Universidade de Aveiro Departamento de Biologia
Ano 2011

SARA CALÇADA NOVAIS

Dos genes à população: efeitos de substâncias tóxicas em *Enchytraeus albidus*

From genes to population: effects of toxicants on *Enchytraeus albidus*

Universidade de Aveiro Departamento de Biologia
Ano 2011

SARA CALÇADA NOVAIS

Dos genes à população: efeitos de substâncias tóxicas em *Enchytraeus albidus*

From genes to population: effects of toxicants on *Enchytraeus albidus*

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Mónica João de Barros Amorim (Investigadora Auxiliar do Departamento de Biologia & CESAM da Universidade de Aveiro) e do Doutor Wim Monique Ivo De Coen (Professor do Departamento de Biologia da Universidade de Antuérpia).

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através de uma Bolsa de Doutoramento atribuída a Sara Calçada Novais (SFRH / BD / 36253 / 2007)

o júri

presidente

Doutor Vitor José Babau Torres
Professor catedrático do Departamento de Física da Universidade de Aveiro

Doutor Amadeu Mortágua Velho da Maia Soares
Professor catedrático do Departamento de Biologia da Universidade de Aveiro

Doutor Wim Monique Ivo De Coen (co-orientador)
Professor do Departamento de Biologia da Universidade de Antuérpia

Doutor Janeck James Scott-Fordsmand
Cientista sénior do Departamento de Biociências da Universidade de Aarhus

Doutor Carlos Alexandre Sarabando Gravato
Investigador auxiliar do Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR) da Universidade do Porto

Doutora Mónica João de Barros Amorim (orientador)
Investigadora auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro

agradecimentos

Este trabalho não teria sido possível sem a ajuda de várias pessoas e instituições, às quais não poderia deixar de agradecer.

Em primeiro lugar agradeço ao Departamento de Biologia e ao CESAM da Universidade de Aveiro, assim como ao laboratório EBT da Universidade de Antuérpia, por me acolherem e disponibilizarem todas as condições necessárias. À Fundação para a Ciência e Tecnologia pelo financiamento sob a forma de uma Bolsa de Doutoramento (SFRH/BD/36253/2007).
Ao meu co-orientador, professor Wim De Coen, que me transmitiu o gosto pelas ferramentas moleculares e, em especial, à minha orientadora, Mónica Amorim, por todo o acompanhamento, transmissão de conhecimentos, disponibilidade, paciência, pelos incentivos e pela força que foram fundamentais para que conseguisse levar o trabalho a bom porto, e pela amizade ao longo de todos estes anos. Ao professor Amadeu Soares que me iniciou nesta área da ecotoxicologia e me acolheu no seu grupo de investigação.

Ao laboratório de ecotoxicologia do CIIMAR pela disponibilidade para uma colaboração e por me ter recebido de braços abertos. Um agradecimento muito especial a todos os que lá trabalham e em particular ao Carlos, à Joana, à Cris e à Patrícia, que me fizeram sentir em casa. Ao Carlos, não podia deixar também de agradecer por tudo o que me ensinou, pela ajuda preciosa no laboratório e na discussão dos resultados, assim como pelo companheirismo e amizade.
A todos do laboratório EBT pela ajuda e companhia durante as minhas estadias em Antuérpia, e em especial à Rosa Hermans por todo o apoio logístico, e à Tine por tudo e mais alguma coisa.

Ao Abel, porque torna a nossa vida sempre tão mais fácil. Às minhas Moniquetes, pelo apoio incondicional, pelo espírito de entreajuda, por estarem sempre lá, pelos desabafos e por serem mesmo, mesmo umas queridas!
A todos os colegas e amigos do LETAL, que tornaram esta jornada tão mais fácil e mais "gostosa"! Não posso, no entanto, deixar de salientar aqueles que foram os meus companheiros desta, mas também de outras aventuras: Clara, Pestana, Marco, Rike, Miguel, Salomé, Vanessa, Carla, Zé, Janeco, Rui, Gonçalo, Maria e Fabi. Obrigada pela boa disposição, pelo convívio, por estarem sempre prontos a animar a malta ("que é o que faz falta!") e pelos momentos muito bem passados!

À minha "família Aveirense" que está sempre presente, dando-me toda a força para seguir mais esta viagem: Carla, Maria João, Ana, Ricardo M., Inês, Ricardo – a nossa "bolha"! – Mariana, Clara (sim Clara, faz todo o sentido agradecer-te duas vezes), Jardel, Maria, Patinhos…e aos "casais amigos" com quem ultimamente tenho falhado e que prometo compensar!

À minha família, pelo carinho incondicional e pela loucura, que todos temos um pouco e fazemos questão de a viver em conjunto, fazendo de nós esta família especial e unida. Obrigada pelo apoio desde o primeiro dia, mesmo achando "isto das minhocas tudo muito esquisito".

Por fim, ao Manel, e à nossa kakau (claro!), pelo apoio, paciência, força, compreensão, por todo o tempo que não estivemos juntos, pelas gargalhadas, por todas as palavras certas no momento certo (talvez aqui não se aplique à kakau)...por tudo! E agora, vamos dar a volta ao mundo?

palavras-chave

expressão genética, microarray, biomarcadores, stress oxidativo, antioxidantes, alocação de energia celular, sobrevivência, reprodução, comportamento de “evitamento”, metais, pesticidas, Oligochaeta.

resumo

Actualmente, em ecotoxicologia, uma abordagem ao nível da biologia de sistemas representa não só um desafio, como se acredita ser a forma ideal para a compreensão dos mecanismos de resposta dos organismos. A integração de respostas de diferentes níveis de organização biológica, permite a melhor percepção dos mecanismos envolvidos, e assim possibilita a previsão de efeitos de agentes tóxicos num contexto alargado. O objectivo principal desta tese foi a avaliação dos mecanismos de resposta de *Enchytraeus albidus* a stressores químicos. Desta forma, um grande investimento foi feito de forma a enriquecer a biblioteca genómica desta espécie, como explicado mais adiante. Em suma, os efeitos de compostos químicos pertencentes a duas classes diferentes (metais e pesticidas) foram avaliados a níveis de organização biológica distintos: desde parâmetros populacionais até bioquímicos e moleculares com a alteração de expressão genética. Os compostos seleccionados foram: 1) os metais cádmio e zinco; 2) o insecticida dimetoato, herbicida atrazina e fungicida carbendazim. Ao nível sub-cellular e genético, os efeitos do tempo de exposição e da dose aplicada também foram avaliados. Os testes de ecotoxicologia tradicionais – sobrevivência, reprodução e evitamento – mostraram uma maior toxicidade dos pesticidas em relação aos metais. O evitamento é um parâmetro de extrema importância do ponto de vista ecológico, apesar de não recomendado para avaliação de risco. O estudo dos indicadores de stress oxidativo revelou que o Zn e o Cd induziram efeitos significativos na actividade de várias enzimas antioxidantes e níveis de substratos, provocando igualmente danos oxidativos nas membranas celulares. De forma a aumentar as potencialidades da nossa ferramenta molecular disponível para avaliar respostas ao nível da transcrição, a biblioteca de cDNA existente foi enriquecida com genes de resposta a metais e a pesticidas, usando o método de SSH. A partir das sequências obtidas foi desenvolvido um microarray de oligonucleótidos (Agilent), assim como uma base de dados de utilização gratuita na internet que reúne todos os dados moleculares disponíveis para *E. albidus* e constitui uma ferramenta interactiva de acesso a informação. Com a aplicação do novo microarray, foram obtidos dados novos e relevantes acerca dos mecanismos de toxicidade, tendo sido possível a identificação de diferentes vias metabólicas afectadas por cada composto químico. Os resultados obtidos permitiram a identificação de mecanismos de acção destes compostos em *E.albidus* que, em grande parte, coincidem com mecanismos descritos para mamíferos, sugerindo que os modos de acção são conservados em várias espécies e sublinhando a utilidade deste invertebrado como espécie modelo. De um modo geral, as respostas bioquímicas e moleculares foram influenciadas pelo tempo de exposição e concentração do composto tóxico, permitindo seguir a evolução dos eventos. Os resultados da determinação da alocação energética confirmaram as evidências de um aumento de gastos energéticos, sugeridas pelo microarray, e que podem explicar parcialmente o decréscimo na reprodução verificado numa fase posterior. As correlações encontradas no decorrer desta tese entre parâmetros de vários níveis de organização biológica, contribuíram para uma melhor compreensão da toxicidade de metais e pesticidas nesta espécie.

keywords

gene expression, microarray, biomarkers, oxidative stress, antioxidants, cellular energy allocation, survival, reproduction, avoidance behaviour, metals, pesticides, Oligochaeta.

abstract

Nowadays, a systems biology approach is both a challenge as well as believed to be the ideal form of understanding the organisms' mechanisms of response. Responses at different levels of biological organization should be integrated to better understand the mechanisms, and hence predict the effects of stress agents, usable in broader contexts. The main aim of this thesis was to evaluate the underlying mechanisms of *Enchytraeus albidus* responses to chemical stressors. Therefore, there was a large investment on the gene library enrichment for this species, as explained ahead. Overall, effects of chemicals from two different groups (metals and pesticides) were assessed at different levels of biological organization: from genes and biochemical biomarkers to population endpoints. Selected chemicals were: 1) the metals cadmium and zinc; 2) the insecticide dimethoate, the herbicide atrazine and the fungicide carbendazim. At the gene and sub-cellular level, the effects of time and dosage were also adressed. Traditional ecotoxicological tests - survival, reproduction and avoidance behavior - indicated that pesticides were more toxic than metals. Avoidance behaviour is extremely important from an ecological point of view, but not recommended to use for risk assessment purposes. The oxidative stress related experiment showed that metals induced significant effects on several antioxidant enzyme activities and substrate levels, as well as oxidative damage on the membrane cells. To increase the potential of our molecular tool to assess transcriptional responses, the existing cDNA library was enriched with metal and pesticide responding genes, using Suppression Subtractive Hybridization (SSH). With the sequencing information obtained, an improved Agilent custom oligonucleotide microarray was developed and an EST database, including all existing molecular data on *E. albidus,* was made publicly available as an interactive tool to access information. With this microarray tool, most interesting and novel information on the mechanisms of chemical toxicity was obtained, with the identification of common and specific key pathways affected by each compound. The obtained results allowed the identification of mechanisms of action for the tested compounds in *E. albidus,* some of which are in line with the ones known for mammals, suggesting across species conserved modes of action and underlining the usefulness of this soil invertebrate as a model species. In general, biochemical and molecular responses were influenced by time of exposure and chemical dosage and these allowed to see the evolution of events. Cellular energy allocation results confirmed the gene expression evidences of an increased energetic expenditure, which can partially explain the decrease on the reproductive output, verified at a later stage. Correlations found throughout this thesis between effects at the different levels of biological organization have further improved our knowledge on the toxicity of metals and pesticides in this species.

INDEX

Chapter I

General Introduction

I – GENERAL INTRODUCTION

1. Soil Ecotoxicology

Pollution is a worldwide problem that is identified by the European Union as one of the major threats to the normal functioning of soil (Rodrigues et al., 2009; Nota et al., 2010). Soil is often the ultimate "sink" for pollutants due to the number of surfaces, chemical groups and organic particles present in the clay minerals and humic materials to which chemical compounds can attach (van Straalen and Roelofs, 2008). Additionally, in highly polluted scenarios, the chemical compounds can reach groundwater and also affect aquatic ecosystems due to the surpassed limits of filtering and buffer capacity of soil.

The soil compartment is responsible for several important ecological functions like supporting vegetation and biomass production, being a place of intense biological activity with the degradation of organic matter, recycling of nutrients and humus synthesis. Moreover, soil systems are essential for human agricultural practices and for natural resource industries which depend on soil fertility, hence on the proper ecological functioning of soil. Contamination on this environmental compartment poses a problem to society with the adverse effects on ecological systems, agricultural functions and human health. As referred by Lanno and co-authors (2003) to maintain soil quality and protect the environment and human health, it is vital to understand the effects of chemical pollutants on soil organisms and develop regulatory strategies for soil protection.

Traditionally in ecotoxicology, toxic effects are measured using standardized methods, based mainly on acute (e.g mortality) and chronic responses (e.g. reproduction) of a sensitive biological indicator. Although these methods are very important to screen the toxicity of polluted soils, they provide limited information regarding the underlying mechanisms of the observed stress response. In addition, those tests are time-consuming and often problems have a time pressure where more immediate answers are demanded for a timely action to be taken. Hence, several alternative testing options have been optimized and suggested to use in ecotoxicology and ecological risk assessment (ERA). An ideal

stress biomarker is one that responds before effects are already visible phenotypically on individuals, i.e. that take place at e.g. the cellular level, and that can be linked to the individual effects. Ultimately, these can provide an early warning for relevant effects occurring at higher levels of biological organization (Forbes et al., 2006). The establishment of a firm link between sub-cellular or molecular responses and effects at higher levels of biological organization is of great ecological importance and crucial for the application of such biomarkers in ERA. This is the more regulatory perspective of the use of molecular markers, yet the advantages are endless if we think about the possibility of understanding the mechanisms of action of stress agents.

Currently in ecotoxicology, a great challenge is a systems biology approach, where responses at different levels of biological organization can be integrated, to better understand the mechanisms of toxic action and predict the effects of chemicals in a broader context (fig. 1).

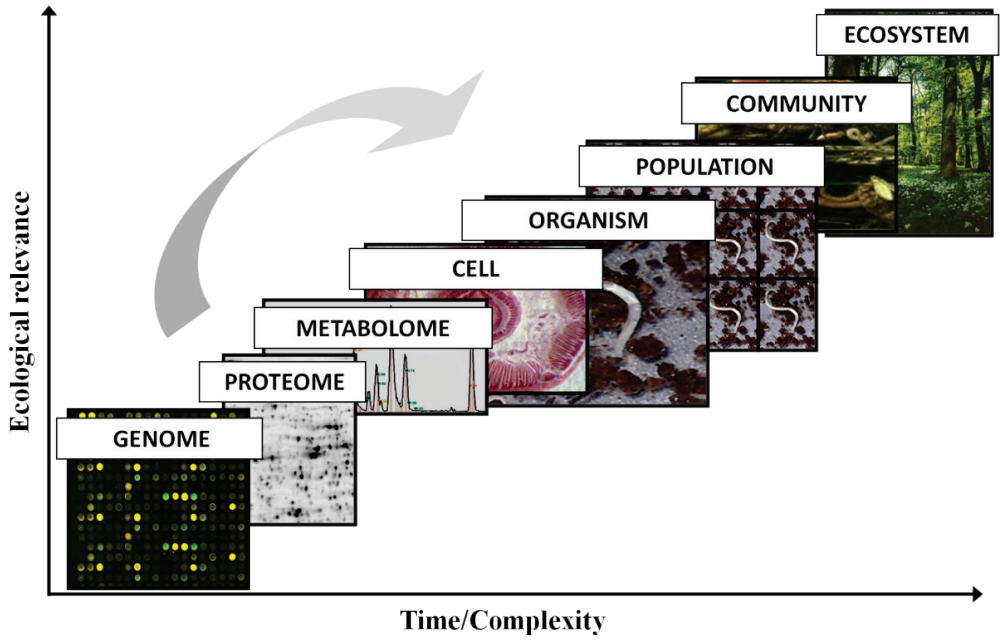

Figure 1 – Levels of biological organization where effects of toxicants can be measured, as a function of their complexity and ecological relevance.

1.1 Traditional endpoints at the population level

Several tests have been used in terrestrial ecotoxicology to evaluate and predict effects of chemical substances in the environment and to assess soil quality. International organizations such as the Organization for Economic Cooperation and Development (OECD) or the International Organization for Standardization (ISO) have standardized guidelines for such purpose.

Effects measured by these guidelines can be of short duration such as survival (acute tests) or long term in the case of sublethal parameters such as reproduction or bioaccumulation (chronic tests). Acute tests are usually employed as a 'screening tool' with a broad range of high toxic concentrations, whereas chronic tests are often applied to evaluate prolonged exposures to lower, sublethal chemical concentrations. Avoidance test is a behavioural endpoint performed as a first assessment of toxicity, in a short period of time, using the reaction of the organisms as an endpoint. In this period of time, the ability of the organisms to choose between the control and contaminated soil is evaluated (Amorim et al., 2005).

At present, standardized toxicity tests with soil invertebrates like earthworms (*Eisenia andrei or Eisenia fetida*), collembolans (*Folsomia candida*), enchytraeids (*Enchytraeus albidus*) or predatory mites (*Hypoaspis aculeifer*) are available to measure the following endpoints: survival (OECD, 1984; ISO, 1993, 2004), reproduction (ISO, 1998, 1999, 2004; OECD, 2004b, a, 2008, 2009), bioaccumulation (OECD, 2010) and avoidance behaviour (ISO, 2006, 2011).

1.2 Biochemical biomarkers at the cell level

Antioxidant defenses are among the most used subcellular biomarkers. These antioxidant defenses include water and lipid soluble low molecular weight free radical scavengers (like glutathione – GSH) and specific antioxidant enzymes which play a major role on cell homeostasis avoiding DNA damage, enzymatic inactivation and peroxidation of cell

constituents due to increased reactive oxygen species (ROS) production (Halliwell and Gutteridge, 1999).

Reactive oxygen species are partially reduced forms of molecular oxygen (O_2). During normal animal metabolism, with processes like food oxidation or energy generation, O_2 undergoes tetravalent reduction to water. However, partial reduction of O_2 results in the formation of radical species such as superoxide anion radical ($O_2^{\cdot-}$), peroxide (O_2^{2-}) and hydroxyl radical ($OH^{\cdot}$), or non-radical species like hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge, 1999). In the normal healthy cells, ROS can be eliminated by the antioxidant defences. However, large increases in ROS production can overcome antioxidant defences, resulting in increased oxidative damage to macromolecules and alterations in critical cellular processes, which is designated oxidative stress.

Reduced GSH has its role in the cellular antioxidant defence by (fig. 2): 1) acting as a key conjugate of electrophilic intermediates, mainly via glutathione-S-transferase (GST) activities in phase II of xenobiotic biotransformation; 2) being the substrate for glutathione peroxidase (GPx); 3) being linked directly to pro-oxidants like transition metals, thus functioning as non-enzymatic antioxidant (Meister, 1995; Saint-Denis et al., 1999).

