required by laws, guidelines and regulations, and the performance of many toxicity tests is routine, without case-by-case scientific justification, or even any scientific justification at all (Balls and Fentem 1999). However, techniques involving *in vitro* tests are increasingly used as alternatives to whole animal toxicity tests due to their reduced use of experimental animals, low cost, and rapid performance (Bhanushali *et al.* 2010). Moreover, *in vitro* models allow the use of specific endpoints to determine the targets of toxic effects with great precision and reproducibility (Olabarrieta *et al.* 2001).

In vitro cell, tissue and organ cultures play an important role in science, medicine and industry then assuming vast applications (van der Valk *et al.* 2004, Hartung *et al.* 2002). Actually animal cell culture was acknowledge as an important tool as soon as it was first established for the production of viral vaccines in the 1940s/50s (Falkner *et al.* 2006). Cultured human and animal cells are increasingly used as the basis for simplified, direct test systems that have the potential to be more controllable and more reproducible than test systems employing animals (Hartung *et al.* 2002, Falkner *et al.* 2006).

The accumulation of metals in earthworm tissues depend on their feeding behaviour and the selection of soil fine fractions. The selectivity of ingested materials by earthworms depends on their different ecological behaviour or ecological categories (AbdulRida and Bouche 1997). This is one of the major issues that can be surpassed by using *in vitro* cell cultures. In respect to coelomocytes, direct *in vitro* exposure provides a more sensitive complement to *in vivo* exposure assays, capable of detecting the potential immunotoxicity of contaminants at very low concentrations and in very small volumes of material. A single coelomocyte biomarker offers a wide range of sensitivity, and should be useful in the identification and/or assessment of the toxicity of different contaminants present in soils (Burch *et al.* 1999).

In conclusion, measuring responses to pollutants with coelomocytes *in vitro* might provide a powerful tool for ecotoxicological studies (Brousseau *et al.* 1997).

1.7. Viability/Cytotoxicity assays

The viability of coelomocytes is one of the most promising surrogate assays to assess immunotoxic risks (Homa, Niklinska and Plytycz 2003). Cytotoxicity assays have been developed to use different parameters associated with cell death and proliferation (Weyermann, Lochmann and Zimmer 2005). Many viability assays have been used to evaluate the cell “well-being” as it is generally known as “cell viability”. Some of those viability assays include the famous trypan blue dye exclusion test where viable cells prevent the accumulation of the dye inside them which is analysed through microscope observation. The most convenient, modern assays have been optimized for the

use of microtiter plates (multi-well format) allowing the reading of many samples which are therefore analysed rapidly and simultaneously (Weyermann *et al.* 2005).

At the cellular level, biotic systems have evolved control mechanisms to minimize accumulation of reactive metal species and to facilitate optimal utilization of essential metals. This is achieved by the various chemical forms in which metals can be present in an organism, including the following:

- (1) free ionic form or complexed ion species (e.g., CdCl^2 , CdCl^+ , CdCl^3);
- (2) bound in the active center of functional proteins (e.g., haemoglobin, hemocyanine) and low molecular weight peptides (e.g. zinc finger proteins);
- (3) bound into the active center of enzymes (e.g. cytochromes, carbonic anhydrase, superoxide dismutase);
- (4) bound to low molecular weight organic acids (e.g. citrate);
- (5) bound to metallothionein, to transport proteins (e.g. ferritin), or other sequestration proteins;
- (6) precipitated in extracellular granules, mineral deposits, residual bodies, and exoskeletons;
- (7) bound to cellular constituents potentially causing dysfunction (enzymes, ion channels, DNA);
- (8) bound in vesicles of the lysosomal system, as intracellular granules (Vijver *et al.* 2004). In fact it has been reported that the first detectable alterations caused by pollutants are linked to lysosomes before any other effect on physiological parameters can be observed (Cancio *et al.* 1995).

Although cytotoxicity tests evaluating cellular dysfunction or cell death with coelomocytes from single earthworm can be planned, a pool of cells from different animals could also be used. However, when pooling coelomocytes from different earthworms of the same species, one may have to consider possible inter-individual incompatibilities as reported for *L. terrestris* (Diogene *et al.* 1997).

1.7.1. Neutral Red based assay

Perhaps the most applied assay that demands the use of NR is the *neutral red retention time assay* (NRRT assay). Evidence from published literature on the NRRT assay supports it as a suitable monitoring tool for assessing the effects of pollutants in soils (Svendsen *et al.* 2004). Notwithstanding another similar assay

was developed called neutral red retention assay (NRR assay) is the technique used in this work (Borenfreund and Puerner 1985) which.

The NRR assay was developed with the purpose of obtaining a simple, rapid and sensitive assay based both on morphological and spectrophotometrical criteria in order to easily determine the survival rate of viable cells upon termination of experimental procedures. An attractive added value of the method is that sometimes an inexpensive assay like this is sufficient when more expensive test kit fails (Weyermann *et al.* 2005). The photometrically conducted assay is widely used for the prediction of contaminants toxicity, conveniently carried out in 96-well microtiter plates (Asensio *et al.* 2007). In contrast to the NRRT assay, the NRR assay is linearly dependent on the cell density and is therefore applied preferably on cells in culture (Maleri *et al.* 2008).

1.7.1.1. Lysosomes and membrane stability

Lysosomes are a membrane-delimited organelles which contain an array of enzymes capable of degrading biological polymers (proteins, nucleic acids, carbohydrates, lipids) functioning as a digestive system of the cell, serving both to degrade material taken up from outside the cell and to digest obsolete components of the cell itself (Cooper 2000).

Lysosomal perturbations have been widely used as early indicators of adverse effects to various factors including environmental pollutants (Moore, Allen and McVeigh 2006). In fact, lysosomes are one of the main cell compartments for metal toxicity (Fig. 4). Metals as a relevant pollutant are known to be associated with metal binding proteins which may enter lysosomes and follow the catabolic pathway as any other cellular protein. However, excessive concentration of metal compounds can cause alterations of structure, permeability, and integrity of lysosomal membranes when the storage capacity of the lysosomes is overloaded (Etxeberria *et al.* 1994). Diverse sources of environmental stress (chemical pollution, changes in pH, temperature, malnutrition, reproductive stress) are known to provoke an increase in the size of digestive cell lysosomes (Marigomez, Soto and Kortabitarte 1996). Occasionally, together with the increased lysosomal size, the activity of hydrolases and the

number of lysosomes may result augmented as well (Moore and Viarengo 1987, Marigomez *et al.* 1996).

As for metals, lysosomes have been particularly shown to be involved also in the sequestration of a variety of weakly basic molecules (Duvvuri and Krise 2005). And this is particularly useful considering that its internalization can be measured according to the lysosome membrane stability serving as a reference for cellular “well-being” (Moore *et al.* 2006).

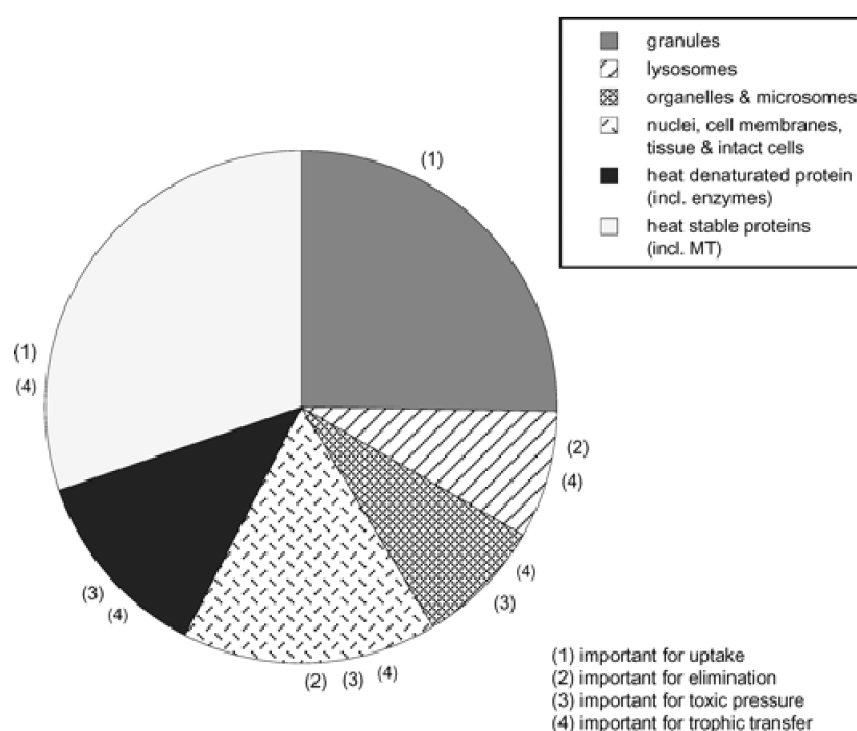


Figure 4. General hypothesis concerning the importance of internal metal fractions in organisms for the description of accumulation patterns, toxicity, and trophic transfer. The size of fractions displayed corresponds to the distribution of Cd commonly found in organisms (Vijver *et al.* 2004).