Antioxidant enzymes, representing the defence system to eliminate oxyradicals, include superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR).

SODs are a group of metalloenzymes that catalyse the conversion of reactive $O_2^{\cdot-}$ to yield H_2O_2 (fig. 2). H_2O_2 is subsequently detoxified by two types of enzymes: CAT and GPx. CAT facilitate the removal of H_2O_2, reducing it to molecular oxygen and water while GPx catalyses the metabolism of H_2O_2 to water, involving a concomitant oxidation of reduced GSH to its oxidized form – GSSG (Apel and Hirt, 2004). GR is important in maintaining GSH/GSSG homeostasis under oxidative stress conditions (Winston and Di Giulio, 1991). It catalyses the transformation of the oxidized disulfide form of glutathione to the reduced form, with the concomitant oxidation of NADPH to $NADP^+$ (fig. 2). A direct effect of certain pollutants consists in the decrease in thiol status, i.e. the ratio of reduced to oxidized glutathione (GSH:GSSG), due to either direct radical scavenging or increased peroxidase activity (Stegeman *et al.*, 1992).

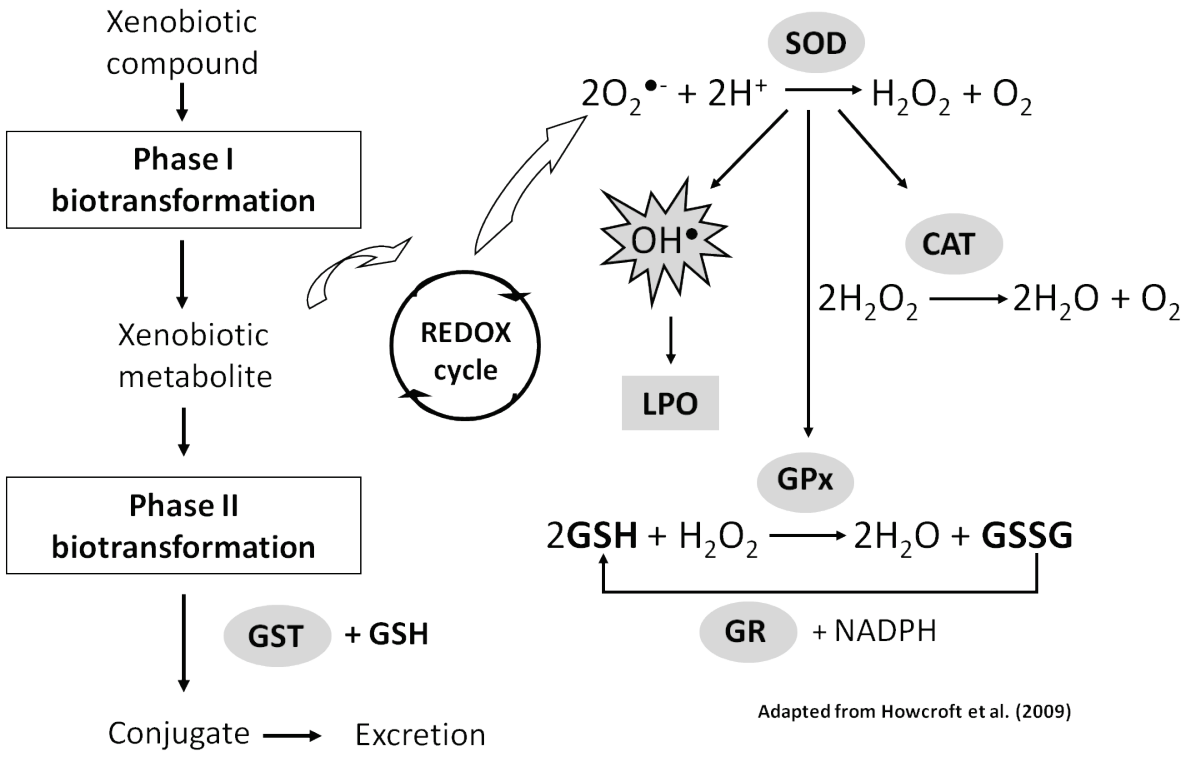

Figure 2 – Enzymatic pathways involved in xenobiotic biotransformation and antioxidant defences. Adapted from Howcroft et al. (2009).

Some environmental stressors like pesticides are known to inhibit acetylcholinesterase activity (AChE), making the activity of this enzyme one of the most used biomarkers in environmental studies. AChE is an important nervous system enzyme responsible for the degradation of the neurotransmitter acetylcholine. The role of AChE is essential for proper muscle function, ensuring that the signal does not over stimulate the post-synaptic membrane and hence preventing continuous nerve firings (Nachmansohn and Wilson, 1951).

Other important biomarkers are the ones used for measuring the metabolic effects, since responses of the organisms to environmental stressors are considered to be metabolic costly (Calow, 1991; De Coen and Janssen, 2003). Under normal conditions organisms use energy for their growth, reproduction and basal metabolism. In case of toxic stress a larger amount of the assimilated energy is used to cope with chemical detoxification and to

maintain or compensate basal metabolism, leaving less energy available for growth and reproduction. De Coen and Janssen (1997, 2003) have proposed a methodology to evaluate the effects of toxic stress on the metabolic balance – the cellular energy allocation (CEA) assay. With this approach, available energy reserves and energy consumption are quantified on a cellular level and integrated into a general stress indicator. Energy reserves are quantified as carbohydrate, protein and lipid content of the test organism, while the energy consumption is estimated by measuring the electron transport activity (ETS) at the mitochondrial level. The difference between energy reserves and energy consumption represents the net cellular energy allocation (CEA) of the test organism (De Coen and Janssen, 2003).

1.3 Molecular endpoints at the gene level – Transcriptomics

Every toxic event is starting from a primary signal that can be a molecule or a receptor that is affected by the stressor, leading to a chain reaction of events orchestrated by the cell to maintain homeostasis (De Coen et al., 2000). By measuring toxicological effects at the molecular level a better insight can be generated into chemical modes of action which could eventually lead to improved risk assessment and mitigation of these chemicals.

The discipline within ecotoxicology interested in defining how the regulation and expression of genes, proteins and metabolites are correlated with the toxicological effects induced by an environmental stressor is denominated ecotoxicogenomics. This term was first proposed by Snape et al. (2004) to describe the integration of genomic techniques and bioinformatics into ecotoxicology. Transcriptomics is the term used to describe the study of specifically gene expression in a certain tissue or organism, in a particular time and condition. The main goal of transcriptomics is to unravel toxic modes and mechanisms of action of environmental pollutants in ecologically relevant species. This information can complement traditional ecotoxicological endpoints and has the potential to improve risk assessment of chemicals.

Different tools to evaluate transcriptomic responses include Suppression Subtractive Hybridization (SSH), microarrays and quantitative real-time PCR (qPCR). DNA

microarrays are a very popular tool used in transcriptomics, first described by Schena et al. (1995). This technique allows simultaneous assessment of the expression of thousands of genes and its development has opened up tremendous new possibilities in the study of gene expression alterations. With microarray technology, different transcriptomes can be compared (e.g. gene expression of control organisms versus gene expression of chemical exposed organisms) and from this analysis altered genes in response to the treatment can be identified (as up- or down-regulated). An overview of the microarray methodology is presented in figure 3.

This tool represents a privileged platform to elucidate unknown mechanisms of response and molecular pathways affected by an environmental stress. When it was first introduced in ecotoxicology, the major disadvantage was that the majority of standard test species used in the field did not have fully sequenced genomes or even had any genomic information. Nevertheless, the combination of SSH and microarray technology proved to be successful to allow identification of gene expression changes in ecotoxicological standard test species without prior genomic information (Snell et al., 2003).

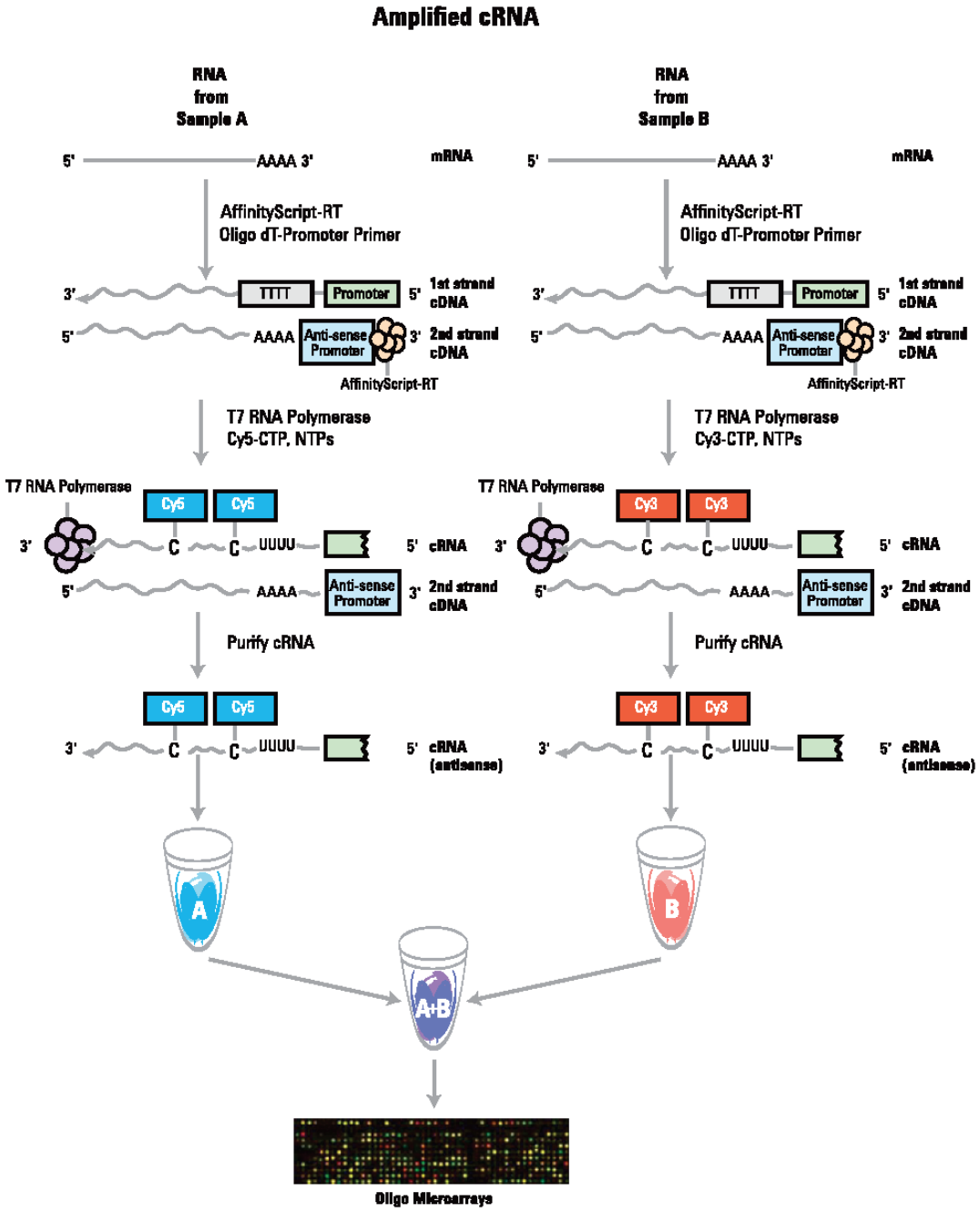

Figure 3 – Schematic of amplified cRNA procedure. Generation of cRNA for a two-color microarray experiment is shown. For a one-color microarray experiment, only the Cy3-labeled "B" sample is produced and hybridized. Adapted from Agilent Technologies (Palo Alto, CA, USA).

SSH-PCR is a technique developed by Diatchenko et al. (1996) that combines high subtraction efficiency with an equalized representation of differentially expressed sequences. An overview of the methodology is presented in figure 4. This method is based on a specific form of PCR – suppression PCR – that allows exponential amplification of target cDNAs (differentially expressed genes between two mRNA populations), whereas amplification of undesirable sequences (equally abundant transcripts) is suppressed (Moens et al., 2003).

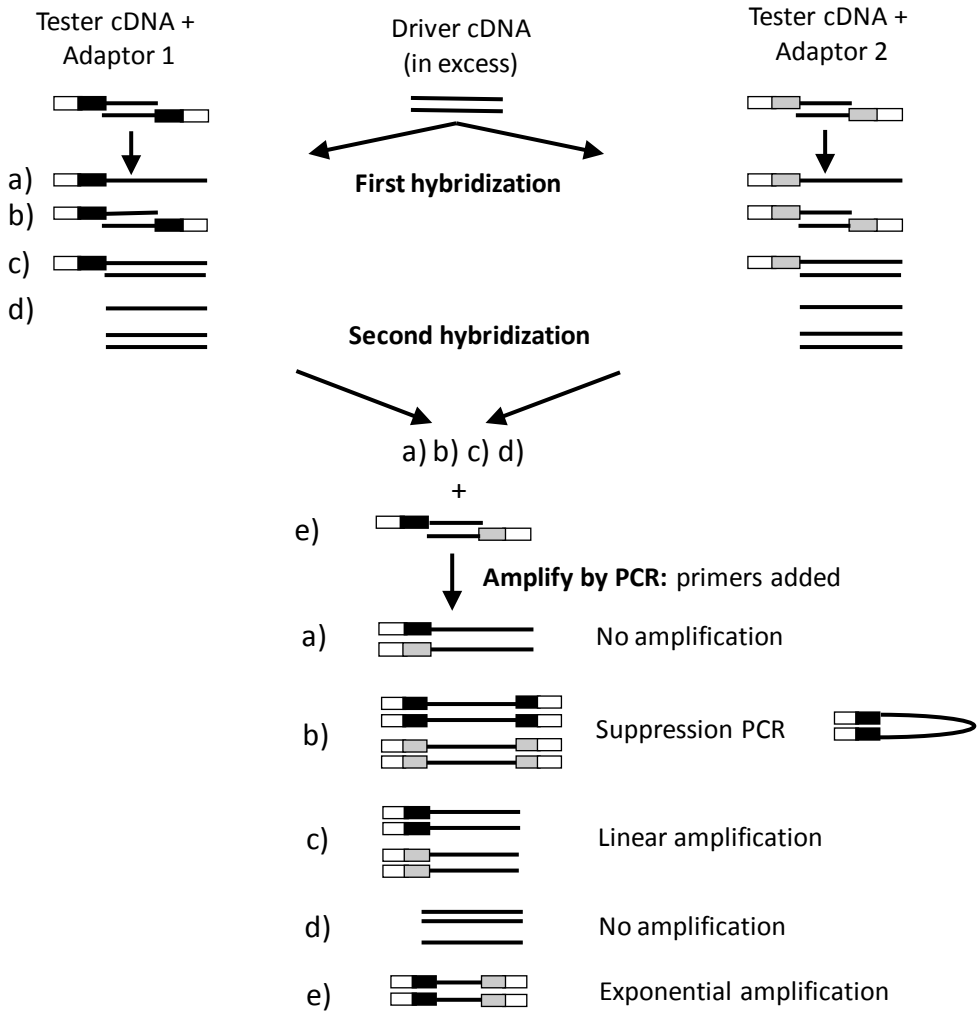

Figure 4 – Schematic representation of the Suppression Subtractive Hybridization method. Adapted from Diatchenko et al. (1996).

Quantitative real-time PCR can also be used to evaluate differential gene expression and it has been generally the method applied to validate data obtained by microarray analysis. It is a rapid, accurate and quantitative method that evolved from the polymerase chain reaction (PCR) technique which allows the amplification of specific DNA sequences. Different reagents and adapted equipment for qPCR analysis of fluorescence has been developed and the two most used approaches to measure this fluorescence are based on: (1) the binding of fluorescence molecules (e.g. SYBR Green I) to the amplified target gene; (2) probes labelled with both a reporter dye and a quencher dye (e.g. TaqMan system) (Moens et al., 2003). For both methodologies when fluorescence is plotted against the cycle number a typical PCR reaction profile is obtained with an initial lag phase (below the sensitivity of the fluorimeter), a log-linear phase and a final plateau phase (fig. 5).

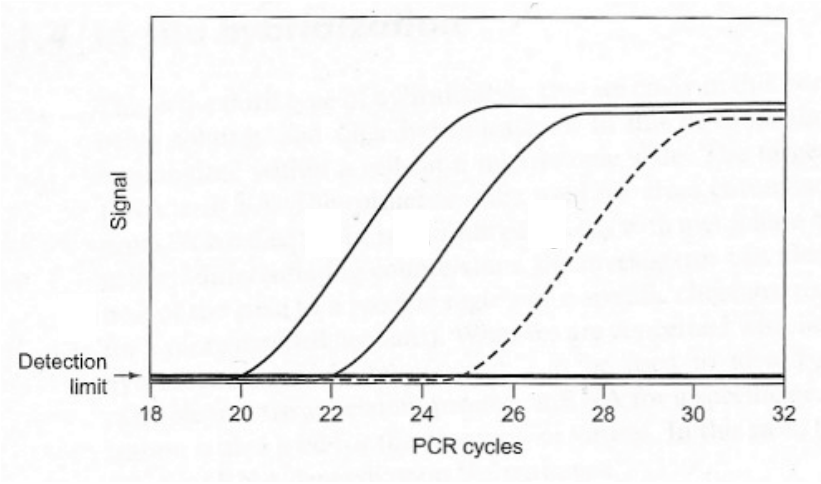

Figure 5 – Schematic representation of quantitative real-time PCR amplification plots. Adapted from Dale and von Schantz (2002).

The real-time kinetic quantification allows measurements to be made during the log-linear phase of a PCR, being the unit used for quantification the crossing point, or C_T value. The C_T value is the cycle at which a statistically significant increase in the fluorescence signal is first detected (Moens et al., 2003). The increase in signal is associated with the beginning of the exponential growth of the PCR product. The qPCR quantification can be relative, when samples are normalized to the expression of a housekeeping gene, or absolute using a standard of known copy number. Obtaining quantitative expression information is the main advantage of using real-time PCR, in addiction to reproducibility.

2. *Enchytraeus albidus* (Enchytraeidae: Oligochaeta) as test organism

The family Enchytraeidae belongs to the phylum Annelida, and class Oligochaeta. Enchytraeids are typical inhabitants of many soils worldwide and belong to the saprophagous mesofauna of the litter layer and the upper mineral soil. These organisms contribute to vital processes of soil environmental compartment such as the decomposition of organic matter and nutrient cycling, and also improve the pore structure of the soil (Amorim et al., 2005; Jansch et al., 2005).