1.7.1.2. Neutral red as a “lysosomotrophic” agent

The neutral red dye (NR; 3-amino-*m*-dimethylamino-2-methyl-phenazine hydrochloride) is a weak base chemical ($pK_a=6,5$) used for a variety of purposes (Fig. 5). The first reference made to its use seems to go back to 1901 for distinguishing bacteria species (Hunter 1901). But ever since it has been exploited for biological staining, medical purposes to investigate viruses, pH indicator in biochemical systems, in the determination of DNA using optical and electrochemical methods, for the evaluation of naturally-derived and synthetic biomaterials, for the development of optical sensors, etc (Pauliukaite and Brett 2008).

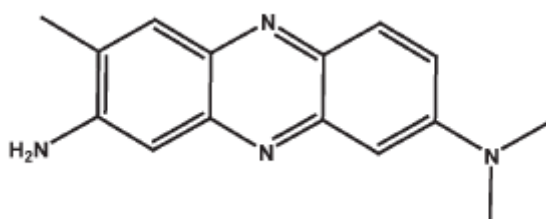


Figure 5. Chemical structure of neutral red monomer (Pauliukaite and Brett 2008).

NR

was the first and probably one of the best known “lysosomotrophic” agents (Ohkuma and Poole 1981). Actually the NR would be better renamed as a “acidotropic” since the main characteristic of this compound is the conversion of the membrane permeable free form (NR) into a non-permeable protonated form (NRH⁺) when exposed to acid environments (Deduve 1983). In this respect assays have been developed to take advantage of this capability.

1.7.1.3. General mechanism underlying the NRR assay

The general mechanism by which the NR is measured by the NRR assay is relatively simple (Fig. 6): the cells previously exposed to a noxious compound are

incubated with NR for a enough period of time for it to accumulate mainly in the lysosomes since its acid compartment is constantly maintained by H^+ -pumps. This accumulation phenomenon is due to the fact that NR after crossing the biological membranes till the lysosomes gets trapped due to its conversion to NRH^+ – ion-trapping mechanism. After the incubation period the cells are washed and then exposed to a NR extraction solution. Finally the relative amount of NR retained by the cells is photometrically measured.

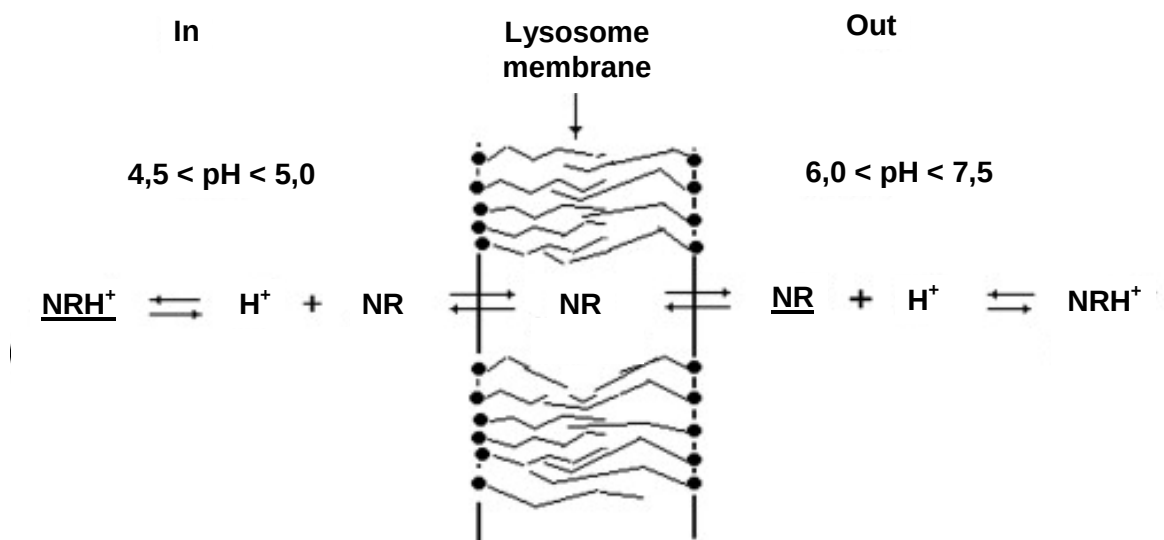


Figure. 6. Schematic mechanism responsible for the entry and accumulation of neutral red in lysosomes. Since the $pK_a = 6,5$ for neutral red tends to predominate in the protonated form inside the lysosomes and in its undissociated form outside. (NR, neutral red; NRH^+ , protonated form of neutral red; $_$, predominant form of neutral red). Adapted from Manente *et al.* (2008).

Chapter 2 – Hypothesis and Objectives

2.1. Hypothesis

The establishment of an *in vitro* culturing protocol for coelomocytes of *E. fetida* could become a reliable tool in order to assess the health of field soils as well as to analyse the toxicity of different chemical compounds using an optimized neutral red retention assay.

2.2. Objectives

In order to fulfil this hypothesis the present work has two main objectives:

1) To develop an *in vitro* protocol for culturing and maintenance of *E. fetida* coelomocytes (immune cells) in order to assess the health status of soils (from the field or laboratory made) and the toxicity profiles of chemical compounds through the neutral red retention assay. In order to fulfil this objective several aspects of the neutral red uptake assay for primary *in vitro* cultures of coelomocytes will be analysed:

- coelomocyte extrusion method
- earthworm pre-depuration
- neutral red incubation time with coelomocytes
- adequate cell concentration for the applied assay
- use of different sera in culture medium
- culture medium renewal

2) To validate the reliability of using the previously developed protocol together with the NRR assay in earthworm coelomocytes to assess the effects produced after a short-term *in vitro* experiment to a wide range of concentrations of essential and toxic metals (cadmium, copper, nickel, lead).

Chapter 3 – Materials and Methods

3.1. Earthworms

E. fetida earthworms were obtained from the stock population in the laboratory that was formerly purchased from a commercial source (Manchaverde SL, Ciudad Real, Spain). The conditions of the stock maintenance were: 19° C and 12:12 light/dark cycle, fed with horse manure as required. The earthworms used in the experiment were all healthy, clitellated and of similar size, (300-600 mg fresh weight). Before using the earthworms, the content of the gut intestinal tracts were cleaned by massaging the posterior body of the animals.

3.2. NRR assay

The cells are incubated for a 1 hr period (except in second experiment) with working solution of NR freshly prepared in an cell incubator at 18 °C with a CO₂-free atmosphere. Then the cells are washed twice with HBSS followed by a 15 min exposure to extraction solution and then shaken for 5 min. Finally the plaque is placed in a spectrophotometer so the NR absorbance can be measured at 540 nm.

3.3. Coelomocyte extrusion methods comparison to determine the extrusion method that yielded more coelomocytes with the greater viability (first experiment).

Ultrasound method: Three animals were placed in individual polystyrene 15 ml falcon tubes with 3 ml of extrusion solution. Then they were placed in an ultrasound bath (4,5 A, 1000 W, 220 V, 1~50 Hz) enough time till the solution became yellow turbid. The worms were removed and the extruded coelomocytes in solution were cleaned through two cycles of centrifugation (212 RCF, 4 °C, 10 min). The remain pellet was re-suspended in HBSS. The coelomocytes counting was carried out by an automatic cell counting equipment (Z2™ Coulter Counter, Beckman Coulter, USA). The NRR assay was carried out following the protocol explained below for 2 x10⁵ cells/well.

Electric method: Three animals were placed in glass Petri dishes with 1 ml/worm of extrusion solution followed by the appliance of an electric current with a 9 V battery for 10 cycles of 3 seconds. The worms were removed and the extruded coelomocytes were cleaned through two cycles of centrifugation (212 RCF, 4 °C, 10 min) using the cleaning solution. The remain pellet was resuspended in HBSS. The coelomocyte quantification was carried out by an automatic cell counting equipment (Z2™ Coulter Counter, Beckman Coulter, USA). The NRR assay was carried out following the protocol explained below for 2×10^5 cells/well.

Table. 1. Solutions used in the the development of this work.

Solutions	Composition
Extrusion solution	HBSS, 0,2 g/L EDTA, ~7,3 pH
Cleaning solution	HBSS, amphotericin B (10 ml/l of 250 µg/ml solution), gentamicin sulfate (5 ml/l of 10 mg/ml solution), penicillin-streptomycin solution (10ml/l of 10.000 units penicillin-G and 10mg streptomycin per ml), ~7,3 pH
Culture medium	L-15 medium, amphotericin B (10 ml/l of 250 µg/mL solution), gentamicin sulfate (5 ml/l of 10 mg/ml solution), penicillin-streptomycin solution (10ml/l of 10.000 units penicillin-G and 10mg streptomycin per ml, l-glutamine (0,3 g/l), ~7,3 pH, ~300 mOsm.
Neutral red stock solution (NRSS)	NR in distilled water (5mg/ml), ~7,3 pH
Neutral Red working solution	NRSS in HBSS (0,5 mg/ml), ~7,3 pH
Extraction solution	1% acetic acid, 50% ethanol

3.4. Assessment of earthworm depuration conditions, coelomocyte number estimation and neutral red incubation time for the optimization of NRR assay (second experiment).

The purpose of this experiment was to determine the most suitable earthworm pre-extrusion treatment (depurated or not depurated), optimum cell-incubation time with neutral red and cell number estimation for using the NRR assay in *in vitro* exposures.

Two sets of earthworms were prepared before coelomocyte extrusion as follows:

- 1 – depurated for 24h in wet filter paper;
- 2 – non depurated before coelomocytes extrusion.

Animals from both sets were then placed in glass Petri dishes with 1ml/worm of extrusion solution (HBSS, Table 1) followed by the appliance of an electric current with a 9 V battery for 10 cycles of 3 seconds. The worms were removed and the extruded coelomocytes in solution were cleaned (HBSS cleaning solution, Table 1) through two cycles of centrifugation (212 RCF, 4 °C, 10 min). The remaining pellet was re-suspended in culture medium (Table 1) the final concentration matching the desired cell concentrations per 200 µl (2×10^5 , 1×10^5 , 5×10^4 and 2.5×10^4 cells/µl). Then, 200 µl of each coelomocyte suspension were deposited in a 96-well plate with five replicas per each cell concentration NR measurement (see below) were done after 0.5, 1, 1.5, 2 and 2.5hr of incubation (18 °C, CO₂-free atmosphere) with cells. A negative control (without cells) was performed for all the previous conditions.

3.5. Assessment of serum containing medium in coelomocyte cultures to determined the best serum for the coelomocyte cultures (third experiment)

After harvesting coelomocytes, according to the previous electric extrusion method, different coelomocyte cultures where established by allowing cells to acclimatize for 24hr in the plaques after their extrusion. Different types of serum supplements as well as a negative control 96-well plaque (without cells) for a

period of two days were tested. The mediums were supplemented with FBS and NCS at 5% and 10% each.

For the NRR assay the culture mediums where replaced by NR working solution and proceeded following the protocol explained above.

3.6. Assessment of culture medium renewal to confirm or not this requirement (fourth experiment)

Following the electric extrusion of coelomocytes they were cultured in the selected medium derived from the third experiment. This medium was daily renewed for 3 days. Another cell culture plaque was used as control where the culture medium was not changed for 3 days. The NRR assay was daily performed.

3.7. *In vitro* metal exposure to Cd, Cu, Ni and Pb to study their cytotoxicity (fifth experiment)

The coelomocyte cultures were established according to the previously tested conditions (experiments first to fourth). Four metals solutions (50 µL of Cd, Cu, Ni and Pb supplied as chloride) were tested in coelomocyte cultures to raise the following final concentrations in the wells for each metal: 0.1, 0.5, 1, 5, 10, 50, 100, 500 and 1000 µg/ ml of metal. Five replicas were carried out for each metal concentration. After 24hr exposure the NRR assay was applied as described above.

3.8. Statistical analysis

For the second experiment a general linear model for correlated data (clustered data) was performed. For the remaining experiments one-way analysis of variance and Student's *t*-test (*, $p < 0,05$) were applied.

Chapter 4 – Results and Discussion

4.1. Extrusion of coelomocytes

A variety of extrusion methods have been used so far to obtain coelomocytes from earthworms. The most used methods are: electric excitation (in 1973), puncture through the integument (in 1974), ethanol irritation (since 1991), ethanol extrusion combined with a low-vacuum holding device (since 1997) and the most recent ultrasound extrusion (since 2004) (Hendawi *et al.* 2004). Each of them has their advantages and limitations but the most used one is the electric shock system. Even though the ultrasound method was described to extrude a greater number of cells and has the advantage of being more cost-effective, it did not exhibit any significant differences when compared with the other techniques (Hendawi *et al.*, 2004). Both methods were compared in our laboratory.

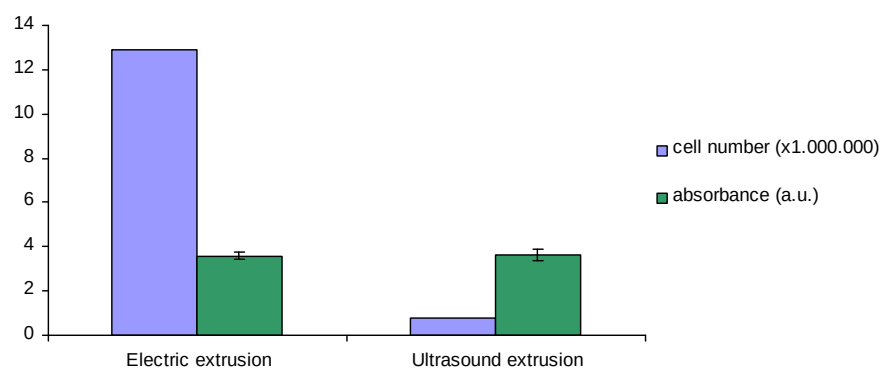


Figure 7. Number of coelomocytes retrieved using different extrusion techniques and the correspondent neutral red retention capacity for 2×10^5 cells/well. a.u., arbitrary units. Mean values are given $\pm$ SD.

The total amount of coelomocytes extruded by the electric extrusion method was clearly higher (~17 times) than the number of cells obtained by the ultrasound method (Fig. 7). These results do not match the ones obtained by Handawi *et al.* (2004) since we did not have access to the setting of the ultrasound equipment used by Hendawi and collaborators given that they are not specified in the published material.

Another drawback was the presence of contamination (fungi, bacteria ...) on the cell cultures after coelomocyte extrusion. The ultrasound method rendered

larger contamination probably due to the damage produced in some earthworms which could release some “inner-bacteria” along with the coelomocytes. After electric extrusion the coelomocytes contamination seemed to be much more controllable together with the earthworm depuration period and the use of antibiotics.

Nevertheless, when comparing both extrusion methods the capability of the cells (2×10^5 cells/well were tested for each method) for retaining NR was very similar suggesting that the viability of the coelomocytes is not affected by the type of extrusion method. Therefore, we strongly recommend the electric extrusion method against the ultrasound method for coelomocyte culturing.