Enchytraeus albidus is the best-known and one of the largest species of the genus with an average size of about 20 mm. Worldwide it occurs mainly in places where a large amount of organic material is present (Westheide and Muller, 1996; Jansch et al., 2005). *E. albidus* reproduces sexually but self-fertilization and parthenogenesis have been shown to exist (Gavislov, 1935; Westheide and Muller, 1996; Jansch et al., 2005).

E. albidus is considered a suitable test species for the assessment of soil quality due to the following reasons (Didden and Rombke, 2001; Rombke, 2003):

- high ecological relevance
- plays a key role in the functioning of the soil ecosystem
- covers a variety of exposure routes (via the soil solution, the solid phase, and the gaseous phase in soil)
- sensitivity to chemicals and other environmental stressors
- are easily collectable and maintained in culture
- methods for measuring oxidative stress (Howcroft et al., 2009) and energy reserves (Amorim et al., 2011a) were optimized for this species
- part of its transcriptome has been sequenced (Amorim et al., 2011b), opening new possibilities for genomic research with this species.

This species has been used frequently in terrestrial ecotoxicological studies and were subjected to various standardization procedures (ISO, 2004; OECD, 2004b, 2010).

3. Aims and outline of the thesis

The main aim of this thesis was to evaluate the mechanisms behind the responses of *Enchytraeus albidus* to chemical stressors. For that, effects of chemicals from two different groups (metals and pesticides) were assessed at different levels of biological organization: from genes and biochemical biomarkers to population endpoints. The goal was to explore the possibility of gaining more and ecologically relevant information on the mechanisms underlying toxic responses, using biochemical and molecular endpoints.

This thesis is divided in eight chapters, including the current general introduction (Chapter I), six chapters describing the experimental studies to achieve the main goal (Chapters II to VII), and a final chapter that summarizes the general conclusions of this study (Chapter VII). In detail:

In Chapter II: "Can avoidance in *Enchytraeus albidus* be used as a screening parameter for pesticides testing?", published in Chemosphere, the effects of a range of pesticides were assessed on survival and reproduction and compared to the avoidance behaviour results. The various endpoints were compared in terms of sensitivity and dose-response curves with the goal of assessing whether avoidance could be predictive of effects on the survival and reproduction.

In Chapter III: "Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures", published in Environmental Pollution, effects of the selected metals were assessed on survival and reproduction of enchytraeids. Moreover, the activity and levels of their antioxidant defences were determined, at three different exposure times (2, 4 and 8 days) and at concentrations that affected reproduction, to assess metals oxidative stress potential and neurotoxic effects.

In Chapter IV: "*Enchytraeus albidus* microarray (oligonucleotide): enrichment, design, annotation and database (EnchyBASE)", under review in PloS ONE, description is given

on how the existing cDNA library for *E. albidus* was enriched with metal and pesticide exposure responsive genes, using suppressive subtractive hybridization (SSH). With the sequencing information, an Agilent custom oligonucleotide microarray was developed for transcriptomic studies and an EST database including all genomic data on *E. albidus* was made publicly available.

In Chapter V: "Transcriptional responses in *Enchytraeus albidus*: comparison between cadmium and zinc exposure and linkage to reproduction effects", submitted to BMC Genomics, the newly developed microarray was used to assess and compare the transcriptional responses induced by the selected metals, at two concentrations of effect on reproduction (EC_{50} and EC_{90}) and three exposure periods (2, 4 and 8 days).

In Chapter VI: "Differences between Cd and Zn exposure in *Enchytraeus albidus* - linking changes in cellular energy allocation to transcriptional, enzymatic and reproductive responses", submitted to Environmental Pollution, effects on the total net energy budget of the organisms were assessed after exposure to the reproduction effect concentrations of Cd and Zn (EC_{50} and EC_{90}). Cellular Energy Allocation (CEA) was determined over periods of time from 0 to 8 days and results were discussed linked with effects obtained at other levels (chapters III and V).

In Chapter VII: "Gene expression responses in *Enchytraeus albidus* – exposure to the reproduction EC_{10}, EC_{20}, EC_{50} and EC_{90} of dimethoate, atrazine and carbendazim", submitted to Toxicological Sciences, transcription profiles after exposure to a range of concentrations of an insecticide, an herbicide and a fungicide were compared and key biological processes were found to be affected by each compound. CEA was assessed along with the microarray experiment, for the same 2 days of exposure.

In Chapter VIII: "General Discussion and Concluding Remarks", results and findings of preceding chapters are discussed, and future research perspectives formulated.

References

Amorim, M., Rombke, J., Soares, A., 2005. Avoidance behaviour of *Enchytraeus albidus*: Effects of Benomyl, Carbendazim, phenmedipham and different soil types. Chemosphere 59, 501-510.

Amorim, M.J.B., Gomes, S.I.L., Soares, A.M.V.M., Scott-Fordsmand, J.J., 2011a. Energy basal levels and allocation among lipids, proteins and carbohydrate in *Enchytraeus albidus*: changes related to exposure to Cu-salt and Cu-nanoparticles. Water, Air, Soil Pollut., In press.

Amorim, M.J.B., Novais, S.C., Van der Ven, K., Vandenbrouck, T., Soares, A.M.V.M., De Coen, W., 2011b. Development of a Microarray for *Enchytraeus albidus* (Oligochaeta): Preliminary Tool with Diverse Applications. Environ. Toxicol. Chem. 30, 1395-1402.

Apel, K., Hirt, H., 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373-399.

Calow, P., 1991. Physiological Costs of Combating Chemical Toxicants - Ecological Implications. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology 100, 3-6.

Dale, J.W., von Schantz, M., 2002. From Genes to Genomes: concepts and applications of DNA technology. John Wiley & Sons Inc., New York.

De Coen, W., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV.Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J. Aquat. Ecosyst. Stress Recovery 6, 43-55.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22, 1632-1641.

De Coen, W.M., Janssen, C.R., Giesy, J.P., 2000. Biomarker applications in ecotoxicology: bridging the gap between toxicology and ecology. New Microbiotests for Routine Toxicity Screening and Biomonitoring, 13-25

Diatchenko, L., Lau, Y.F.C., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D., Siebert, P.D., 1996. Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. U. S. A. 93, 6025-6030.

Didden, W., Rombke, J., 2001. Enchytraeids as Indicator Organisms for Chemical Stress in Terrestrial Ecosystems. Ecotoxicol. Environ. Saf. 50, 25-43.

Forbes, V.E., Palmqvist, A., Bach, L., 2006. The use and misuse of biomarkers in ecotoxicology. Environ. Toxicol. Chem. 25, 272-280.

Gavislov, C., 1935. Contributions à l'étude de l'autofécondation chez les Oligochète. Acta Zoologica 16, 21.

Halliwell, B., Gutteridge, J.M.C., 1999. Free radicals in biology and medicine. 2nd ed, Oxford: Clarendon Press, p. 543.

Howcroft, C.F., Amorim, M.J.B., Gravato, C., Guilhermino, L., Soares, A.M.V.M., 2009. Effects of natural and chemical stressors on *Enchytraeus albidus*: Can oxidative stress parameters be used as fast screening tools for the assessment of different stress impacts in soils? Environ. Int. 35, 318-324.

ISO, 1993. Soil quality -- Effects of pollutants on earthworms (*Eisenia fetida*) -- Part 1: Determination of acute toxicity using artificial soil substrate. Guideline nº11268-1, ISO (International Organization for Standardization). Geneve, Switzerland.

ISO, 1998. Soil quality -- Effects of pollutants on earthworms (*Eisenia fetida*) -- Part 2: Determination of effects on reproduction. Guideline nº11268-2, ISO (International Organization for Standardization). Geneve, Switzerland.

ISO, 1999. Soil quality - Inhibition of reproduction of Collembola (*Folsomia candida*) by soil pollutants. Guideline nº11267, ISO (International Organization for Standardization). Geneve, Switzerland.

ISO, 2004. Soil quality - Effects of pollutants on Enchytraeidae (Enchytraeus sp.) - Determination of effects on reproduction and survival. Guideline no. 16387, ISO (International Organization for Standardization). Geneve, Switzerland.

ISO, 2006. Soil quality -- Avoidance test for determining the quality of soils and effects of chemicals on behaviour -- Part 1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*). Guideline no. 17512-1, ISO (International Organization for Standardization). Geneve, Switzerland.

ISO, 2011. Soil quality -- Avoidance test for determining the quality of soils and effects of chemicals on behaviour -- Part 2: Test with collembolans (*Folsomia candida*). Guideline nº17512-2, ISO (International Organization for Standardization). Geneve, Switzerland.

Jansch, S., Amorim, M.J.B., Rombke, J., 2005. Identification of the ecological requirements of important terrestrial ecotoxicological test species. Environ Rev 13, 51-83.

Lanno, R.P., Fairbrother, A., Gaudet, C., Loehr, R., Saterback, A., Tabak, H., 2003. Introduction. . In: Lanno, R.P. (Ed.). Contaminated Soils: From Soil-Chemical Interactions to Ecosystem Management. Society of Environmental Toxicology and Chemistry (SETAC), p. 427.

Meister, A., 1995. Glutathione Metabolism. Biothiols, Pt A 251, 3-7.

Moens, L.N., van der Ven, K., Caturla, M., De Coen, W., 2003. Applications of toxicogenomics in ecotoxicology. In: Mothersill, C., Austin, B. (Eds.). In vitro methods in aquatic toxicology. Kluwer Publishers, pp. 185-218.

Nachmansohn, D., Wilson, I.B., 1951. The Enzymic Hydrolysis and Synthesis of Acetylcholine. Advances in Enzymology and Related Subjects of Biochemistry 12, 259-339.

Nota, B., Verweij, R.A., Molenaar, D., Ylstra, B., van Straalen, N.M., Roelofs, D., 2010. Gene Expression Analysis Reveals a Gene Set Discriminatory to Different Metals in Soil. Toxicol. Sci. 115, 34-40.

OECD, 1984. Guideline for Testing Chemicals. Earthworm, acute toxicity tests. No. 207, OECD (Organization for Economic Cooperation and Development). Paris, France.

OECD, 2004a. Guidelines for the testing of chemicals. Earthworm Reproduction Test (*Eisenia fetida/Eisenia andrei*). No. 222, OECD (Organization for Economic Cooperation and Development). Paris, France.

OECD, 2004b. Guidelines for the testing of chemicals. Enchytraeid Reproduction Test. No. 220, OECD (Organization for Economic Cooperation and Development). Paris, France.

OECD, 2008. Guidelines for the testing of chemicals. Predatory mite (*Hypoaspis (Geolaelaps) aculeifer*) reproduction test in soil. No. 226, OECD (Organization for Economic Cooperation and Development). Paris, France.

OECD, 2009. Guidelines for the testing of chemicals. Collembolan Reproduction Test in Soil. No. 232, OECD (Organization for Economic Cooperation and Development). Paris, France.

OECD, 2010. Guidelines for the testing of chemicals. Bioaccumulation in Terrestrial Oligochaete. No. 317, OECD (Organization for Economic Cooperation and Development). Paris, France.

Rodrigues, S.M., Pereira, M.E., da Silva, E.F., Hursthouse, A.S., Duarte, A.C., 2009. A review of regulatory decisions for environmental protection: Part I - Challenges in the implementation of national soil policies. Environ. Int. 35, 202-213.

Rombke, J., 2003. Ecotoxicological laboratory tests with enchytraeids: A review. Pedobiologia 47, 607-616.

Saint-Denis, M., Narbonne, J.F., Arnaud, C., Thybaud, E., Ribera, D., 1999. Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil: effects of benzo(a)pyrene. Soil Biology & Biochemistry 31, 1837-1846.

Schena, M., Shalon, D., Davis, R.W., Brown, P., 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270, 467-470.

Snape, J.R., Maund, S.J., Pickford, D.B., Hutchinson, T.H., 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. Aquat. Toxicol. 67, 143-154.

Snell, T.W., Brogdon, S.E., Morgan, M.B., 2003. Gene expression profiling in ecotoxicology. Ecotoxicology 12, 475-483.

van Straalen, N., Roelofs, D., 2008. Genomics technology for assessing soil pollution. J. Biol. 7, 19.

Westheide, W., Muller, M., 1996. Cinematographic documentation of enchytraeid morphology and reproductive biology. Hydrobiologia 334, 263-267.

Winston, G.W., Di Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. Aquat. Toxicol. 19, 137-161.

Chapter II

Can avoidance in *Enchytraeus albidus* be used as a screening parameter for pesticides testing?

II – Can avoidance in *Enchytraeus albidus* be used as a screening parameter for pesticides testing?

Sara C Novais, Amadeu MVM Soares & Mónica JB Amorim

*Published in Chemosphere **79** (2010), 233-237 (IF = 3.155)*

Abstract

Enchytraeids are important inhabitants of a wide variety of soil types and suitable test organisms for environmental risk assessment through the determination of effects on survival and reproduction. In this study, the effect of pesticides with different modes of action is assessed in the standard test species *Enchytraeus albidus.* Main goals were a) to test the toxicity of a range of pesticides to *E. albidus* in a natural soil (LUFA 2.2), b) to assess whether avoidance gave a prediction of effects on the survival and reproduction, and c) to investigate if the organisms' response to different toxics can be grouped into the respective chemical classes. Pesticides selected were the: (1) herbicides: phenmedipham and atrazine; (2) fungicides: carbendazim and pentachlorophenol; and (3) insecticides: dimethoate and lindane. All tested pesticides caused effects in the survival and reproduction of *E. albidus* and the compounds that showed a higher toxicity were carbendazim, dimethoate and atrazine. The effect concentrations were not chemical class dependent. In general, survival and reproduction showed similar response patterns. Avoidance behaviour showed trends of response similar to these other measured endpoints, but was less sensitive and more variable (data from a previous study). It was not possible to establish a clear correlation between survival, reproduction and avoidance endpoints. From an ecological point of view, avoidance tests are relevant but due to lower sensitivity and higher variability it is recommended that the enchytraeid avoidance test should not be used for risk assessment purposes.

Keywords: Enchytraeid worms, toxicity, soil, survival, reproduction

1. Introduction

Soil organisms play an important role as decomposer communities, contributing significantly to the organic matter decomposition, nutrient cycling and soil formation (Lee and Pankhurst, 1992; Martikainen, 1996). In particular, enchytraeids (Oligochaeta) are dominant members of the soil biocenosis in many temperate biotopes and are distributed in soils worldwide. These organisms are indirectly involved in regulating the degradation of organic matter, as well as improving the pore structure of the soil (Didden, 1993; Amorim et al., 2005b). *Enchytraeus albidus* is the best-known and one of the largest species of the genus *Enchytraeus*. The fact that they inhabit the upper layers of the soil (being specially exposed to pesticides) makes these organisms' suitable test-species for environmental risk assessment of pesticides. Furthermore, the use of enchytraeids to test the effects of pesticides is of ecological relevance since they can be negatively affected by this group of contaminants either by a direct impact of the chemicals dissolved in pore water or adsorbed to organic particles, or via a reduction in food supply – directly by fungicides or indirectly by herbicides (Didden and Römbke, 2001).

There are currently standardized tests available to evaluate various toxicity parameters for enchytraeids, such as survival and reproduction (ISO, 2003; OECD, 2004). Avoidance behaviour can be also evaluated following the earthworms ISO (International Organization for Standardization) guideline (ISO, 2007). These tests were originally validated with OECD (Organization for Economic Co-operation and Development) artificial soil (OECD, 1984), and therefore most of the existing information is from studies performed in this artificial soil (Lock et al., 2002; Römbke, 2003). Although the use of a standard artificial soil has advantages, its properties have been shown to differ clearly from those of natural soils, in particular the higher organic matter (OM) content in comparison to the majority of field soils.

In Europe, regulatory risk assessment of pesticides consists of a tiered, stepwise approach, starting with single-species tests carried out in laboratory under worst-case exposure conditions (lower-tier). If laboratory studies indicate unacceptable risk, further testing under more ecologically realistic conditions is carried out (higher-tier) (Jänsch et al.,

2006). It has been suggested (Natal-da-Luz et al., 2004; Natal-da-Luz et al., 2010) that the avoidance test with earthworms could be used as a first tier testing, due to its rapidness (48 h), followed by survival and reproduction tests. This seems only valid if the avoidance test somehow can predict survival and reproduction effects.

To test whether avoidance can predict responses on the survival and reproduction, 6 compounds were chosen. It is unknown what chemical composition causes avoidance in enchytraeids. Thus, compounds were selected so that they could represent different modes-of-action and high tonnage used pesticides. Therefore, the following two compounds of three classes of pesticides are selected: phenmedipham and atrazine as herbicides; carbendazim and pentachlorophenol as fungicides; dimethoate and lindane as insecticides. Phenmedipham and atrazine have the function of inhibiting photosynthesis in plants photosystem II. Their modes of action in animals are not yet well described but atrazine is known to act as an endocrine disruptor in frogs and fish (Hecker et al., 2005a; Hecker et al., 2005b). Carbendazim is a systemic broad spectrum benzimidazole carbamate fungicide known for affecting the nucleus division by inhibiting the beta-tubulin assembly and it is also known for inhibiting the activity of the enzyme acetylcholinesterase (Cuppen et al., 2000). The chlorinated hydrocarbon pentachlorophenol is used as a general disinfectant, termiticide or molluscicide but the greatest use is as a wood preservative (fungicide). It is widely believed that pentachlorophenol affects uncoupling mitochondrial oxidative phosphorylation, thereby causing accelerated aerobic metabolism and increasing heat production (FAO/UNEP, 1996). As for the insecticides studied, the organophosphate dimethoate is known as a cholinesterase inhibitor acting at the cholinergic synapses (IPCS, 1989) and the organochloride lindane is known as a GABA (γ-aminobutyric acid)-gated chloride channel antagonist acting as a central nervous system stimulant (Casida, 1993), and also an inducer of DNA fragmentation (Olgun et al., 2004).

Because of the extensive use (past or present) of these pesticides in agriculture and human activities, they are present in many European soils making it essential to know their effects on soil organisms. In this study, the main goals were a) to test the toxicity of a range of pesticides to *E. albidus* in a natural soil (LUFA 2.2), b) to assess whether avoidance gave a prediction qualitatively or quantitatively of effects on the survival and reproduction, and c)

to investigate if the organisms' response to different toxics can be grouped into the respective classes (herbicides, fungicides and insecticides).