One way of determining the cell concentration of the coelomic fluid extruded from the earthworm is the time-consuming manual counting of the cells by a hemocytometer (Neubauer chamber). However, if such a procedure has to be conducted prior to the microplate assays, the advantage of the simple and rapid determination of cytotoxicological effects of many samples simultaneously is nullified (Maleri *et al.* 2008). Thus it was used an automatic particle counting apparatus.

4.2. Assessment of earthworm depuration conditions, coelomocyte number estimation and neutral red incubation time

The NRR assay has been previously applied in different experiments to assess the health of chronically polluted soils and study the effect of different chemical compounds (metals and organic) under laboratory conditions (Borenfreund and Puerner 1985, Asensio *et al.* 2007). This method was concluded to be a reliable tool under *in vivo* exposures, although some limitations were pointed out. However, the alternative use of this method under *in vitro* exposure conditions (exposure of coelomocytes) requires some modifications.

One of the main problems concerns the depuration period of the earthworms prior to extrusion of coelomocytes. Among the advantages of the 24hr depuration of the earthworms prior to the extrusion of coelomocytes is the cleaning of the attached soil particles and bacteria to the tegument and gut of the animal. This process prevents the contamination of the suspension of extruded coelomocytes. However, the reduction of this depuration time for very short-term *in vitro* exposures might be feasible since microorganism contamination and/or

proliferation could not occur at such time intervals. The reduction of depuration time was verified with the NRR assay.

Another issue of interest deals with the period of incubation of the cells with NR. In a previous study, in an overnight incubation no neutral red dye remained in the lysosomes and no dye was detected photometrically after washing off the excess dye in cytosol and solution (Maleri *et al.* 2008). Spurgeon and colleagues found that the NR incubation times, kept under controlled conditions, differed substantially from species to species (Spurgeon *et al.* 2000). This could indicate that the optimum incubation time for different earthworm species might differ from the 3hr experienced as the most suitable for example to *E. andrei* (Maleri *et al.* 2008). It has also been reported that NR uptake is significantly higher for coelomocytes from *L. terrestris* and *Octolasion tyrtaeum* than that of *E. fetida* (Diogene *et al.* 1997).

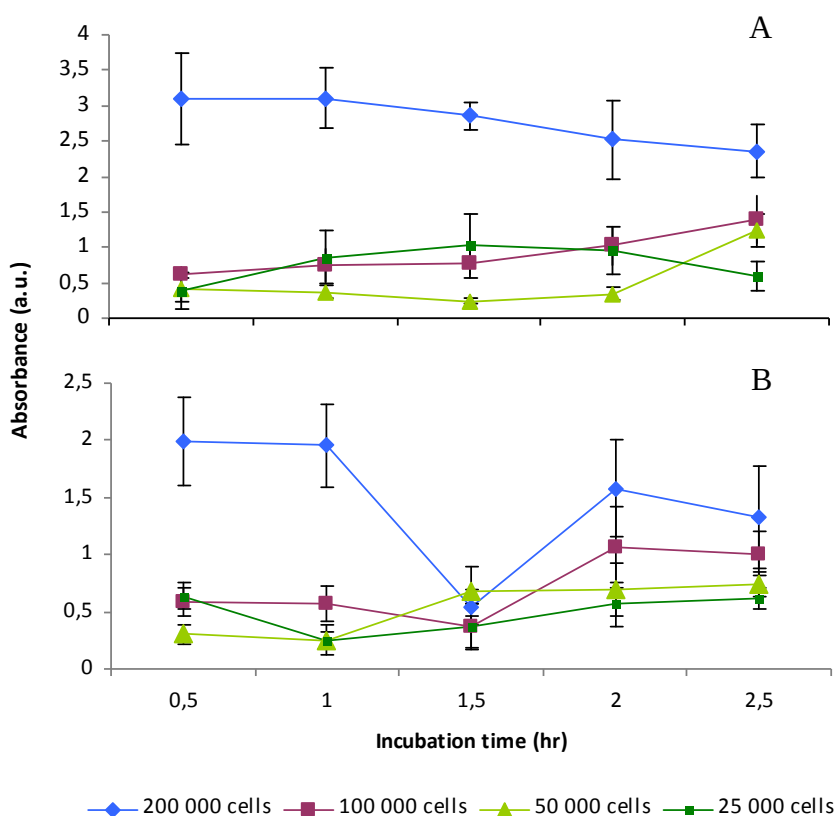


Figure 8. Coelomocytes NR retention capacity from depurated (A) and non-depurated (B) *Eisenia fetida* earthworms at different NR incubation times according to cell concentrations. a.u., arbitrary units. hr, hour. Mean values are given $\pm$ SD.

According to the Fig. 8A and 8B the wells with 2×10^5 cells, retained more NR (ca. 3 times fold) than all the other cell concentrations at 1hr incubation in depurated specimens, as previously reported by (Borenfreund and Puerner 1985). Further on, the NR signal decreases at 2×10^5 cells/well progressively unlike the other cell concentrations that show a general increase absorbance tendency. The abnormal NR signal obtained at 1.5hr for 2×10^5 cells from non-depurated *E. fetida* was most likely due to a handling mistake. Nevertheless, the lower cell concentrations exhibited slight more unstable absorbance values with time suggesting the use of the higher cell concentration for the assay. In addition, the use of the high cell concentration renders a more distinguishable NR signal that could more easily discriminate the effects produced by unfamiliar toxics with unknown cytotoxic potentials. Some possible explanations for the difference between the signal of the depurated and non-depurated earthworms coelomocytes might be the different relative proportion of coelomocytes (eleocytes/amoebocytes) with different NR retention capability or distinct stress-induced coelomocyte production (Homa *et al. in press*).

On the other hand, it seems that there is a proportion between the NR signal of the different cell concentration at each incubation period (Fig. 9A and 9B). According to the coefficients of determination there is a good linear correlation between the cell number and their NR absorbances. Interestingly, at a certain interval of cells (ca. 1×10^5 – 1.5×10^5) the trendlines for each incubation period seem to converge. This could suggest that at that equilibrium point the NRR would not be affected by the incubation period for such number of incubated cells. This could be a way of using less coelomocytes and therefore fewer earthworms for the assay.

Other studies applied incubation times ranging from 1 to 4hr without specifying the reason. The trendlines show that the distance between them is less with increasing incubation periods (eg. the trend lines 1hr and 1.5hr are no closer than the 2hr and 2.5hr trend lines). If this is true then it would mean that at a particular period of incubation, beyond the 2.5 hr period used herein, the NRR signal would be cell concentration independent affecting the relevance of the assay. Accordingly, Maleri and colleagues reported that if the incubation period extends for too long (>4hr), the dye will be washed off prior to the colorant extraction and thus no colorant could be detected photometrically (Maleri *et al.* 2008).