2. Materials and methods

The avoidance studies have already been published (Amorim et al., 2008). For comparison purposes, the survival and reproduction studies were performed under identical conditions to the avoidance ones.

2.1 Test species

The test species used was the Oligochaete *E. albidus*, Henle 1837. Organisms were maintained in laboratory cultures, being bred in moist soil (50% OECD soil, 50% natural garden soil) at 17 ºC with a photoperiod of 16:8 h light:dark and fed twice a week with finely ground and autoclaved rolled oats (Cimarron, Portugal). Details of the culturing process are given in Römbke and Moser (2002).

2.2 Test soil

All exposures were performed in the natural standard soil LUFA 2.2 (Løkke and van Gestel, 1998). This soil type is commercially available. The properties of this soil can be summarised as follows: pH = 5.5, OM = 3.9%, texture = 6% clay; 17% silt; 77% sand).

2.3 Test substances and spiking

The substances tested were the following pesticides from different classes: a) herbicides: phenmedipham (Betosip, Stähler Agrochemie, 157 g L^{-1} of active ingredient (a.i.)) and atrazine (Sigma-Aldrich, 97.4% a.i.); b) fungicides: carbendazim (Sigma-Aldrich, 97% a.i.) and pentachlorophenol (Sigma-Aldrich, 98% a.i.); c) insecticides: dimethoate [Sigma-Aldrich (Riedel-de Haën), 99.8% a.i.] and lindane (γ-HCH, Sigma-Aldrich, 97% a.i.).

The chemical compounds dimethoate and phenmedipham were spiked into the pre-moistened soil as aqueous solutions, each test concentration into the whole batch of soil. In the case of chemicals non water soluble (atrazine, pentachlorophenol, carbendazim and lindane) acetone was used as a solvent being this solution homogeneously mixed with the soil. Solvent was left to evaporate overnight and then deionised water was added to moisten the soil to approximately 50% of the water holding capacity (WHC). In this case, a solvent control was added – control soil spiked with the same amount of acetone. After homogeneous mixing, the soil was introduced into the test vessels. All pesticides were tested in at least 5 concentrations given as active ingredient per kg of soil dry weight. The ranges of concentrations were between 0.1 and 100 mg kg^{-1}.

2.4 Experimental procedure

The assays to determine the survival and reproduction of *E. albidus* were performed in accordance to the standardized guideline for enchytraeids reproduction testing (ISO, 2003). Ten adult worms with well developed clitellum were introduced in a glass vessel, each containing 25 g moist soil (40–60% of the maximum WHC) plus food supply. Four replicates per treatment were used. The tests ran at 20 ºC with 16:8 h light/dark photoperiod, for 6 wk. Soil moisture content was adjusted each week by replenishing weight loss with the appropriate amount of deionised water. At the end of the test, the organisms were immobilized with alcohol and stained with Bengal red. After 1 h, the organisms were stained and the soil solution was spread in a box and observed under a

binocular for counting. Mortality of adults and the number of juveniles were evaluated. Avoidance was performed under identical conditions (Amorim et al., 2008).

2.5 Statistical analysis

Reproduction effect concentrations (ECx), the lethal concentration to 50% of the organisms (LC_{50}), the no-observed-effect concentrations (NOECs) and the lowest-observed-effect concentrations were calculated using ToxRat statistical software (ToxRat, 2003). For the calculation of the NOEC, data were checked for normality (Shapiro-Wilk's test) and variance homogeneity (Levene's test). Depending on the results, either the Welch *t* test for inhomogeneous variances with Bonferroni adjustment or the Williams multiple sequential *t*-test for homogeneous variances were used. To determine the LC_{50}, the regression model used was Probit and for the ECx determinations, two regressions models were used: 2-parameters Logit and Weibull as mentioned in Table 1. The regression model with the best fit for each individual data sets generated in each toxicity tests was used. For comparisons between control and solvent control, Student's *t*-test was performed.

3. Results

All the tests fulfilled the validity criteria as described by the ISO and OECD guidelines (ISO, 2003; OECD, 2004). No significant changes occurred in soil pH due to chemical spiking or test duration. In the cases where an extra acetone control was used, the statistical differences between the two controls were evaluated. For atrazine and pentachlorophenol, no significant differences were found between the two controls and all further analysis was performed versus a pool of the two controls. Significant differences occurred between control and solvent control for carbendazim and lindane ($p < 0.05$), with the solvent causing an increase in the reproductive rate hence in these cases all further analysis was performed versus the solvent control.

Responses on the survival and reproduction of *E. albidus* exposed to the different classes of pesticides are shown in Fig. 1 and compared to avoidance behaviour previously published (Amorim et al., 2008).

The results show that both survival and reproduction of *E. albidus* were affected by exposure to the tested concentrations of all six pesticides. The effect concentrations for survival and reproduction were calculated and are shown in Table 1.

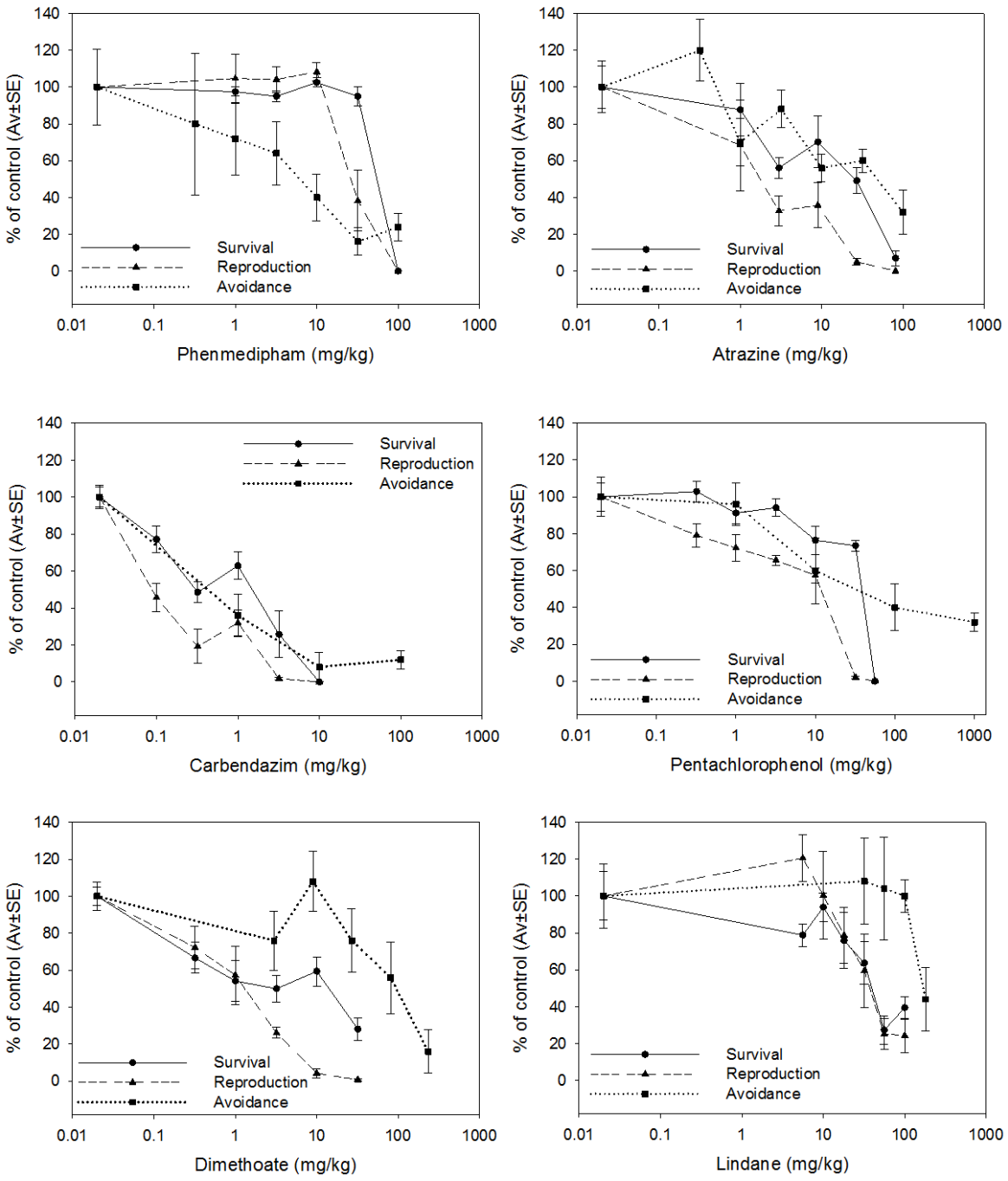

Figure 1: Results on the survival, reproduction and avoidance behaviour of *Enchytraeus albidus* when exposed in LUFA 2.2 soil to phenmedipham, atrazine, carbendazim, pentachlorophenol, dimethoate and lindane. Results express average values ± standard error (SE). * (Amorim et al., 2008).

Table 1: Effect Concentrations for survival and reproduction of *Enchytraeus albidus* exposed to phenmedipham, atrazine, carbendazim, pentachlorophenol, dimethoate and lindane. Results of the avoidance behaviour from literature are also presented.

Pesticide	Parameter										
	Survival				Reproduction						Avoidance
	LC_{50} (95%-CL)	NOEC	LOEC	R.M.	EC_{10} (95%-CL)	EC_{20} (95%-CL)	EC_{50} (95%-CL)	NOEC	LOEC	R.M	EC_{50}
Herbicides											
Phenmedipham	50 (n.d.)	32.0	100.0	Probit	14 (13 - 16)	19 (17 - 20)	28 (27 - 30)	10.0	32.0	Weibull	7[a]; 51[b]
Atrazine	12 (n.d.)	1.0	3.0	Probit	0.1 (n.d. - 1)	0.2 (n.d. - 1)	2 (0 - 7)	1.0	3.0	Weibull	38[a]
Fungicides											
Carbendazim	1 (0 - 2)	<0.1	<0.1	Probit	n.d.	n.d.	0.1 (n.d.)	<0.1	<0.1	Logit-2P	0.1[a]
Pentachlorophenol	38 (n.d.)	3.2	10.0	Probit	0.1 (0 - 1)	0.4 (0 - 2)	4 (0 - 15)	<0.3	<0.3	Weibull	703[a]
Insecticides											
Dimethoate	7 (n.d.)	0.32	1.0	Probit	0.1 (0 - 1)	0.2 (0 - 1)	1 (1 - 2)	1.0	3.2	Weibull	58[a]
Lindane	47 (6 - 95)	<5.6	5.6	Probit	9 (4 - 14)	14 (8 - 19)	33 (25 - 43)	18.0	32.0	Logit-2P	173[a]

*All values are in mg of active ingredient per kg of dry weight LUFA 2.2 soil. LC_{50} = 50% Lethal Concentration; EC_{10} = 10% Effect Concentration; EC_{20} = 20% Effect Concentration; EC_{50} = 50% Effect Concentration; 95%-CL = 95% Confidence Limits; NOEC = No-Observed-Effect Concentration; LOEC = Lowest Observed Effect Concentration; R.M. = Regression Model used; n.d. = not determined. [a] (Amorim et al., 2008); [b] (Amorim et al., 2005b).

Taking into account the concentrations of effect of the tested herbicides (Table 1), atrazine showed to have a higher toxicity to *E. albidus* (EC_{50} = 2 mg kg^{-1}; LC_{50} = 12 mg kg^{-1}) than phenmedipham (EC_{50} = 28 mg kg^{-1}; LC_{50} = 50 mg kg^{-1}). The response curves of these two compounds (Fig. 1) were very similar for the three endpoints studied: survival, reproduction and avoidance behaviour.

Regarding the studied fungicides, carbendazim was more toxic (EC_{50} = 0.1 mg kg^{-1}; LC_{50} = 1 mg kg^{-1}) than pentachlorophenol (EC_{50} = 4 mg kg^{-1}; LC_{50} = 38 mg kg^{-1}). The response pattern of the three endpoints was very similar in both fungicides: the sensitivity was decreasing in the following order, reproduction > avoidance > survival.

In the case of the tested insecticides, dimethoate showed a higher toxicity with an EC_{50} for reproduction of 1 mg kg^{-1} and a mortality LC_{50} of 7 mg kg^{-1} when compared to the ECs of lindane: EC_{50} = 33 mg kg^{-1} and LC_{50} = 47 mg kg^{-1} (Table 1). For both insecticides, the least sensitive endpoint was the avoidance behaviour.

4. Discussion

Effect concentrations found in literature with the same compounds in OECD standard soil were comparable to the present results. Except for the effect in reproduction with Lindane (Lock et al., 2002), in all of the tested compounds the toxicity was higher in LUFA 2.2 then in OECD standard soil. Tests with the artificial soil showed higher EC_{50}s and LC_{50}s: phenmedipham: EC_{50} = 53 mg kg^{-1}; LC_{50} = > 100 mg kg^{-1} (Amorim et al., 2005a); carbendazim: EC_{50} = 3 mg kg^{-1}; LC_{50} = 6 mg kg^{-1}(Römbke, 2003); pentachlorophenol: LC_{50} = 136 mg kg^{-1} (Römbke, 1989); the LC_{50} for lindane: LC_{50} = 200 mg kg^{-1} (Lock et al., 2002).The lower toxicity observed in OECD soil is in accordance with the fact that soils with a higher content of OM leads to a decrease in the bioavailable fraction of the chemical (Kuperman et al., 2006; Römbke et al., 2006). No literature data on the effects of atrazine and dimethoate on *E. albidus* reproduction in OECD soil was found. Nevertheless, a study with dimethoate and other *Enchytraeus* species, *E. crypticus*, in OECD soil (Martikainen, 1996), showed a reproduction NOEC of 8.1 mg kg^{-1}, 8 times higher than the

reproduction NOEC for *E. albidus* obtained in the present study. In the same study by Martikainen, the author has also seen an increasing toxicity of dimethoate in soils with lower OM content, which supports the previous results.

The toxicity tests conducted with these pesticides showed that the rank order of chemical toxicity (on reproduction and survival) to this enchytraeid species was, from the most toxic: carbendazim > dimethoate > atrazine > pentachlorophenol > phenmedipham > lindane (Table 1). For avoidance behaviour, the rank order of toxicity was: carbendazim > phenmedipham > atrazine > dimethoate > lindane > pentachlorophenol (Table 1). The toxicity was not related to the chemical class, i.e., it was not possible to define one class of pesticides as the most toxic to *E. albidus*. This is in accordance with the study by Frampton and co-authors (2006) where the sensitivities of different groups of soil invertebrates towards pesticides with different modes of action were addressed. In the mentioned study, no clear relation between the sensitivity of a certain group of organisms and the class of chemicals was found. However, it was observed that oligochaetes were more sensitive to fungicides (especially carbendazim), which is in accordance with the present results with *E. albidus*. The most sensitive and robust endpoint was reproduction. Interestingly, except for dimethoate where it is not so prominent, in all tested compounds survival and reproduction followed exactly the same patterns, i.e., the decrease in reproduction was preceded by an equal effect on survival (Fig. 1). This seems to indicate that the effect on reproduction caused by pesticides (at least the tested in the present study) could be caused via a decreased number of adults.

Despite the fact that, overall, avoidance behaviour showed similar dose-response patterns to the other measured endpoints, this was less sensitive and more variable, being either within the EC ranges of reproduction or survival. There was a tendency to have higher EC values in the following rank order: reproduction < survival < avoidance. Nevertheless there was no consistency on the degrees of magnitude that characterize the differences, nor a constant rank order. The present results confirm the previous knowledge that survival and reproduction do not always have a correlation, and that there seem to be no clear relation to avoidance. Moreover, the different results obtained for avoidance with phenmedipham (Table 1) shows the high variability of this endpoint, also visible by the error bars.

Difference in EC values may also be indicative of the different sensitivity of different batches of organisms.

Exposure to pentachlorophenol, dimethoate and lindane caused a mortality approximately 20, 10 and 4 times lower than the avoidance EC_{50} value (Table 1). Therefore, this means that the organisms cannot avoid the soil with these compounds in concentrations that already cause severe effects on their reproduction (see Fig. 1). In the case of lindane and dimethoate, there are evidences that such a difference in sensitivity between avoidance and survival might be explained by the effect of these compounds at the nervous system of the organisms, impairing their capacity of escaping from the contaminated soil. This is in agreement with the described mode of action of these two compounds. Lindane is known as a GABA-gated chloride channel antagonist that can thus over-stimulate the central nervous system. As to dimethoate, it inhibits acetylcholinesterase activity, which can lead to neuromuscular paralysis due to the excessive acetylcholine accumulation.

Although it was not possible to observe a clear relation between survival, reproduction and avoidance behaviour, the response curves of the three endpoints seem to be identical at least for the compounds belonging to the classes of insecticides and fungicides. For insecticides, the avoidance behaviour was the least sensitive endpoint, probably due to the interaction with the nervous system, whereas reproduction was the most sensitive one. For the fungicides, effects were more pronounced on reproduction whereas survival was the endpoint least affected. Reproduction was the most sensitive endpoint in the majority of pesticides tested, which is in accordance with the results by Frampton and co-authors (2006) where they concluded that chronic reproduction was preferred in comparison to acute lethality to provide the most protective risk assessment.

The present study highlights the ecological relevance of avoidance, i.e., if an organism is not present in a field that may not only be due to mortality but also due to avoidance. Additionally, the present study also showed that avoidance is of little use as a rapid screening for hazard assessment when using enchytraeids, as it does not predict survival and reproduction effects.

5. Conclusions

The compounds that showed a higher toxicity to *E. albidus* were carbendazim, dimethoate and atrazine, belonging to different classes of pesticides. Effect concentrations in *E. albidus* were not chemical class dependent.

The overall results showed that the toxicity of organic compounds was higher in the natural LUFA 2.2 soil than in the OECD soil. This indicates the underestimation of the risk assessment of compounds when using data from studies with OECD soil. It was not possible to see a correlation between survival, reproduction and avoidance within the selected range of pesticides. Nevertheless, overall avoidance behaviour was less sensitive than reproduction. This deserves special attention, since it means that if the organisms do not avoid contaminated soils in the field, the likelihood that effects are caused on their reproduction and/or on survival is higher. From an ecological point of view, avoidance tests are very relevant but due to their variability of effects, we do not recommend the enchytraeid avoidance test to be use in risk assessment.