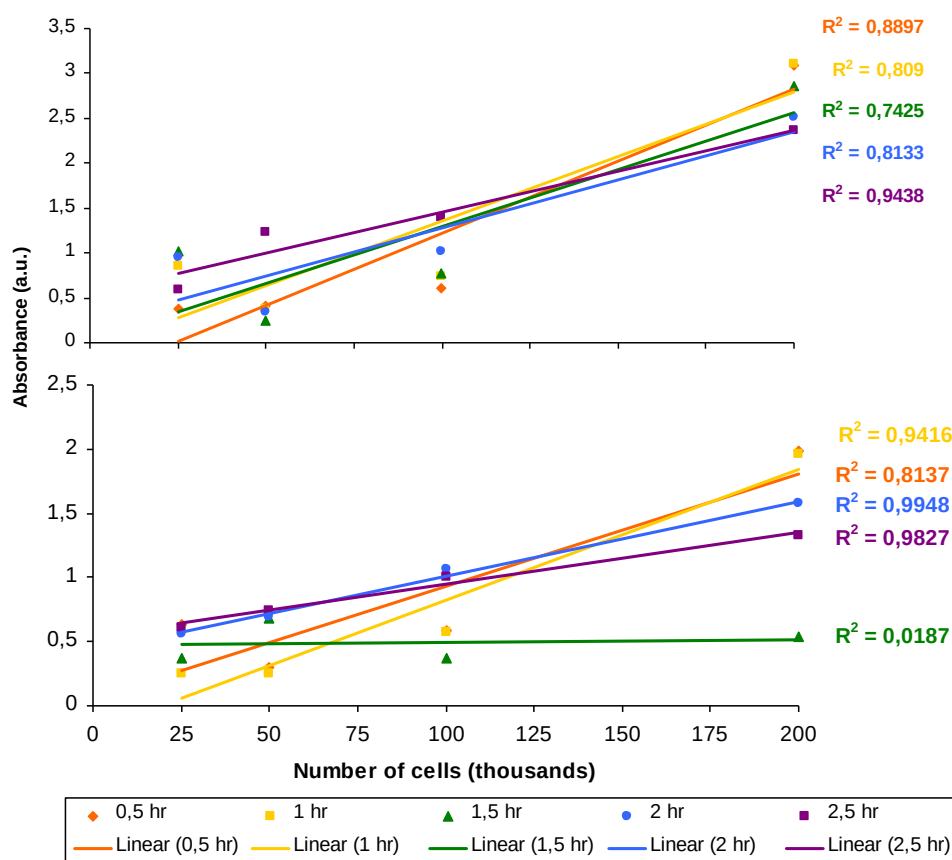


Figure 9. Linear correlation trendlines and correspondent coefficients of determination between absorbance of neutral red retained by coelomocytes from depurated (A) and non-depurated (B) *Eisenia fetida* earthworms at different cell concentrations and each neutral red incubation time tested. a.u., arbitrary units. hr, hour.

Despite the differences amongst coelomocytes from depurated and non-depurated earthworms the depurated methodology was preferred for the forthcoming experiments because it returned higher absorbance which is preferred since it gives a broader cytotoxic effect range of detection. For practical purposes this can be useful for testing unknown lysosomal harmful effects of unknown soil toxic compounds.

4.3. Assessment of serum containing medium in coelomocyte cultures

To obtain good experimental reproducibility, the composition of the cell culture medium is essential (van der Valk *et al.* 2010). Medium supplements, particularly sera from various animal species or from different suppliers, are inevitably complex and cannot be defined. In fact, all the components of culture media comprise a significant potential source of variability (Hartung *et al.* 2002). It has been already reported that the benefits of using animal serum in *in vitro* cell cultures are limited and also a source of unknown risks (Falkner *et al.* 2006). For example, as much as 20-50% of commercial fetal bovine serum (FBS) is virus-positive (van der Valk *et al.* 2010). Besides, the serum influences the bioavailability of toxic compounds like metals (Fischer 1985, Seibert, Morchel and Gulden 2002).

Therefore, two different sera, FBS and NCS (newborn calf serum), widely used in cell culture, were tested on coelomocytes cell cultures in order to verify its effect on the NRR assay. In fact, a partial inhibition of the signal has been already reported, probably due to some binding of NR to serum components (Pick and Avron 1976).

As far as our knowledge goes, the work of Toupin and colleagues is the only one focused in determining the coelomocyte culture components (Toupin *et al.* 1977). They also found different *L. terrestris* coelomocyte viabilities for a period of 10 days with different quantities of FBS. Concentrations of 0 and 20% of FBS affected far more the cells viability than 5 or 10% as it was visible as early as the second day of culture. Mortality at the highest serum dose could be due to excessively high osmotic stress. However, mortalities recorded at low concentrations of FBS were possibly due to the lack of essential nutrients or too low osmotic tension (Toupin *et al.* 1977).

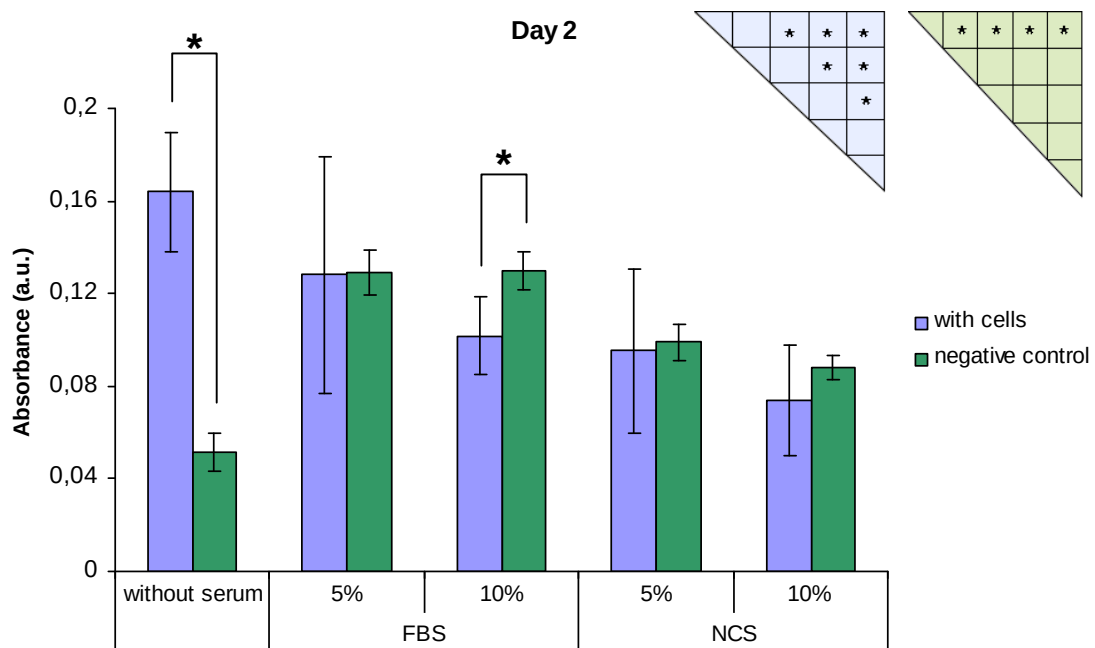
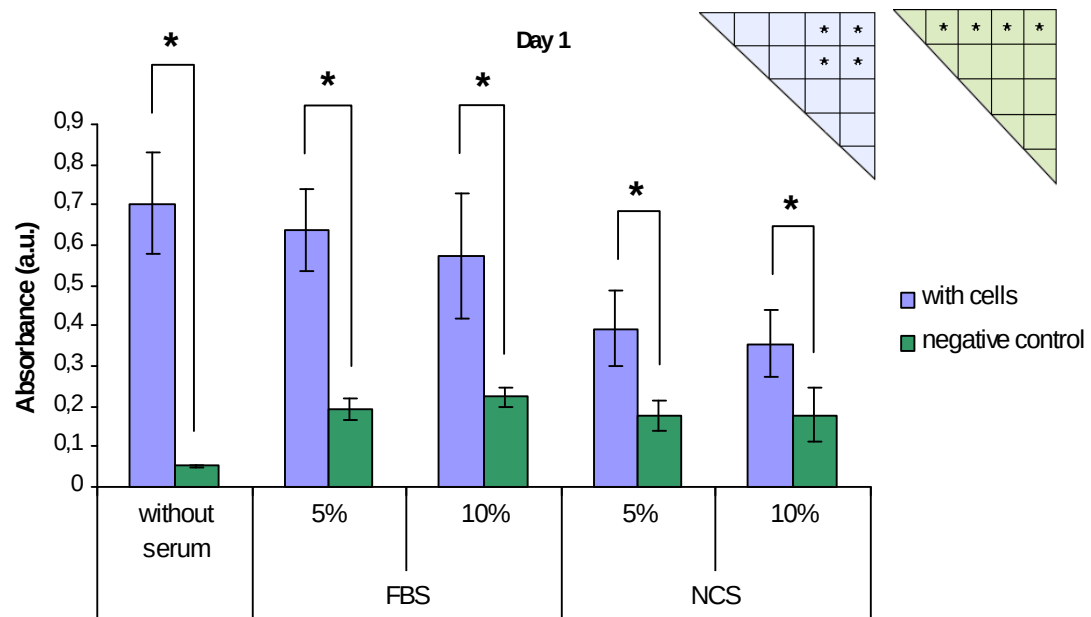


Figure 10. Neutral red retention capacity of cultured coelomocytes at different serum supplemented culture mediums (without serum; FBS, fetal bovine sereum; NCS, newborn calf serum) after 24hr and 48hr incubation. Negative controls correspond to wells without cells. a.u., arbitrary units. Mean values are given $\pm$ SD; *, $p < 0,05$.