References

Amorim, M., Römbke, J., Scheffczyk, A., Soares, A., 2005a. Effect of different soil types on the enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* using the herbicide phenmedipham. Chemosphere 61, 1102-1114.

Amorim, M., Römbke, J., Soares, A., 2005b. Avoidance behaviour of *Enchytraeus albidus*: Effects of benomyl, carbendazim, phenmedipham and different soil types. Chemosphere 59, 501-510.

Amorim, M.J.B., Novais, S., Römbke, J., Soares, A.M.V.M., 2008. *Enchytraeus albidus* (enchytraeidae): A test organism in a standardised avoidance test? Effects of different chemical substances. Environ. Int. 34, 363-371.

Casida, J.E., 1993. Insecticide action at the gaba-gated chloride channel - recognition, progress, and prospects. Arch. Insect Biochem. 22, 13-23.

Cuppen, J.G.M., Van den Brink, P.J., Camps, E., Uil, K.F., Brock, T.C.M., 2000. Impact of the fungicide carbendazim in freshwater microcosms. I. Water quality, breakdown of particulate organic matter and responses of macroinvertebrates. Aquat. Toxicol. 48, 233-250.

Didden, W., Römbke, J., 2001. Enchytraeids as indicator organisms for chemical stress in terrestrial ecosystems. Ecotox. Environ. Safe. 50, 25-43.

Didden, W.A.M., 1993. Ecology of terrestrial echytraeidae. Pedobiologia 37, 2-19.

FAO/UNEP, 1996. Pentachlorophenol and its Salts and Esters, Decision Guidance Documents, Operation of the Prior Informed Consent Procedure For Banned Or Severely Restricted Chemicals In International Trade. Joint FAO/UNEP Programme for the Operation of Prior Informed Consent. Rome-Geneva.

Frampton, G.K., Jänsch, S., Scott-Fordsmand, J.J., Römbke, J., Van den Brink, P.J., 2006. Effects of pesticides on soil invertebrates in laboratory studies: A review and analysis using species sensitivity distributions. Environ. Toxicol. Chem. 25, 2480-2489.

Hecker, M., Kim, W.J., Park, J.W., Murphy, M.B., Villeneuve, D., Coady, K.K., Jones, P.D., Solomon, K.R., Van Der Kraak, G., Carr, J.A., Smith, E.E., du Preez, L., Kendall, R.J., Giesya, J.P., 2005a. Plasma concentrations of estradiol and testosterone, gonadal aromatase activity and ultrastructure of the testis in *xenopus laevis* exposed to estradiol or atrazine. Aquat. Toxicol. 72, 383-396.

Hecker, M., Park, J.W., Murphy, M.B., Jones, P.D., Solomon, K.R., Van Der Kraak, G., Carr, J.A., Smith, E.E., du Preez, L., Kendall, R.J., Giesy, J.P., 2005b. Effects of atrazine on cyp19 gene expression and aromatase activity in testes and on plasma sex steroid concentrations of male african clawed frogs (*Xenopus laevis*). Toxicol. Sci. 86, 273-280.

IPCS, 1989. Environmental Health Criteria For Dimethoate, Environmental Health Criteria. World Health Organization, Published Under the Joint Sponsorship of the United Nations Environment Programme, the International Labour Organisation and the World Health Organization, Geneva.

ISO, 2003. Soil Quality - Effects of Pollutants on Enchytraeidae (*Enchytraeus sp.*) - Determination of Effects on Reproduction and Survival. Guideline no. 16387, ISO (International Organization for Standardization). Geneve, Switzerland.

ISO, 2007. Soil Quality - Avoidance Test For Testing the Quality of Soils and Effects of Chemicals on Behaviour - Part 1: Test With Earthworms (*Eisenia fetida* and *Eisenia andrei*). Guideline 17512-1. ISO (International Organization for Standardization). Geneve, Switzerland.

Jänsch, S., Frampton, G.K., Römbke, J., Van den Brink, P.J., Scott-Fordsmand, J.J., 2006. Effects of pesticides on soil invertebrates in model ecosystem and field studies: A review and comparison with laboratory toxicity data. Environ. Toxicol. Chem. 25, 2490-2501.

Kuperman, R.G., Amorim, M.J.B., Römbke, J., Lanno, R., Checkai, R.T., Dodard, S.G., Sunahara, G.I., Scheffczyk, A., 2006. Adaptation of the enchytraeid toxicity test for use with natural soil types. Eur. J. Soil Biol. 42, S234-S243.

Lee, K.E., Pankhurst, C.E., 1992. Soil organisms and sustainable productivity. Aust. J. Soil Res. 30, 855-892.

Lock, K., De Schamphelaere, K.A.C., Janssen, C.R., 2002. The effect of lindane on terrestrial invertebrates. Arch. Environ. Con. Tox. 42, 217-221.

Løkke, H., van Gestel, C.A.M., 1998. Handbook Of Soil Invertebrate Toxicity Tests. Wiley & Sons Ltd, Chichester.

Martikainen, E., 1996. Toxicity of dimethoate to some soil animal species in different soil types. Ecotox. Environ. Safe. 33, 128-136.

Natal-da-Luz, T., Ribeiro, R., Sousa, J., 2004. Avoidance tests with collembola and earthworms as early screening tools for site-specific assessment of polluted soils. Environ. Toxicol. Chem. 23, 2188-2193.

Natal-da-Luz, T., Tidona, S., Van Gestel, C.A.M., Morais, P.V., Sousa, J.P., 2009. The use of collembola avoidance tests to characterize sewage sludges as soil amendments. Chemosphere 77, 1526-1533.

OECD, 1984. Guideline For Testing Chemicals. Earthworm Acute Toxicity Tests. No. 207, OECD (Organization for Economic Cooperation and Development). Paris, France.

OECD, 2004. Guidelines For The Testing Of Chemicals. Enchytraeid Reproduction Test. No. 220, OECD (Organization for Economic Cooperation and Development). Paris, France.

Olgun, S., Gogal, R.M., Adeshina, F., Choudhury, H., Misra, H.P., 2004. Pesticide mixtures potentiate the toxicity in murine thymocytes. Toxicology 196, 181-195.

Römbke, J., 1989. Entwicklung eines reproduktionstests an bodenorganismen-enchytraeen. Bericht fu.. R das umweltbundesamt fe 106 03 051/01. Teil A: Entwicklung einer Testrichtlinie, 36 S. Teil B: Beschreibung der Entwicklungsarbeiten, 90 S. Battelle Institut e.V., 2. Auflage 1991.

Römbke, J., 2003. Ecotoxicological laboratory tests with enchytraeids: A review. Pedobiologia 47, 607-616.

Römbke, J., Jänsch, S., Junker, T., Pohl, B., Scheffczyk, A., Schallnass, H.J., 2006. Improvement of the applicability of ecotoxicological tests with earthworms, springtails, and plants for the assessment of metals in natural soils. Environ. Toxicol. Chem. 25, 776-787.

Römbke, J., Moser, T., 2002. Validating the enchytraeid reproduction test: Organisation and results of an international ringtest. Chemosphere 46, 1117-1140.

ToxRat, 2003. Toxicity Response Analysis And Testing - Software. ToxRat Solutions GmbH.

Chapter III

Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures

III – Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures

Sara C. Novais, Susana IL Gomes, Carlos Gravato, Lúcia Guilhermino, Wim De Coen, Amadeu MVM Soares & Mónica JB Amorim

Published in Environmental Pollution (2011) ***159,*** *1836-1843 (IF = 3.395; 2010)*

Abstract

To better understand chemical modes of action, emphasis has been given to stress responses at lower levels of biological organization. Cholinesterases and antioxidant defenses are among the most used biomarkers due to their crucial role in the neurocholinergic transmission and in cell homeostasis preventing DNA damage, enzymatic inactivation and lipid peroxidation. The main goal of this study was to investigate the effects of zinc and cadmium on survival and reproduction of *E. albidus* and to assess metals oxidative stress potential and neurotoxic effects at concentrations that affected reproduction. Both metals affected the enchytraeids' survival and reproduction and induced significant changes in the antioxidant defenses as well as increased lipid peroxidation, indicating oxidative damage. This study demonstrates that determining effects at different levels of biological organization can give better information on the physiological responses of enchytraeids in metal contamination events and further unravel the mechanistic processes dealing with metal stress.

Keywords: Enchytraeids; antioxidants; survival; reproduction; metals

1. Introduction

Heavy metals, like zinc and cadmium, are present in many soils worldwide mainly due to metal industry activities. These are persistent compounds, found in soils long after the source of pollution shut down. Zinc is the second most important trace metal in the body after iron, being involved in the biological function of several proteins and enzymes (Maity et al., 2008). Although zinc is an essential element, it is known to be toxic to most organisms above certain concentrations (Hopkin, 1989). Cadmium is not known to have an essential function on living organisms and is widely recognized as an environmental contaminant of soil (Lair et al., 2008; Zhang et al., 2009).

Toxicity of metal contaminated soils has been assessed with various bioassays and different species, focusing mainly on their effects on survival and reproduction of selected organisms. These tests, however, do not provide information about the biochemical responses occurring at the subcellular level, which may help to elucidate the mechanisms involved in heavy metal toxicity. For this reason, the use of biomarkers to evaluate the effects of contaminants on the environment has been increasing over the past few years (Scott-Fordsmand and Weeks, 2000).

Metals can be involved in the inhibition of enzymes due to their ability to bind to macromolecules and react with sulfhydryl groups (e.g. Olson and Christensen, 1980; Labrot et al., 1996). It was also shown by Frasco and co-authors (2005) that metals, including zinc and cadmium, inhibit in vitro acetylcholinesterase activity (AChE), a crucial enzyme in the nervous system of vertebrates and invertebrates.

Several studies suggested that exposure to metals lead to the generation of reactive oxygen species (ROS). Oxygen free radicals, like hydrogen peroxide (H_2O_2), superoxide ($O_2 -$) and hydroxyl ($OH^{\cdot}$) radicals, are among the most reactive compounds produced during heavy metal stress (Dazy et al., 2009). In order to scavenge ROS and avoid oxidative damage on lipids, proteins or DNA, cells protect themselves using enzymes and small molecular-weight antioxidants, such as glutathione (van der Oost et al., 2003; Valavanidis et al., 2006). Glutathione can have a key role in the cellular antioxidant defence

mechanisms in different ways: 1) being the substrate for gluthatione peroxidase (GPx) which metabolises hydrogen peroxide (H_2O_2) and organic hydroperoxides; 2) being conjugated to electrophilic xenobiotics by glutathione-S-transferase (GST); 3) being linked directly to pro-oxidants (like transition metals) (Meister, 1995; Saint-Denis et al., 1999). The glutathione reductase enzyme (GR) also plays an important role in cellular protection by reducing GSSG (oxidized form) to GSH (reduced and active form) (Saint-Denis et al., 2001). Superoxide dismutase (SOD) and Catalase (CAT) are other important enzymatic antioxidants in the response to oxidative stress: SOD metabolises the superoxide anion (O_2 –) into molecular oxygen and hydrogen peroxide (H_2O_2), which is then deactivated by CAT, thus preventing oxidative damage (Apel and Hirt, 2004; Howcroft et al., 2009).

Under the terrestrial environment, earthworms have been the most studied invertebrates regarding the biochemical responses towards toxic compounds. Effects of organic compounds like carbaryl, benzo(a)pyrene, atrazine and acetochlor on the biochemical responses of earthworms have been investigated (Saint-Denis, et al., 1999; Ribera et al., 2001; Xiao et al., 2006; Song et al., 2009), as well as metals like zinc, cadmium, copper and lead (Saint-Denis, et al., 2001; Laszczyca et al., 2004; Lukkari et al., 2004; Maity, et al., 2008; Zhang, et al., 2009). Studies at this level on enchytraeids (Oligochaeta), in particular *Enchytraeus albidus*, an important test species in terrestrial risk assessment, have only been recently addressed in a few publications where the effects of chemical substances on the oxidative stress and neurotransmission were studied (Gomes et al., 2011; Howcroft et al.,2009; Howcroft et al., 2011).

Although a complicated issue, if biochemical responses are to be used to predict the responses at the population level than it is important to establish links between different levels of biological organization, since toxicity induced at sub-cellular level may then be a marker for population level effects (Kammenga et al., 2000; Scott-Fordsmand and Weeks, 2000; Vasseur and Cossu-Leguille, 2006; Gravato and Guilhermino, 2009). It should be noted that population responses are easily detected in laboratory but difficult in the field, whereas biomarkers can be detected both places (not forgetting the issue of confounding factors (see eg Svendsen et al., 2007)). Recently, some attempts have been made to demonstrate the relationship between some biomarkers acting at the sub-cellular level (e.g. acetylcholinesterase, lipid peroxides, glutathione redox status) and ecologically relevant

parameters, such as behaviour, growth, reproduction, survival and feeding (Engenheiro et al., 2005; Moreira-Santos et al., 2005; Gravato and Guilhermino, 2009; Howcroft, et al., 2009).

For specific metals more specific pathways may be present; for example, it is well known that cadmium induces metallotheionein (Morgan et al., 2004; Spurgeon et al., 2004; Brulle et al., 2007; Demuynck et al., 2007). It is not the intention of this study to go into the latter more specific markers of cadmium, e.g. metallotheionein, although the induction of these can be connected to glutathione depletion (Sato et al., 1995).

The main goals of this study were: a) to assess the effects of zinc and cadmium on survival and reproduction of *E. albidus*; b) to investigate the potential effects of metals in biomarkers of oxidative stress and neurotransmission ; c) to test the influence of exposure time (2, 4 and 8 days) on the biomarker responses.

2. Materials and methods

2.1 Test species

The test species used was the Oligochaete *Enchytraeus albidus* (Henle, 1837). Organisms were maintained in laboratory cultures, being bred in moist soil (50% OECD soil, 50% natural garden soil) at 17ºC with a photoperiod of 16:8h light:dark and fed twice a week with finely ground and autoclaved rolled oats (Cimarron, Portugal). Details of the culturing process are given in Römbke and Möser (2002).

2.2 Test Soil

All exposures were performed in the natural standard soil LUFA 2.2 (Løkke and van Gestel, 1998). This soil type is commercially available from the German institution LUFA

Speyer. The properties of this soil can be summarised as follows: pH ($CaCl_2$) =5.5, Organic Matter = 4.4%, texture = 6% clay; 17% silt; 77% sand. This standard natural soil was selected instead of the OECD artificial standard due to the fact that organisms show better performance (Amorim et al., 2005a; Amorim et al., 2005b). Additionally, LUFA 2.2 soil properties (lower pH, clay and organic matter content) support relatively higher bioavailability of metals.

2.3 Spiking of the test substances

Two test substances were tested individually in this study, one essential and one non-essential metal: zinc chloride [$ZnCl_2$, Sigma-Aldrich (Riedel-de Haën), 98%] and cadmium chloride ($CdCl_2$, Fluka, 99%), respectively. Potassium chloride (KCl, MERCK, 99.5%) was used to assess the effect of the chloride ions in both Zn and Cd assays. The chemicals were spiked into pre-moistened batches of soil as aqueous solutions, each test concentration into the whole batch of soil for all replicates. The spiked soil was allowed to equilibrate three days before test start as recommended for heavy metals testing (McLaughlin et al., 2002). For the reproduction bioassays, the two metals were tested in 6 concentrations given as active ingredient (a.i.) per kg of dry weight soil. The effect of the chloride ions was tested by spiking the soil with KCl in a concentration resembling the amount of chloride ions present in the highest metal concentration tested (control+KCl).

For the oxidative stress biomarkers, the organisms were exposed to concentrations of each metal in the range of the reproduction EC_{20}, EC_{50} and EC_{90}. Similarly, one extra control spiked with KCl was also performed (control+KCl).

2.4 Experimental procedure

2.4.1 Survival and reproduction

The assays to determine the survival and reproduction of *E. albidus* were performed in accordance to the standardized guideline for enchytraeids reproduction testing (ISO, 2004; OECD, 2004). Ten adult worms with well developed clitellum were introduced in vessels, each containing 25 g moist soil (40 – 60% of the maximum water holding capacity (WHC)) plus food supply (50 mg of finely ground and autoclaved rolled oats, being half of the amount supplied every week). Four replicates per treatment were used. The tests ran at 20ºC with 16:8 light/dark photoperiod, for six weeks. Soil moisture content was adjusted each week by replenishing weight loss with the appropriate amount of deionised water. At the end of the test, the organisms were immobilized with alcohol and coloured with a few drops of Bengal red (1% solution in ethanol), a dye that colors organic material (reddish color) and facilitates observation and counting. After one hour, the organisms were coloured and the soil solution was spread in a box and observed under the binocular for counting. Mortality of adults and the number of juveniles were evaluated.

2.4.2 Oxidative stress and neurotransmission biomarkers

Fifteen adult worms with well developed clitellum were introduced in the test vessels, each containing 25 g moist soil (40 to 60% of the maximum WHC). The vessel was covered with a lid (containing small holes) and the worms were exposed for two, four and eight days at 20ºC and a 16:8h photoperiod. Seven replicates per treatment were used. At test end, animals of each replicate were carefully removed, rinsed in deionised water, weighed and frozen in liquid Nitrogen. Organisms from day zero of exposure were equally frozen and all the samples were stored at -80ºC till further analysis.

2.5 Biochemical Analysis

Fifteen organisms were removed from the soil in each replicate and homogenized (1:15) in 0.1 M K-phosphate buffer (pH 7.4). Part of this tissue homogenate was used to determine the extent of endogenous lipid peroxidation (LPO) by measuring the thiobarbituric acid reactive substances (TBARS) according to Ohkawa et al. (1979) and Bird and Draper (1984), with the adaptations of Wilhelm et al. (2001) and Torres et al. (2002). The remaining tissue homogenate was centrifuged for 20 min at 10000 g (4ºC) to obtain the post-mitochondrial supernatant (PMS). GST activity was determined following the conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) at 340 nm and 25ºC (Habig et al., 1974). SOD activity was determined based on the reduction of cytochrome c by superoxide radicals generated by the xanthine-xanthine oxidase system at 550nm and 25ºC (Mccord and Fridovic, 1969) adapted to microplate (Lima et al., 2007). CAT activity was measured following the decrease in absorbance at 240 nm (25ºC) representing the substrate (H_2O_2) consumption (Clairborne, 1985). GPx activity was determined by measuring the decrease in NADPH at 340 nm and 25ºC , using H_2O_2 as a substrate (Mohandas et al., 1984). GR activity was assayed by measuring the decrease in NADPH levels at 340 nm and 25ºC according to Cribb et al. (1989). TG content (GSH+GSSG) and oxidized glutathione (GSSG) were determined at 412 nm and 25ºC , using a recycling reaction of GSH with 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) in the presence of GR excess (Tietze, 1969; Baker et al., 1990). 2-Vinyl-pyridine was used to conjugate GSH for the GSSG determination (Griffith, 1980). GSH was calculated by subtracting GSSG from the TG levels. Cholinesterases (ChEs) activity were measured using the Ellman method (Ellman et al., 1961) adapted to microplate (Guilhermino et al., 1996) at 414 nm and 25ºC. The protein concentration of PMS was determined according to the Bradford method (Bradford, 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as a standard.

2.6 Statistical Analysis

The reproduction effect concentrations (ECx), the lethal concentration to 50% of the organisms (LC_{50}), the no-observed-effect concentrations (NOECs) and the lowest-observed-effect concentrations (LOECs) were calculated. Probit regression model was used to determine the LC_{50} (SPSS, 1999), and for the ECx determinations the 2-parameters Logit regression model was used because it showed the best fit for both individual data sets, based on the probabilities of Chi^2 and F, which are a goodness of fit measure (ToxRat, 2003). NOECs and LOECs were based on results from One way Analysis of Variance (ANOVA) with Dunnett's method for multiple comparisons, performed for both endpoints to assess significant differences between the control and spiked soils (SigmaPlot, 1997).

Correspondence Analysis - CA (multivariate analysis) was performed to explore the general correspondence between the biomarker expressions and the exposures, using standardized data (Proc Corresp, SAS 9.1.3). This was then used to support the uni-variate analysis and interpretation.

For the biomarkers uni-variate analysis, a two-way ANOVA (SigmaPlot, 1997) was performed for differences between treatments and times of exposure. Holm–Sidak test was used to discriminate statistical significant differences by performing multiple comparisons relatively to the control group. Data was transformed due to lack of normal distribution and homogeneity of variance. For comparisons between day 0 and control from day 2, a Student's *t*-test was performed (SigmaPlot, 1997).

In both reproduction and biomarkers experiments, for comparisons between control and control+KCl, a Student's *t*-test was performed (SigmaPlot, 1997).

3. Results

3.1 Survival and reproduction

Tests fulfilled the validity criteria as described by the standard guidelines (ISO, 2004; OECD, 2004). Soil pH did not change significantly due to chemical spiking or test duration.

Effects on the survival and reproduction of *E. albidus* exposed to zinc and cadmium chloride are shown in figure 1.

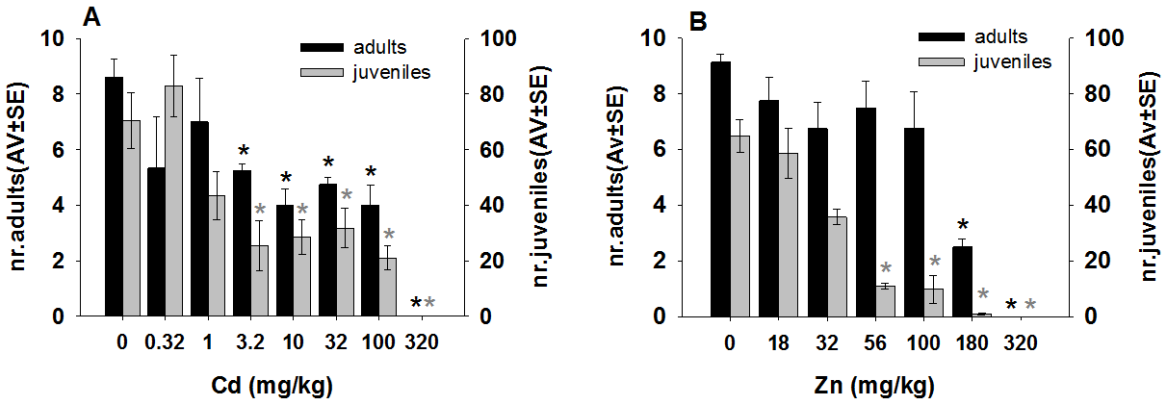

Fig. 1: Results on the survival and reproduction of *Enchytraeus albidus* when exposed in LUFA 2.2 soil to the chloride salts of cadmium (A) and zinc (B). Results express average (AV) values ± standard error (SE); n=4 (* indicates statistically significant differences between control and spiked soil, Dunnett's $p<0.05$). Control shows the pool of control and KCl values.

No statistically significant differences were found between control and the spiked soil with KCl for both tests ($p < 0.05$). Therefore, all statistical analyses were performed against the mean of both controls (pool).

The results show that both metals affected the number of adults and juveniles, shown by a significant decrease within the concentrations tested. The survival of adults and the number

of juveniles were significantly decreased for Cd concentrations equal to or greater than 3.2 mg/Kg (Figure 1A). Concerning Zn, the survival of adults was significantly decreased for concentrations equal or higher than 180 mg/Kg, whereas a significant decrease of the number of juveniles was observed for concentrations equal to or greater than 32 mg/Kg (Figure 1B). The effect concentrations for survival and reproduction can be seen in Table 1.

Table 1: Effect concentrations (ECx), NOEC and LOEC for survival and reproduction of *Enchytraeus albidus* exposed to cadmium or zinc chloride.

	Survival			**Reproduction**						
	LC_{50} (95%-CL)	**NOEC**	**LOEC**	**EC_{10} (95%-CL)**	**EC_{20} (95%-CL)**	**EC_{50} (95%-CL)**	**EC_{90} (95%-CL)**	**NOEC**	**LOEC**	**R.M.**
Cadmium ($CdCl_2$)	14.1 (4.0-123.0)	1.0	3.2	0.02 (n.d)	0.2 (n.d.)	6.2 (n.d.)	186.9 (n.d.)	1.0	3.2	Logit-2P r^2=0.554 $p(Chi^2)$=0,998 p(F)=0,09
Zinc ($ZnCl_2$)	72.6 (58.1-84.9)	100	180	16.3 (7.9-21.7)	21.6 (13.2-26.8)	35.0 (28.6-42.4)	76.0 (56.7-150.9)	18.0	32.0	Logit-2P r^2=0.899 $p(Chi^2)$=0,999 p(F)= 0,004

*All values are in mg of active ingredient (a.i.) per kg of dry weight LUFA 2.2 soil. EC_{10} = 10% Effect Concentration; EC_{20} = 20% Effect Concentration; EC_{50} = 50 % Effect Concentration; EC_{90} = 90 % Effect Concentration; 95%-CL = 95% Confidence Limits; NOEC = No-Observed-Effect Concentration; LOEC = Lowest Observed Effect Concentration; R.M. = Regression Model used; n.d. = not determined.

The comparison of LOECs for survival and reproduction (Table 1) showed that effects occur at lower concentrations with cadmium than with zinc.

3.2 Oxidative stress and neurotransmission biomarkers

3.2.1 Control versus control+KCl

The effect of the chloride ions was tested for all biomarkers measured in animals of control+KCl and compared to control. In most cases, no significant differences were found between the control and control+KCl. Thus, further analyses were performed versus a pool of the two controls. In the few cases where significant differences occurred between control and control+KCl ($p < 0.05$), the subsequent statistical analysis was performed versus the control+KCl.

3.2.2 Effects of zinc and cadmium

Results showed several significant differences in most biomarkers induced by zinc and cadmium chloride through the exposure periods (2, 4 and 8 days).

On an exploratory level, Correspondence Analysis (CA) showed, when analysing all Zn data together (Fig. 2-A), that there was no pronounced correspondence between individual markers and the exposure groups, which probably reflect a change in response pattern over time. The latter is confirmed in an analysis performed on the level of exposure day (not shown) and the individual analysis (see Fig 3-6), although the number of data for the individual days was lower indicating a risk of over fitting. However, there was a tendency to high GSSG when low TG and GSH, mainly expressed at the 2 day exposure (not shown). This tendency was not as pronounced as for cadmium where there was a clearer correspondence between high GSSG and high Cd exposure (Fig. 2-B). Again there was a tendency to high GSSG when low GSH, LPO, GST and GPx in cadmium exposures. Also here the trend became clear when analysing on the level of individual days (see example Fig. 2-C), although over fitting is an issue here.

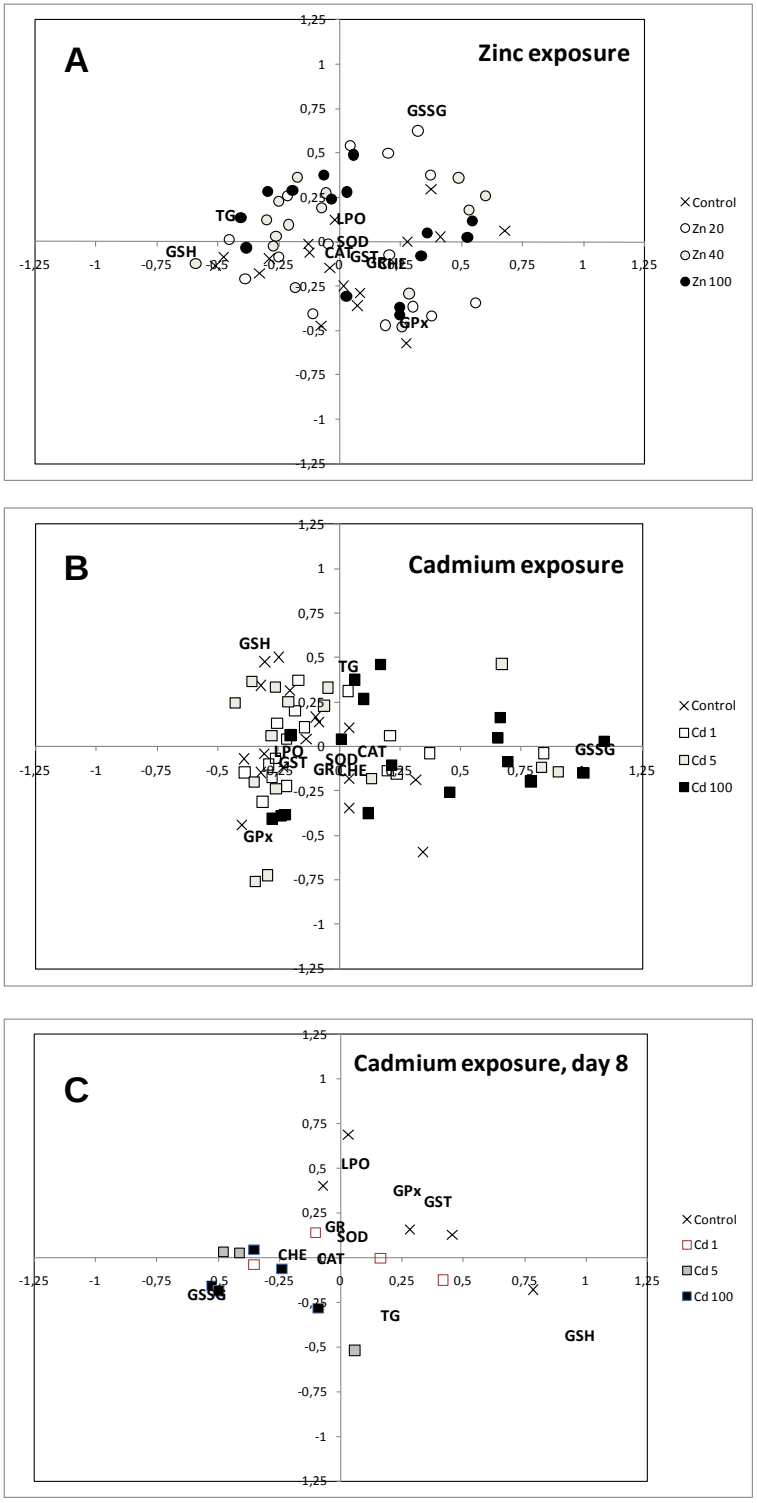

Fig. 2: Plots showing correspondence analysis of (all exposures marked differently) A: All data with Zn. B: All data with Cd. C: Day 8 data with Cd.

At 2 days of exposure, SOD activity was significantly increased by all concentrations of Cd (1, 5 and 100 mg/Kg) and by 40 and 100 mg/kg of Zn (Fig. 3-B). The activity of GR was also significantly increased at 2 days, but only for the lowest concentration of Zn (20 mg/Kg) and the highest concentration of Cd (100 mg/Kg) (Fig. 3-E). Nevertheless, no oxidative damage was observed at 2 days exposure, since the level of LPO was not significantly altered by exposure to metals (Fig. 5).

At 4 days of exposure, CAT and GR activities were significantly decreased in animals exposed to either metal. Inhibition of CAT activity was observed for all the concentrations of Cd and Zn tested (Fig. 3-C), whereas inhibition of GR activity was only observed at 40 mg/kg of Zn (Fig. 3-E). The activity of SOD was significantly increased after exposure to 20 mg/kg of Zn (Fig 3-B). During this time point (4 days) significantly increased levels of TG were observed when animals were exposed to the highest concentrations of Cd (100 mg/Kg) and to Zn (20 and 100 mg/Kg) (Fig. 4-A). Increased levels of GSSG were also observed in organisms exposed to the highest concentrations of Cd and Zn (100 mg/Kg) (Fig.4-B). Moreover, significantly increased levels of LPO were seen at 4 days of exposure to 100 mg/kg of Zn and to 5 mg/kg and 100 mg/kg of Cd (Fig. 5).

At 8 days of exposure, most of the enzymes involved in the antioxidative stress response showed a decrease of their activities. SOD and GPx activities were significantly decreased by all concentrations of both metals (Fig. 3-B and D). Moreover, inhibition of GR activity was observed at 100 mg/kg of Zn (Fig. 3-E) as well as the inhibition of GST activity at 100 mg/kg of both Zn and Cd (Fig. 3-A). Furthermore, at 8 days of exposure the level of the oxidized form of glutathione (GSSG) was significantly increased at 20 mg/kg of Zn and at all concentrations of Cd, whereas the levels of reduced glutathione (GSH) were significantly decreased at 100 mg/kg of Zn and Cd (Fig. 4-B and C). No oxidative damage was observed at 8 days of exposure since the LPO levels were not increased by exposure to metals. On the contrary, significant lower levels of LPO were observed at 1, 5 and 100 mg/kg of Cd. (Fig. 5).

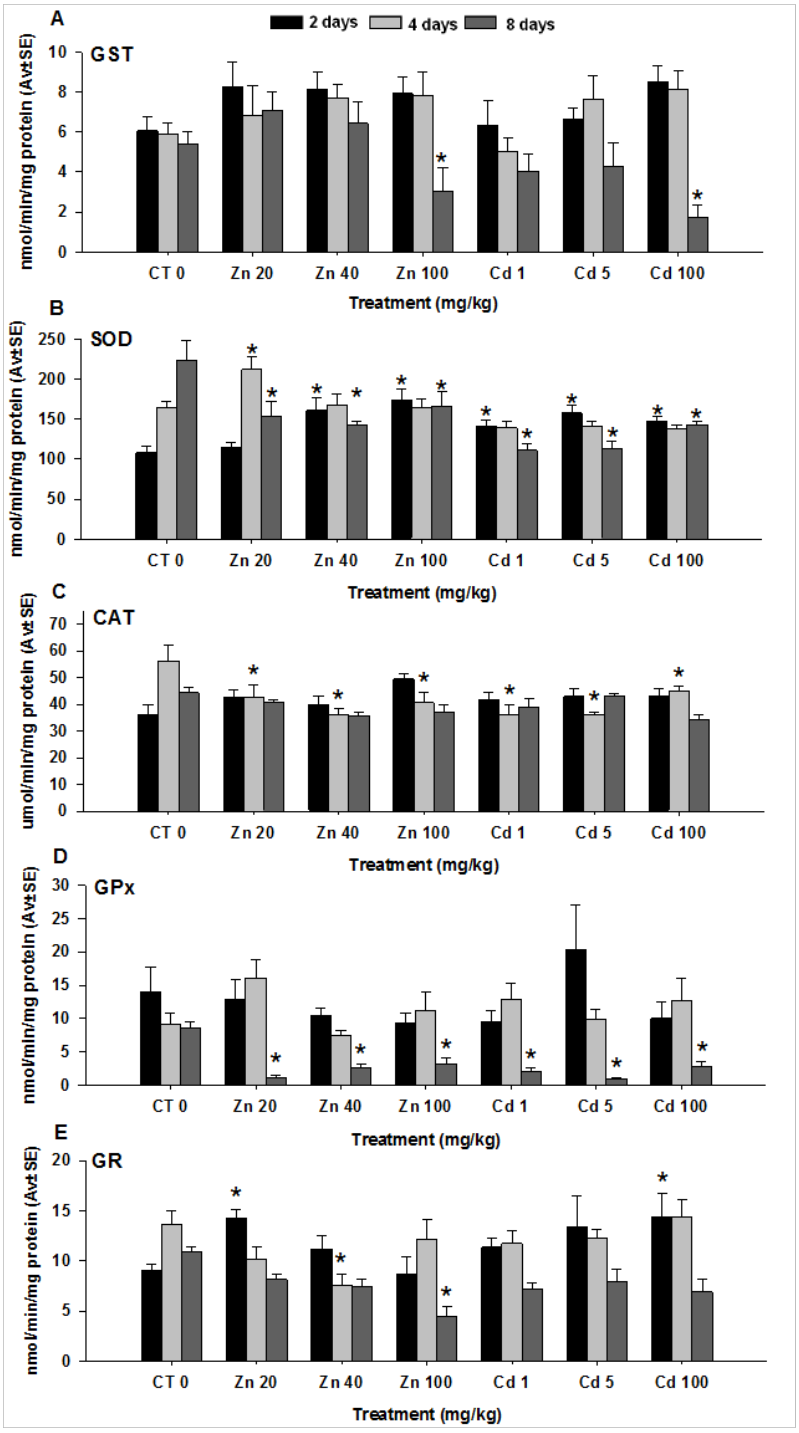

Fig. 3: Results of the detoxification (A-GST) and enzymatic antioxidant defenses (B-SOD, C-CAT, D-GPx and E-GR) of *E. albidus* when exposed to zinc and cadmium chloride in three concentrations (reproduction EC_{20}, EC_{50} and EC_{90} in mg/kg) and three time points (2, 4 and 8 days). CT 0 shows the pool of control and control+KCl values when there were no differences, or control+KCl when this was different from the control (a). Results express average values ± standard error (SE); n=7 (* indicates statistically significant differences between exposed groups and control, Holm-Sydak $p<0.05$).