It has been observed a gradual decrease in NR absorbance with increasing percentages of both sera in the cell cultures at both days (Fig. 10). Interestingly, the negative control wells with different serum conditions at 24hr show the same NR signal. The serum-free negative control wells exhibited much lower NR signal suggesting that NR is capable of attaching to sera. Moreover, despite the sort of serum and their percentage used in the negative controls, the absorbance of NR remains similar indicating the probable total coverage of the well. Thus both sera contain common NR-interacting components that could also be interfering with the NR absorbance spectrum (Liu *et al.* 2010). In the first day the absorbance was mostly due to NR retained in the cells (very low signal in the negative controls), although in the second day the signal of the controls was greatly increased which could be masking the signal of the cells. These results could be due to the fact that there are no longer cells longer present in the wells being the NR signal due to the interaction of the dye with the serum. However, previous microscopical observations did not prove the presence of abnormal cells in the wells. There is no clear explanation for this event since the cells could in fact be heavily damaged. This matter requires future investigations.

Taking into account that proteins in the medium of *in vitro* systems can influence the biological activity of chemicals by reducing their availability to targets due to binding (Seibert *et al.* 2002), and that both the interaction of NR with serum and the possible interaction between testing compounds and the serum (Pick and Avron 1976), it can be suggested the use of culture mediums without serums for toxicity testing.

Finally, the signal of NR retrieved by the primary cultures decreased from the first to the second day. Such a decrease was more marked in the case of NCS (Fig. 9A and 9B). This daily difference could be due to differences in the newly prepared NR solutions Even though the absolute absorbance clearly diminished the relative absorbance between mediums in each day remained analogous.

4.4. Coelomocyte culture medium renewal

Nutrient supplement is an essential aspect of cell culture. If the cells metabolic demands are not well sustained it compromises the quality of the cell population. Different types of cells have different nutritional requirements which

should be kept in mind when deciding the best supplementation system according to the purposes of the cell culture. In order to evaluate the impact of medium renewal on coelomocytes a three day experiment was carried out.

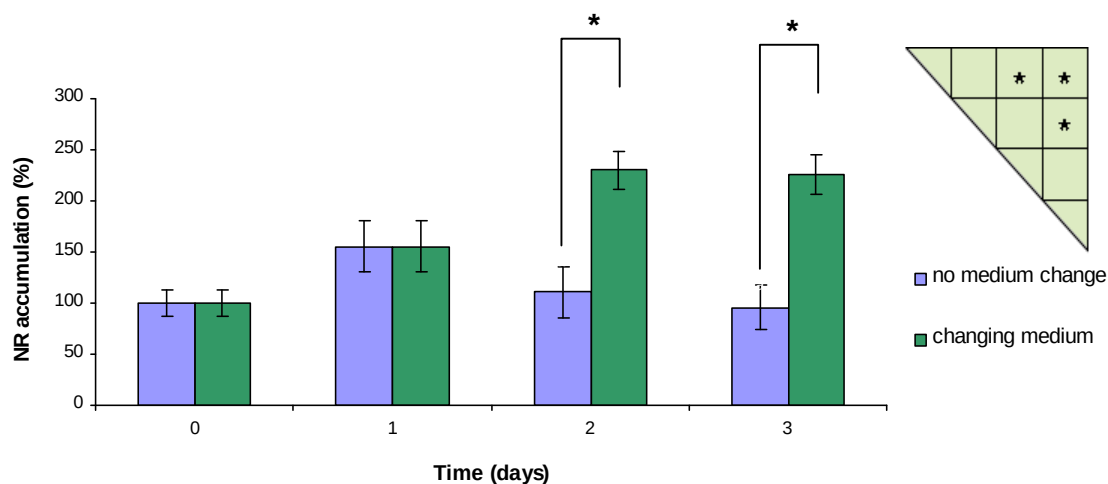


Figure 11. Neutral red accumulation by primary cultures of *Eisenia fetida* coelomocytes with and without renewal of culture mediums in a 3 day experiment. Mean values are given $\pm$ SD; *, $p < 0,05$.

In Fig. 11 the NR signal of coelomocyte cultures was maintained without substituting the culture medium along three days. This eventually means that the nutrient requirements were met being the medium composition adequate. The medium renewal increased coelomocyte NR accumulation at the day 2 remaining stable from this day onwards. It would be reasonable to test if mitotic divisions take place but there was no attempt to measure it since they were neither observed in any coelomocyte type nor in any instance by other studies (Hamed, Kauschke and Cooper 2002, Toupin *et al.* 1977).

In conclusion, the renewal of the medium is not needed in order to perform 1 day experiments (as the ones carried out in the present work). On the other hand, the cells could be maintained for longer periods (at least up to 3 or 4 days) with daily renewal of the medium daily, thus allowing more experiments with the same pool of coelomocytes (extruded from the same earthworms). This could reduce the variability of the NRU when working with different pools of cells.

4.5. *In vitro* metal exposure to Cd, Cu, Ni and Pb

Initial perturbation caused by toxic exposure generate responses within the homeostatically regulated mechanism of the biological units (cell, individual,etc) (De Coen *et al.* 2000). Existing studies correlate higher concentrations of contaminant in soil with lower NR retention capacity of coelomocytes (Xiao *et al.* 2006), and in *in vitro* studies metal compounds caused the decrease of cell viability and activity (Kurek, Homa and Plytycz 2002). In the present work all the amendments previously reported in coelomocyte culturing have to be validated in order to be considered as an animal alternative for toxicity testing (Balls and Fentem 1999). Therefore, it is necessary to prevent false-positive or false-negative results (Weyermann *et al.* 2005). With such a purpose earthworm coelomocytes were *in vitro* exposed to different contaminants and NRR assay was used to assess their toxic responses.

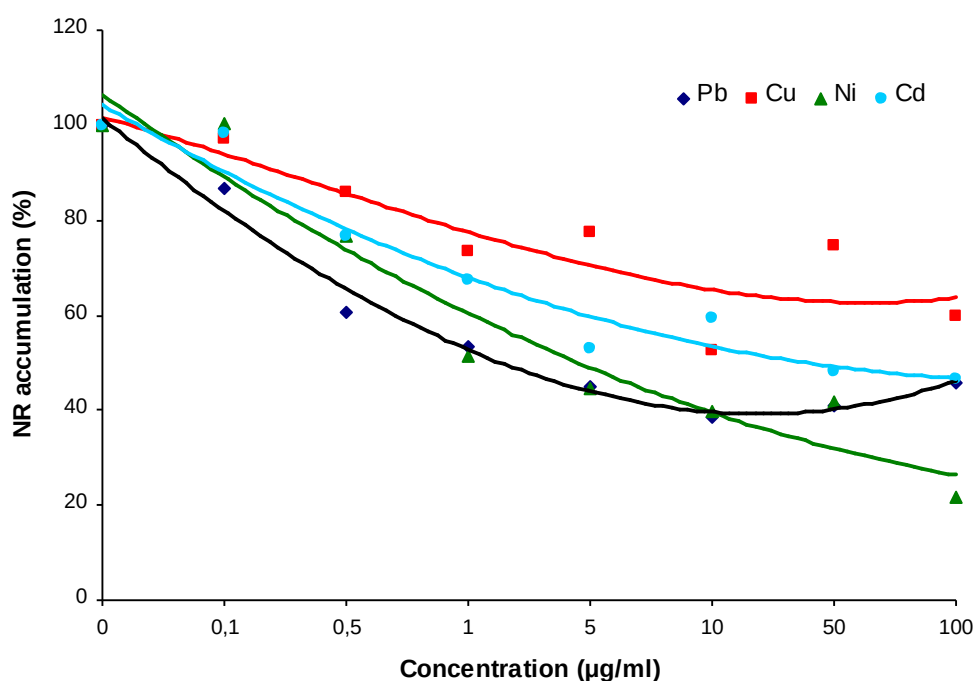


Figure 12. Dose-response curve of NR coelomocytes retention after 24hr exposure to different metals (Cd, Cu, Ni, Pb), supplied as chlorides, at different concentrations (0.1, 0.5, 1, 5, 10, 50 and 100 µg metal/ml). Values for 500 and 1000 µg/ml concentrations have been omitted in this graph (for explanation see the text).