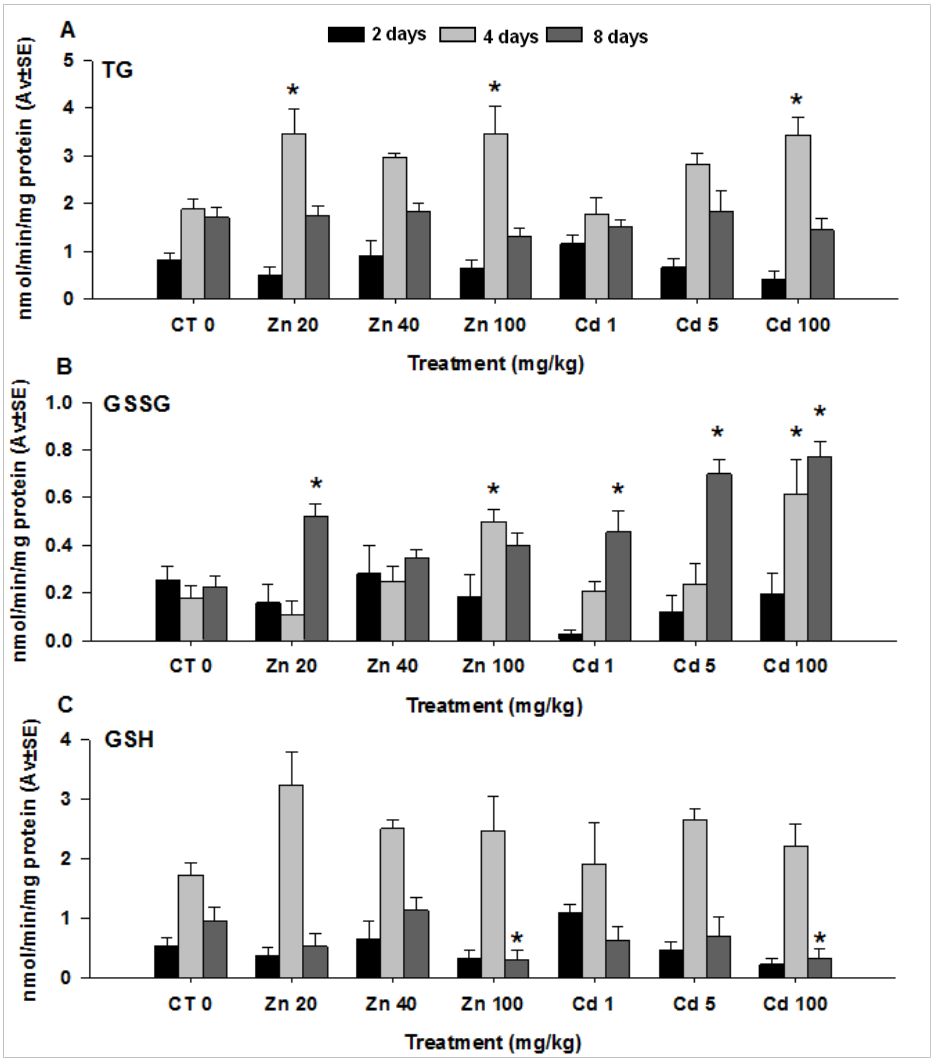

Fig. 4: Results of the non-enzymatic antioxidant defenses (A-TG, B-GSSG and C-GSH) of *E. albidus* when exposed to zinc and cadmium chloride in three concentrations (reproduction EC_{20}, EC_{50} and EC_{90} in mg/kg) and three time points (2, 4 and 8 days). CT 0 shows the pool of control and control+KCl values when there were no differences, or control+KCl when this was different from the control (a). Results express average values ± standard error (SE); n=7 (* indicates statistically significant differences between exposed groups and control, Holm-Sydak $p<0.05$).

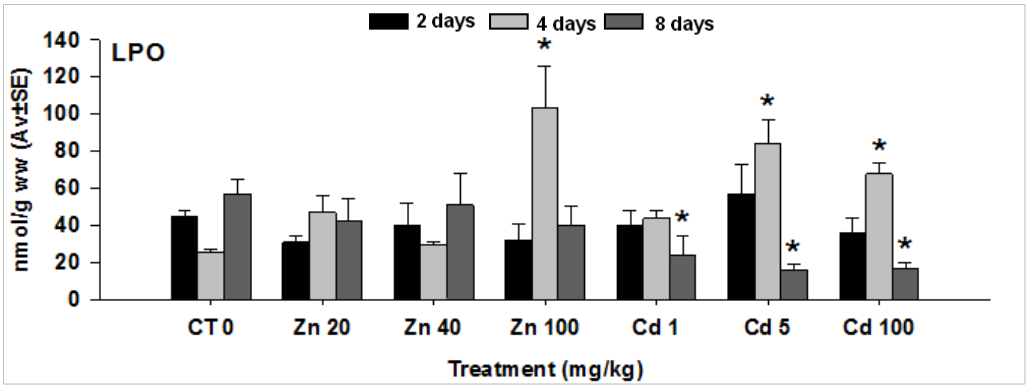

Fig. 5: Results of LPO levels in *E. albidus* when exposed to zinc and cadmium chloride in three concentrations (reproduction EC_{20}, EC_{50} and EC_{90} in mg/kg) and three time points (2, 4 and 8 days). CT 0 shows the pool of control and control+KCl values when there were no differences, or control+KCl when this was different from the control (a). Results express average values ± standard error (SE); n=7 (* indicates statistically significant differences between exposed groups and control, Holm-Sydak $p<0.05$).

Regarding the ChE activity, no significant differences were observed after 2, 4 and 8 days exposure to any of the metals (Fig. 6).

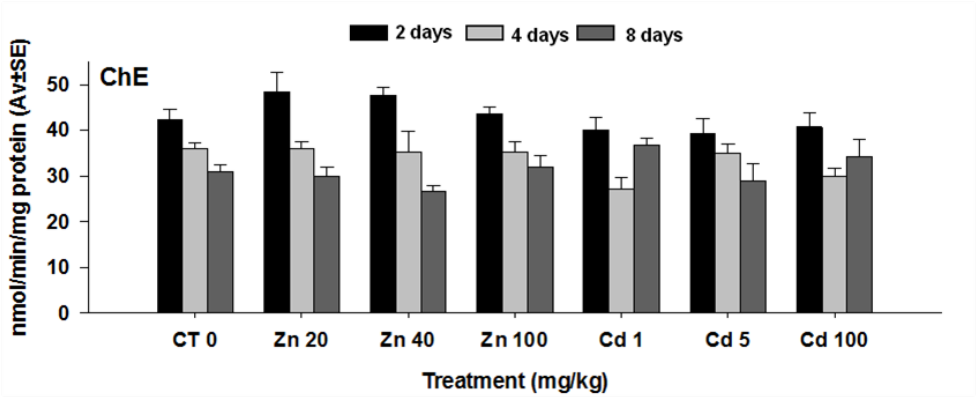

Fig. 6: Results of ChE activity in *E. albidus* when exposed to zinc and cadmium chloride in three concentrations (reproduction EC_{20}, EC_{50} and EC_{90} in mg/kg) and three time points (2, 4 and 8 days). Results express average values ± standard error (SE); n=7.

3.2.3 Effects of time of exposure

No significant differences were found between organisms from day 0 and from the control of 2 days exposure in any of the parameters measured.

However, results showed that the response of biomarkers varied with the time of exposure. In some cases, significant differences were also found between controls from different time points like for example in SOD and ChE activities, where significant differences were found between controls from all three times of exposure.

4. Discussion

Both zinc and cadmium had an effect on the survival and reproduction of *E. albidus*. When testing cadmium, it could be observed that the decrease in reproduction was preceded by an effect on survival (Fig. 1), which seems to indicate a causal effect via a decrease in the number of adults. This response pattern has been observed for other compounds, e.g. pesticides, in which certain compounds caused a more pronounced effect on the survival of *E. albidus* than others (Novais et al., 2010). Such pattern was not observed for zinc since reproduction was affected at concentrations that did not affect survival (32, 56 and 100 mg/Kg – Fig. 1-B). Cadmium affected the organisms at lower concentrations than zinc (Table 1). These results are in good agreement with previous studies with Zn and Cd using OECD artificial soil (Lock and Janssen, 2001a; Lock and Janssen, 2001b) where reproduction EC_{50s} for reproduction were 267 and 158 mg/kg respectively. The lower toxicity observed in OECD soil than in LUFA 2.2 soil is probably related with lower bioavailable fraction of the chemical at higher organic matter content (Kuperman et al., 2006; Rombke, et al., 2006).

In terms of oxidative stress biomarkers, exposure to the reproduction ECs caused effects in most of the tested parameters. As seen from the correspondence analysis, especially GSSG

and LPO/GSH/GPx and GST (in opposite directions) corresponded to changes in exposure, which supports the individual analysis.

GST activity was significantly decreased after 8 days of exposure to the highest concentration of zinc and cadmium. Cunha et al. (2007) suggested that GST inhibition may occur either through direct action of the metal on the enzyme, forming an inactive complex and facilitating the elimination of the toxic agent , which has been shown to happen with cadmium (Dierickx, 1982) or indirectly via: 1) the production of ROS that interact directly with the enzyme; 2) the depletion of its substrate (glutathione – GSH); and/or 3) the down regulation of GST genes through different mechanisms. This down regulation of GST has been observed in vitro under exposure to cadmium and zinc, which was attributed to inhibition of binding of nuclear transcription factors (Nf-κB, AP-1) to the gene promoter region involving generation of reactive oxygen species (Shumilla et al., 1998). An incomplete phase II biotransformation may thus lead to the production and accumulation of ROS, as well as, the formation of electrophilic intermediates, that can inactivate other enzymes, cause lipid peroxidation or DNA damage (Gravato and Guilhermino, 2009).

The ROS accumulation can explain the depletion of GSH in animals exposed to the highest concentrations of the metals during 8 days and the high levels of GSSG observed at 4 and 8 days, indicating a clear imbalance in the cellular redox status concomitant with the inactivation of the enzymes involved in the defenses against the oxidative stress (SOD, CAT, GR, GPx). Ultimately, this ROS accumulation can be seen in the increase of LPO levels at 4 days of exposure to the highest concentrations of both zinc and cadmium, indicating that oxidative damage occurred. The fact that no further increase in LPO was observed at 8 days can be hypothesized as being due to: 1) an extreme damage occurring in the membrane cells that may have led to their disruption and, consequently, to lower levels of lipid peroxidation measured; 2) an attempt of the organisms to eliminate the stress factor and recover from the oxidative state by directly complexing GSH with the metals; 3) an increased metallothionein synthesis related to GSH depletion, acting as a radical scavenger (Sato, et al., 1995). The last two hypotheses can be supported by the significantly decreased levels of GSH after 4 days of exposure.

The direct binding of GSH to the metals seems to be a suitable explanation for the depletion of this antioxidant since the enzymes that use this substrate are inactive at 8 days of exposure. It is the case for GST that uses GSH in the detoxification processes and GPx that uses GSH as a hydrogen donor to eliminate H_2O_2, promoting the oxidation of two molecules of GSH into a molecule of GSSG (Maity, et al., 2008). Similar results of depletion in GSH and enhancement of GSSG levels were observed in the earthworm *Lampito mauritii* exposed to lead during 7 days (Maity, et al., 2008) as well as for *Eisenia andrei,* exposed to benzo(a)pyrene (Saint-Denis, et al., 1999).

Regarding ChEs, a group of enzymes related to neuronal functions, no significant differences in activity were seen during this study with metals. Although Frasco and colleagues (2005) found that AChE (the main cholinesterase present in earthworms (Rault et al., 2007)) is inhibited by zinc and cadmium in vitro, and besides the fact that the response of our species AChE was not studied in vitro, the results here obtained are not surprising when taking in consideration the behavioral responses of these organisms. In a previous study by Amorim and co-authors (2008), it was seen that *E. albidus* were still able to avoid contaminated soils with zinc or cadmium even at very high concentrations. These compounds were not interfering with the ChE activities, indicating that these could be involved in the neuromuscular transmission, but further studies are required to be able to correlate the two endpoints.

In summary, these results confirm that zinc and cadmium induce oxidative stress and membrane damage under concentrations known to cause effects at the reproduction level in 21 days tests. Results also suggest that glutathione may be an important defense against metal contamination, under short-term exposures, of course combined with an induction of metallothionein. This is in agreement with the study of Howcroft and co-authors (2009) where the exposure of *E. albidus* to copper mainly induced the enzymes dependent of glutathione, GPx and GR. The effect of time in the controls was previously observed by other authors (e.g. Saint-Denis, et al., 1999; Arnaud et al., 2000; Conners and Ringwood, 2000; Saint-Denis, et al., 2001; Vlahogianni and Valavandis, 2007; Gomes et al., 2011) and needs further investigation. As demonstrated previously (Saint-Denis et al., 1999, 2001), knowledge of time- and concentration-dependent relationships of the responses is needed to fully understand short-term physiological responses of enchytraeids in metal

contamination events. In the present study, time of exposure influenced the biochemical responses and, in general, the results seem to indicate that at 2 days of exposure the organisms start to respond to the oxidative stress with the slight increase activities of enzymes like SOD and GR, but after 4 days, oxidative damage already occurred.

5. Conclusions

Zinc and cadmium exposure influenced the survival and reproduction of *E. albidus*, with cadmium being more toxic. Moreover, these chemicals disturbed the cellular redox status in *E. albidus*. Both zinc and cadmium induced significant changes in the antioxidant enzyme activities and substrate levels as well as increased lipid peroxidation, indicating oxidative damage. Glutathione seems to play an important role in the antioxidant defense against metals and the levels of GSH and GSSG seem to be good biomarkers for this type of exposure in *E. albidus*. The time intervals studied were important and allowed to see the evolution of oxidative events.

In general, the biochemical responses investigated were sensitive, with effects observed even at the lowest concentration. This confirms that the use of a set of biomarkers, associated with alterations on higher levels of biological organization, is important to evaluate the effects of pollutants on organisms and to understand their mechanistic action.

References

Amorim, M., Rombke, J., Scheffczyk, A., Soares, A., 2005a. Effect of different soil types on the enchytraeids *Enchytraeus albidus* and *Enchytraeus luxuriosus* using the herbicide phenmedipham. Chemosphere 61, 1102-1114.

Amorim, M., Rombke, J., Soares, A., 2005b. Avoidance behaviour of *Enchytraeus albidus*: Effects of benomyl, carbendazim, phenmedipham and different soil types. Chemosphere 59, 501-510.

Amorim, M.J.B., Novais, S., Römbke, J., Soares, A.M.V.M., 2008. *Enchytraeus albidus* (Enchytraeidae): A test organism in a standardised avoidance test? Effects of different chemical substances. Environment International 34, 363-371.

Apel, K., Hirt, H., 2004. Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55, 373-399.

Arnaud, C., Saint-Denis, M., Narbonne, J.F., Soler, P., Ribera, D., 2000. Influences of different standardised test methods on biochemical responses in the earthworm *Eisenia fetida andrei*. Soil Biology & Biochemistry 32, 67-73.

Baker, M.A., Cerniglia, G.J., Zaman, A., 1990. Microtiter plate assay for the measurement of glutathione and glutathione disulfide in large numbers of biological samples. Analytical Biochemistry 190, 360-365.

Bird, R.P., Draper, A.H., 1984. Comparative studies on different methods of malondyhaldehyde determination Methods in Enzymology 90, 105-110.

Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Analytical Biochemistry 72, 248-254.

Brulle, F., Mitta, G., Leroux, R., Lemiere, S., Lepretre, A., Vandenbulcke, F., 2007. The strong induction of metallothionein gene following cadmium exposure transiently affects the expression of many genes in *Eisenia fetida*: A trade-off mechanism? Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 144, 334-341.

Clairborne, A., 1985. Catalase activity, in: Greenwald, R.A. (Ed.), CRC Handbook of Methods in Oxygen Radical Research. CRC Press, Boca Raton, FL, USA, pp. 283-284.

Conners, D.E. and Ringwood, A.H., 2000. Effects of glutathione depletion on copper cytotoxicity in oysters (*Crassostrea virginica*). Aquatic Toxicology 50, 341-349.

Cribb, A.E., Leeder, J.S., Spielberg, S.P., 1989. Use of a microplate reader in an assay of glutathione-reductase using 5,5'-dithiobis(2-nitrobenzoic acid). Analytical Biochemistry 183, 195-196.

Cunha, I., Mangas-Ramirez, E., Guilhermino, L., 2007. Effects of copper and cadmium on cholinesterase and glutathione S-transferase activities of two marine gastropods (*Monodonta lineata* and *Nucella lapillus*). Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 145, 648-657.

Dazy, M., Masfaraud, J.F., Ferard, J.F., 2009. Induction of oxidative stress biomarkers associated with heavy metal stress in *Fontinalis antipyretica* Hedw. Chemosphere 75, 297-302.

Demuynck, S., Grumiaux, F., Mottler, V., Schikorski, D., Lemiere, S., Lepretre, A., 2007. Cd/Zn exposure interactions on metallothionein response in *Eisenia fetida* (Annelida, Oligochaeta). Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 145, 658-668.

Dierickx, P.J., 1982. In vitro inhibition of the soluble glutathione s-transferases from rat-liver by heavy-metals. Enzyme 27, 25-32.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7, 88-&.

Engenheiro, E.L., Hankard, P.K., Sousa, J.P., Lemos, M.F., Weeks, J.M., Soares, A.M.M., 2005. Influence of dimethoate on acetylcholinesterase activity and locomotor function in terrestrial isopods. Environmental Toxicology and Chemistry 24, 603-609.

Frasco, M.F., Fournier, D., Carvalho, F., Guilhermino, L., 2005. Do metals inhibit acetylcholinesterase (AChE)? Implementation of assay conditions for the use of AChE activity as a biomarker of metal toxicity. Biomarkers 10, 360-375.