After a period of 24hr of *in vitro* metal exposure the NRR assay was performed in cultured coelomocytes. None of the tested metals revealed a significant damage in the cells capacity of retaining NR at the lower tested concentration (0.1 µg/ml). In addition, an unexpected effect was observed in all the trials done: an initial decrease in the NR signal followed by a strong increase only for the highest doses (500 and 1000 µg/ml) for all the metals. This response can be defined as a biphasic, J-shaped dose-response curve. The second phase has not been shown in Fig. 12 because values were very high and masked the first response provoked by the toxicants. The results will be firstly discussed focusing in the first curve phase since the effects of the second phase might be the result of a strong toxic effect as explained below.

In respect to the first phase it is notable that a common dose-response is described for all the metals tested. However, although the NR signal decreases gradually with the concentration, each of the metals tested exhibited different rates, as was expected (Weyermann *et al.* 2005, Kurek *et al.* 2002). Ni and Pb produced the highest reduction in the NR signal comparing to Cd and Cu. Moreover, the reduction produced by Ni and Pb was significantly different from the control at concentrations equal or higher to 0.5 µg/ml. Pb was proved to be more cytotoxic than Ni since it affected more the NR retention capacity of coelomocytes at the same concentration. Cd was the second more cytotoxic metal with a significant effect starting at 1 µg/ml. Cu exhibited toxic effects only at a concentration of 10 µg/ml. In summary, the overall cytotoxicity for the selected metals is as follows: Pb > Ni > Cd > Cu. Accordingly, and also in *E. fetida* coelomocytes, Sauve *et al.* (2002) found the same pattern (Pb > Ni > Cu) after 18h of *in vitro* exposure assessing cytotoxic effects through measuring the phagocytic activity. Thus, Pb can be considered as the most toxic compound which impaired both the phagocytic capacity and the NRR capacity.

According to the images obtained from the coelomocyte cultures before the NRR assay was performed (Fig. 13) an alteration in the proportions of the cell sub-populations was detected. It was observed that after metal exposure (≥ 100 µg/ml) the sub-population of eleocytes suffered a clear reduction with increasing metal concentrations. This observation is supported by other authors that stated that metal toxicity can induce drastic changes on the patterns of cell distribution across various aquatic and terrestrial invertebrates, such as the *E. fetida* earthworm (Sauve *et al.* 2002). Hence, different sensitivities to pollutant-related

cytotoxicity could be assumed for different earthworm coelomocyte populations as also suggested by (Calisi, Lionetto and Schettino 2009). In conclusion, the relative proportion of eleocytes seems to decrease simultaneously with NR accumulation. This could indicate a greater NR accumulation capacity by higher-sized cells as reported by (Diogene *et al.* 1997).

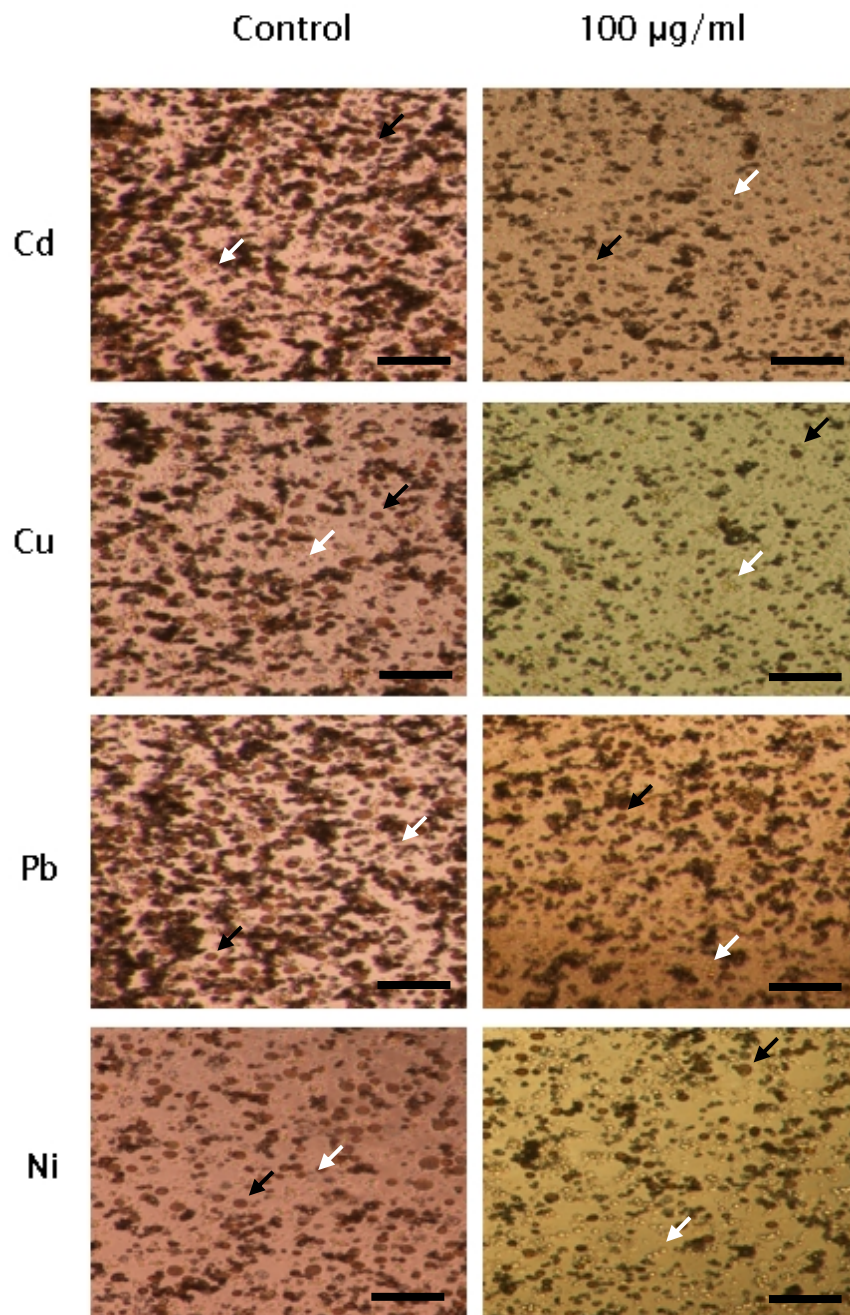


Figure 13. Micrographs of coelomocyte *in vitro* cultures. Control coelomocytes and exposed to Cd, Cu, Pb and Ni (100 µg/ml). Eleocytes: darker and larger cells (black arrow). Amoebocytes: colourless and smaller cells (white arrow). Scale bars: 100 µm.

The design of the experiment could not discard other causes for the decrease of the NR signal and if the negative effects obtained on the cells were a direct interaction of the metals with the coelomocytes or an indirect effect for example due to pH decrease. The decrease of pH values was detected due to the colour shift of the L-15 medium which contained phenol red sensitive to changes in pH levels. Among the factors influencing metal uptake and cytotoxicity *in vitro*, the pH of the medium is of special importance (Fischer 1985). Thus, a large range of pH (2-10) was used to test if NR was attached to the free-cell wells and to discard the possibility of false-positives due to this factor (data not presented). Our results showed that the pH did not have direct influence in the NRR assay results.

In various studies it is common to discuss the metal exposures according to the metal ion and neglecting the corresponding anion. Nevertheless the anions can in some situations contribute to the toxicity of the compounds (Furst, Chien and Chien 1993). The lysosomal membrane is permeable to chloride and other inorganic anions (Klemm *et al.* 1998). An interesting association of toxicity potential can be made since a study conducted by Furst *et al* (1993) revealed nickel chloride as being the most toxic metal compound followed by sulphate. Chloride conduction across the lysosomal membrane is seen as an important functional adjunct to the electrogenic proton pump that maintains the internal acidity of these organelles (Klemm *et al.* 1998). Although there is evidence of passive diffusion on the permeation of chloride it is unlikely that this is the only mechanism. Actually recent reports are reflecting the will to unveil the role of chloride in lysosomes as well as their uptake mechanisms (Smith and Schwappach 2010).

Regarding the second phase of the dose-response curve (from 500 to 1000 µg/ml), we will attempt to discuss its possible causes. The previously reported J-shaped curve has been associated with the phenomenon of hormesis (Calabrese 2008). However, it is unclear whether this event occurs under the present experimental conditions although similar results have been obtained before in other cell cultures (Fotakis *et al.* 2005). First of all it has to be stated that the mechanisms that occurred in the primary coelomocytes culture at the low metal concentrations exposure can differ from the mechanisms that take place at high concentrations of metal. The main hypotheses for this response will be shortly discussed below:

Lysosomal swelling – The treatment of cells with a variety of chemical compounds leads to the formation of many large vacuoles in the cytoplasm (Ohkuma and Poole 1981). Days of exposure of mussels to oil-derived polycyclic aromatic hydrocarbons on hepatopancreatic digestive cells resulted in the decrease of digestive cell volume and increase in the volume of digestive cell lysosomes comparing with the controls (Moore *et al.* 2006). Not just organic compounds but also metal provoke the enlargement of lysosomes in mussels (Etxeberria *et al.* 1994). This enlargement is a consequence of osmotic uptake of water that results in an increase in NR uptake which is measurable by photometry (Olivier *et al.* 1995). Perhaps the same type of phenomenon occurs in the coelomocytes of this study: extended NR retention and the microscopically undetected coelomocyte structure alteration. Lysosomal membrane stability and lysosomal swelling are negatively correlated in mussels which in turn is associated with fusion of lysosomes to produce enlarged secondary lysosomes prone to accumulate larger NR quantities (Etxeberria *et al.* 1994). This phenomenon of lysosomal swelling therefore can lead to an underestimation of the cytotoxicity when the NRR assay test is used. These results demonstrate that the assay must be used with caution when using compounds that cause lysosomal enlargement. It is important the use of several, mechanistically different, cytotoxicity biomarkers for the evaluation of xenobiotic cytotoxicity (Olivier *et al.* 1995).

Coelomocytes distribution and resistance – The dominance of the smaller coelomocyte population in the second-phase curve could also be a simple reflection of different coelomocyte-type viabilities after extrusion. This may lead to the apparent “resistance” of the amoebocytes as these were the majority and maybe it could happen that in the second-phase curve the granules that compose the extinct eleocytes, known to highly accumulated NR, were phagocyted by the other coelomocyte types resulting in a possible indirect uptake of NR by the resistant amoebocytes (Di Marzio *et al.* 2005, Diogene *et al.* 1997).

A curious study by Arai *et al.* (2002) in rat hepatocyte lysosomes revealed that the lysosomal disruption is inhibited by the addition of cytosol due to its content of ASTP (ATP-stimulated translocation promoter). This factor could act as a fusogenic agent, similar to viral proteins, where exposure of the hydrophobic regions of protein and fusion of lysosomes prevents osmotic lysis by increasing

lysosomal volume (Arai *et al.* 2002). In our experiments amoebocytes in exposed cultures could contain this or other factor with similar functions at higher concentrations than eleocytes justifying the dominance of this sub-population after the metal exposure. This matter requires further research.

Nuclear neutral red accumulation – The high pH levels have been pointed out as being capable of inducing an enhancement of nuclear trafficking evidenced by the increased nuclear staining of NR in interphase KB cells (Sit *et al.* 1996). In fact, the dye concentration levels approached those of M-phase nuclei raising the question whether if there is any cell division activity in metal-stress conditions. Nucleic acids are anionic bio-macromolecules, and NR is a small aromatic molecule, which is planar and positively charged, being the interaction between NR and nucleic acids dependent mainly on the electrostatic and hydrophobic forces (Wang *et al.* 1999). So the nucleic acids production increment in response to metal stress could be a plausible contributing factor.

Short- and long-term responses – A decrease in the neutral red retention time was observed in *E. andrei* with increasing concentrations of metal (Maleri *et al.* 2008) as was also observed in our study. However, coelomocytes extruded from long-term exposed worms to metal for various generations showed a high signal of retained NR (Maleri *et al.* 2008). This suggests that the data are the result of the development of an increased tolerance although the underlying mechanisms are largely unknown. Long-term environmental stress appears associated with lysosomal enlargement but also at experiments at high contaminant concentrations (Etxeberria *et al.* 1994). This could indicate that similar mechanisms could be happening either at short and high concentration exposures or longer exposures at lower metal concentrations.

Chapter 5 – Conclusions and Thesis

5.1. Conclusions

1 - The optimum incubation time, tested with NRR assay, was 1 hr since the signal obtained was higher than using longer periods after incubation with 2×10^5 cells/well.

2 - The NR signal decreases at 2×10^5 cells/well progressively unlike the other cell concentrations that show a general increase absorbance tendency. Nevertheless, the lower cell concentrations exhibited slightly more unstable absorbance values with time suggesting the use of the higher cell concentration for the assay. The use of 2×10^5 cells/well renders a more distinguishable NR signal that could more easily discriminate the effects produced by unfamiliar toxics with unknown cytotoxic potentials.

3 - The depuration of earthworms before coelomocyte extrusion was selected for the forthcoming experiments because it produced higher absorbance which is preferred because allows the more accurate detection of a broader range of cytotoxic effects.

4 - There is a good linear correlation between the cell number and their NR absorbances, and at a certain number of cells (ca. 1×10^5) the trendlines for each incubation period converge suggesting an equilibrium point where the neutral red retention would not be affected by the incubation period. This would allow to reduce the number of coelomocytes and therefore of earthworms for the assay. However, it has to be pointed out that the signal of NR is much lower.

5 - The time incubation trendline could suggest that at a particular period of incubation, beyond the 2.5 hr period used herein, the NR signal would be cell concentration independent affecting the relevance of the assay and no colorant could be detected photometrically.

6 - A gradual decrease in NR absorbance with increasing percentages of tested sera, FBS and NCS at 24 and 48 hr of culturing was measured. The serum-free negative control wells exhibited much lower NR signal suggesting that NR is attached to the serum. FBS and NCS contain common NR-interacting components that could also be interfering with the NR absorbance spectrum completely masking the signal of the cells (as seen at 48 hr). Interaction of NR with serum and the possible interaction between the testing compounds and the serum recommend the use of culture mediums without serums for toxicity testing.

7 The total amount of coelomocytes extruded by the electric extrusion method was clearly higher (~17 times) than the number of cells obtained by the ultrasound method. The ultrasound method rendered larger contamination probably due to the damage produced in some earthworms which could release some “inner-bacteria” along with the coelomocytes. The electric extrusion method is recommended against the ultrasound method for coelomocyte culturing.

8 – NR signal of coelomocyte cultures was maintained without substituting the culture medium along three days suggesting that the medium composition was adequate. The medium renewal increased coelomocyte NR accumulation at day 2, remaining stable from this day onwards. The renewal of the medium would not be needed in order to perform a 24 hr experiment. The cells could be maintained for longer times by daily renewal of the medium daily, which allows more experiments with the same pool of coelomocytes.

9 – None of the tested metals revealed a significant damage in the cells capacity of retaining NR at the lowest tested concentration (0.1 µg/ml). An initial decrease in the NR signal followed by a strong increase only for the highest doses (500 and 1000 µg/ml) for all the metals was observed, in a kind of biphasic, J-shaped dose-response curve. The first phase is a common dose-response curve for all the metals. NR signal decreases gradually but at different rates for each of the metals considered. The overall cytotoxicity is as follows: Pb > Ni > Cd > Cu. The second phase, described as increased NR signal at increasing metal concentrations, might be the result of a strong toxic effect.

10 – An alteration in the proportions of the cell sub-populations was observed after metal exposure (≥ 100 µg/ml), with the sub-population of eleocytes suffering a clear reduction with increasing metal concentrations. The relative proportion of eleocytes seems to decrease simultaneously with NR accumulation.

5.2. Thesis

After the amendments and modifications presently done it can be concluded that the primary cultures of coelomocytes extruded from *E. fetida* earthworms can be considered as a reliable tool to assess the toxicity of metals *in vitro* using the NRR assay.

Chapter 6 – References

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