Gomes, S.I.L., Novais, S.C., Gravato, C., Guilhermino, L., Scott-Fordsmand, J.J., Soares, A.M.V.M., Amorim, M.J.B., 2011. Effect of Cu-nanoparticles versus one Cu-salt: analysis of stress biomarkers response in *Enchytraeus albidus* (Oligochaeta). Nanotoxicology, in press.

Gravato, C., Guilhermino, L., 2009. Effects of benzo(a)pyrene on seabass (*Dicentrarchus labrax* L.): biomarkers, growth and behavior. Human and Ecological Risk Assessment 15, 121-137.

Griffith, O.W., 1980. Determination of glutathione and glutathione disulfide using glutathione-reductase and 2-vinylpyridine. Analytical Biochemistry 106, 207-212.

Guilhermino, L., Lopes, M.C., Carvalho, A.P., Soared, A.M.V.M., 1996. Inhibition of acetylcholinesterase activity as effect criterion in acute tests with juvenile *Daphnia magna*. Chemosphere 32, 727-738.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-Transferases - First enzymatic step in mercapturic acid formation. Journal of Biological Chemistry 249, 7130-7139.

Henle, F.G.J., 1837. Ueber Enchytraeus, eine neue Anneliden-Gattung. Archiv für Anatomie, Physiologie und wissenschaftliche Medicn, 74-90.

Hopkin, S.P., 1989. Ecophysiology of metals in terrestrial invertebrates. Elsevier Applied Science, London.

Howcroft, C., Gravato, C., Amorim, M., Novais, S., Soares, A., Guilhermino, L., 2011. Biochemical characterization of cholinesterases in *Enchytraeus albidus* and assessment of in vivo and in vitro effects of different soil properties, copper and phenmedipham. Ecotoxicology 20, 119-130.

Howcroft, C.F., Amorim, M.J.B., Gravato, C., Guilhermino, L., Soares, A.M.V.M., 2009. Effects of natural and chemical stressors on *Enchytraeus albidus*: Can oxidative stress parameters be used as fast screening tools for the assessment of different stress impacts in soils? Environment International 35, 318-324.

ISO, 2004. Soil quality - Effects of pollutants on Enchytraeidae (Enchytraeus sp.) - Determination of effects on reproduction and survival, Guideline no. 16387, ISO (International Organization for Standardization). Geneve, Switzerland.

Kammenga, J.E., Dallinger, R., Donker, M.H., Kohler, H.R., Simonsen, V., Triebskorn, R., Weeks, J.M., 2000. Biomarkers in terrestrial invertebrates for ecotoxicological soil risk assessment. Reviews of Environmental Contamination and Toxicology 164, 93-147.

Kuperman, R.G., Amorim, M.J.B., Rombke, J., Lanno, R., Checkai, R.T., Dodard, S.G., Sunahara, G.I., Scheffczyk, A., 2006. Adaptation of the enchytraeid toxicity test for use with natural soil types. European Journal of Soil Biology 42, S234-S243.

Labrot, F., Ribera, D., SaintDenis, M., Narbonne, J.F., 1996. In vitro and in vivo studies of potential biomarkers of lead and uranium contamination: Lipid peroxidation, acetylcholinesterase, catalase and glutathione peroxidase activities in three non-mammalian species. Biomarkers 1, 21-28.

Lair, G.J., Graf, M., Zehetner, F., Gerzabek, M.H., 2008. Distribution of cadmium among geochemical fractions in floodplain soils of progressing development. Environmental Pollution 156, 207-214.

Laszczyca, P., Augustyniak, M., Babczynska, A., Bednarska, K., Kafel, A., Migula, P., Wilczek, G., Witas, I., 2004. Profiles of enzymatic activity in earthworms from zinc, lead and cadmium polluted areas near Olkusz (Poland). Environment International 30, 901-910.

Lima, I., Moreira, S.M., Rendon-Von Osten, J., Soares, A.M.V.M., Guilhermino, L., 2007. Biochemical responses of the marine mussel *Mytilus galloprovincialis* to petrochemical environmental contamination along the North-western coast of Portugal. Chemosphere 66, 1230-1242.

Lock, K., Janssen, C.R., 2001a. Cadmium toxicity for terrestrial invertebrates: Taking soil parameters affecting bioavailability into account. Ecotoxicology 10, 315-322.

Lock, K., Janssen, C.R., 2001b. Modeling zinc toxicity for terrestrial invertebrates. Environmental Toxicology and Chemistry 20, 1901-1908.

Løkke, H., van Gestel, C.A.M., 1998. Handbook of soil invertebrate toxicity tests. Wiley & Sons Ltd, Chichester.

Lukkari, T., Taavitsainen, M., Soimasuo, M., Oikari, A., Haimi, J., 2004. Biomarker responses of the earthworm *Aporrectodea tuberculata* to copper and zinc exposure: differences between populations with and without earlier metal exposure. Environmental Pollution 129, 377-386.

Maity, S., Roy, S., Chaudhury, S., Bhattacharya, S., 2008. Antioxidant responses of the earthworm *Lampito mauritii* exposed to Pb and Zn contaminated soil. Environmental Pollution 151, 1-7.

Mccord, J.M., Fridovic, I., 1969. Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). Journal of Biological Chemistry 244, 6049-6055.

McLaughlin, M.J., Hamon, R.E., Parker, D.R., Pierzynski, G.M., Smolders, E., Thornton, I., Welp, G., 2002. Soil Chemistry, in: Fairbrother, A., Glazebrook, P.W., Tarazona, J.V., Straalen, N.M.v. (Eds.), Test Methods to Determine Hazards of Sparingly Soluble Metal Compounds in Soils. Society of Environmental Toxicology and Chemistry (SETAC), pp. 5-85.

Meister, A., 1995. Glutathione Metabolism. Biothiols, Pt A 251, 3-7.

Mohandas, J., Marshall, J.J., Duggin, G.G., Horvath, J.S., Tiller, D.J., 1984. Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney - Possible implications in analgesic nephropathy. Biochemical Pharmacology 33, 1801-1807.

Moreira-Santos, M., Fonseca, A.L., Moreira, S.M., Osten, J.R.V., Da Silva, E.M., Soares, A.M.V.M., Guilhermino, L., Ribeiro, R., 2005. Short-term sublethal (sediment and aquatic roots of floating macrophytes) assays with a tropical chironomid based on postexposure feeding and biomarkers. Environmental Toxicology and Chemistry 24, 2234-2242.

Morgan, A.J., Sturzenbaum, S.R., Winters, C., Grime, G.W., Abd Aziz, N.A., Kille, P., 2004. Differential metallothionein expression in earthworm (*Lumbricus rubellus*) tissues. Ecotoxicology and Environmental Safety 57, 11-19.

Novais, S.C., Soares, A.M.V.M., Amorim, M.J.B., 2010. Can avoidance in *Enchytraeus albidus* be used as a screening parameter for pesticides testing? Chemosphere 79, 233-237.

OECD, 2004. Guidelines for the testing of chemicals. Enchytraeid Reproduction Test, No. 220, OECD (Organization for Economic Cooperation and Development). Paris, France.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal-tissues by thiobarbituric acid reaction. Analytical Biochemistry 95, 351-358.

Olson, D.L., Christensen, G.M., 1980. Effects of water pollutants and other chemicals on fish acetylcholinesterase (in vitro). Environmental Research 21, 327-335.

Rault, M., Mazzia, C., Capowiez, Y., 2007. Tissue distribution and characterization of cholinesterase activity in six earthworm species. Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology 147, 340-346.

Ribera, D., Narbonne, J.F., Arnaud, C., Saint-Denis, M., 2001. Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil, effects of carbaryl. Soil Biology & Biochemistry 33, 1123-1130.

Rombke, J., Jansch, S., Junker, T., Pohl, B., Scheffczyk, A., Schallnass, H.J., 2006. Improvement of the applicability of ecotoxicological tests with earthworms, springtails, and plants for the assessment of metals in natural soils. Environmental Toxicology and Chemistry 25, 776-787.

Römbke, J., Moser, T., 2002. Validating the enchytraeid reproduction test: organisation and results of an international ringtest. Chemosphere 46, 1117-1140.

Saint-Denis, M., Narbonne, J.F., Arnaud, C., Ribera, D., 2001. Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil: effects of lead acetate. Soil Biology and Biochemistry 33, 395-404.

Saint-Denis, M., Narbonne, J.F., Arnaud, C., Thybaud, E., Ribera, D., 1999. Biochemical responses of the earthworm *Eisenia fetida andrei* exposed to contaminated artificial soil: effects of benzo(a)pyrene. Soil Biology & Biochemistry 31, 1837-1846.

Sato, M., Sasaki, M., Oguro, T., Kuroiwa, Y., Yoshida, T., 1995. Induction of metallothionein synthesis by glutathione depletion after trans-stilbene and cis-stilbene oxide administration in rats. Chemico-Biological Interactions 98, 15-25.

Scott-Fordsmand, J.J., Weeks, J.M., 2000. Biomarkers in earthworms. Reviews of Environmental Contamination and Toxicology, Vol 165 165, 117-159.

Shumilla, J.A., Wetterhahn, K.E., Barchowsky, A., 1998. Inhibition of NF-kappa B binding to DNA by chromium, cadmium, mercury, zinc, and arsenite in vitro: Evidence of a thiol mechanism. Archives of Biochemistry and Biophysics 349, 356-362.

SigmaPlot, 1997. Statistical Package for the Social Sciences - SigmaPlot for Windows, 11.0 ed, Chicago, IL, USA.

Song, Y., Zhu, L.S., Wang, J., Wang, J.H., Liu, W., Xie, H., 2009. DNA damage and effects on antioxidative enzymes in earthworm (*Eisenia foetida*) induced by atrazine. Soil Biology & Biochemistry 41, 905-909.

SPSS Inc. (1999). SPSS Base 10.0 for Windows User's Guide. SPSS Inc., Chicago IL.

Spurgeon, D.J., Sturzenbaum, S.R., Svendsen, C., Hankard, P.K., Morgan, A.J., Weeks, J.M., Kille, P., 2004. Toxicological, cellular and gene expression responses in earthworms exposed to copper and cadmium. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology 138, 11-21.

Svendsen, C., Hankard, P.K., Lister, L.J., Fishwick, S.K., Jonker, M.J., Spurgeon, D.J., 2007. Effect of temperature and season on reproduction, neutral red retention and metallothionein responses of earthworms exposed to metals in field soils. Environmental Pollution 147, 83-93.

Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione - Applications to mammalian blood and other tissues. Analytical Biochemistry 27, 502-522.

Torres, M.A., Testa, C.P., Gaspari, C., Masutti, M.B., Panitz, C.M.N., Curi-Pedrosa, R., de Almeida, E.A., Di Mascio, P., Wilhelm, D., 2002. Oxidative stress in the mussel *Mytella guyanensis* from polluted mangroves on Santa Catarina Island, Brazil. Marine Pollution Bulletin 44, 923-932.

ToxRat, 2003. Toxicity response analysis and testing - software professional, 2.10 ed, ToxRat Solutions GmbH.

Valavanidis, A., Vlahogianni, T., Dassenakis, M., Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. Ecotoxicology and Environmental Safety 64, 178-189.

van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environmental Toxicology and Pharmacology 13, 57-149.

Vasseur, P., Cossu-Leguille, C., 2006. Linking molecular interactions to consequent effects of persistent organic pollutants (POPs) upon populations. Chemosphere 62, 1033-1042.

Vlahogianni, T.H. and Valavanidis, A., 2007. Heavy-metal effects on lipid peroxidation and antioxidant defence enzymes in mussels *Mytilus galloprovincialis*. Chemistry and Ecology 23, 361-371.

Wilhelm, D., Tribess, T., Gaspari, C., Claudio, F.D., Torres, M.A., Magalhaes, A.R.M., 2001. Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (*Perna perna*). Aquaculture 203, 149-158.

Xiao, N.W., Song, Y., Ge, F., Liu, X.H., Ou-Yang, Z.Y., 2006. Biomarkers responses of the earthworm *Eisenia fetida* to acetochlor exposure in OECD soil. Chemosphere 65, 907-912.

Zhang, Y., Shen, G., Yu, Y., Zhu, H., 2009. The hormetic effect of cadmium on the activity of antioxidant enzymes in the earthworm *Eisenia fetida*. Environmental Pollution 157, 3064-3068.

Chapter IV

Enchytraeus albidus microarray (oligonucleotide): enrichment, design, annotation and database (EnchyBASE)

IV – *Enchytraeus albidus* microarray (oligonucleotide): enrichment, design, annotation and database (EnchyBASE)

Sara C. Novais, Joel Arrais, Pedro Lopes, Tine Vandenbrouck, Wim De Coen,
Dick Roelofs, Amadeu M.V.M. Soares & Mónica J.B. Amorim

Under review in PLoS ONE (IF =4.411; 2010)

Abstract

Enchytraeus albidus (Oligochaeta) is used as standard test organisms for risk assessment. This species is sensitive to chemicals and ecologically relevant. Effects are mainly determined at population level (reproduction and survival as endpoints). To be able to assess transcriptomic responses can be very useful in an ecotoxicological context, e.g., to understand mechanisms of toxicity with gene fingerprinting. In the present paper the following was addressed: 1) development of the suppressive subtractive hybridization (SSH) libraries enriched for metals and pesticides; 2) sequencing and characterization of all cDNA inserts generated; 3) development of a publicly available Expressed Sequence Tags (EST) database including all obtained genomic data on *E. albidus*. A total of 2100 ESTs were isolated, sequenced and assembled into 1124 clusters (947 singletons and 177 contigs). From these sequences, 41% matched known proteins in GenBank (BLASTX, with minimum E-value of 10^{-5}) and 37 % had at least one Gene Ontology (GO) term assigned. In total, 5.5% of the sequences were assigned to a metabolic pathway, based on KEGG. An Agilent custom oligonucleotide microarray was developed, a potential tool for transcriptomic studies. EnchyBASE was developed as a web freely available database containing genomic information on *E. albidus* and will be further extended in the near future for other enchytraeid species. The database so far includes all ESTs generated for *E. albidus* from three cDNA libraries. This information can be downloaded and applied in functional genomics and transcription studies.

1. Introduction

Enchytraeids (Oligochaeta), members of the soil mesofauna, play a key role on the regulation of the composition and activity of soil communities, by improving the pore structure of the soil and being involved in the organic matter decomposition (Jeffery et al., 2010). *Enchytraeus albidus* is present in a wide range of soils and conditions worldwide. *E. albidus* have been increasingly used as indicators of soil health since the standardization of the ecotoxicological tests, where survival, reproduction and, more recently, bioaccumulation effects are measured (ISO, 2004; OECD, 2004, 2010). There is ample literature on chemical and natural stress on enchytraeids at these levels e.g. with heavy metals (Lock and Janssen, 2001a; Amorim et al., 2005b), organic substances (Amorim et al., 2005a; Novais et al., 2010), chemical mixtures (Loureiro et al., 2009) and different soil properties (Lock and Janssen, 2001b; Dodard et al., 2005; Amorim et al., 2008a). Such information is of extreme importance as they provide the tools for risk assessors, and policy makers at a later stage. However, the current ecotoxicology tests are time consuming (e.g. 6 weeks for reproduction) and there is little mechanistic understanding of the impact caused by such stressors. Complementing existing knowledge with the molecular profiling and genomic studies can help considerably to elucidate modes of action, molecular pathways of response or general biological processes affected by stressors. Furthermore, it has been shown by several authors that responses at gene level can be observed in several invertebrates within short time intervals such as 1 or 2 days (Poynton et al., 2007; Heckmann et al., 2008; Nota et al., 2008; Nota et al., 2009; Amorim et al., 2011), presenting a clear advantage in comparison to the more time-consuming population studies.

Promising developments have taken place in this area in soil invertebrates with the establishment of Expressed Sequence Tag (EST) databases and microarrays for a few species of earthworms: *Lumbricus rubellus* (Owen et al., 2008) and *Eisenia fetida* (Pirooznia et al., 2007) and the springtail *Folsomia candida* (Timmermans et al., 2007). The generation of ESTs is of particular interest when studying non-genomic model organisms, which is the case of the referred invertebrate species and also *E. albidus*. This is

an efficient and cost effective way to retrieve sequence information on the protein coding part of the genome (Timmermans et al., 2007).

Regarding *E. albidus*, Amorim and co-authors (2011) started the EST sequencing project with a normalized cDNA library. A cDNA microarray was developed based on this normalized library and has been used to study the effects of phenmedipham, copper, different soil properties or exposure duration (Amorim et al., 2011; Gomes et al., 2011a; Gomes et al., 2011b; Novais et al., 2011b). Subsequently, the existing cDNA library was enriched using suppression subtractive hybridization-PCR (SSH-PCR), a technique that combines high subtraction efficiency with a normalization step to generate differentially expressed sequences equally represented in the library (Diatchenko et al., 1996; Diatchenko et al., 1999).

In the present paper the following main points were addressed: 1) development of two SSH libraries enriched with genes differentially expressed due to exposure to metals and pesticides at different concentrations and exposure times; SSH-metals was developed by exposure to cadmium, zinc, copper and nickel; SSH-pesticides was developed by exposure to dimethoate, atrazine, carbendazim and lindane; 2) Sequencing and characterization of all cDNA inserts generated; 3) development of a publicly available Expressed Sequence Tags (EST) database, including all obtained genomic data on *E. albidus* and allowing the users to search e.g. for sequence similarity (BLAST), gene ontology terms and for sequence information on the differentially expressed genes at the different conditions. After assembling all the information, the existing microarray was enriched and developed into a new oligonucleotide custom Agilent microarray. The present can be used for studies envisaging mechanistic understanding of stress and soil quality assessment.

2. Development and analysis

cDNA libraries construction

SSH procedure was applied for the development of two other cDNA libraries (SSH-metals, SSH-pesticides). For the library enriched for metals exposures, 15 adult organisms with well developed clitellum were exposed, in each replicate, to 25g of LUFA 2.2 standard natural soil (Løkke and van Gestel, 1998), moist to 50% of the water holding capacity according to the standard guidelines (ISO, 2004; OECD, 2004). Soil was spiked with 4 different metals individually: cadmium chloride, zinc chloride, copper chloride and nickel chloride. Enchytraeids were exposed to each metal in two different concentrations in the range of the effective concentrations for 50% (EC_{50}) and 90% (EC_{90}) reduction in reproduction (known based on previous results (Lock and Janssen, 2002; Amorim et al., 2005b; Novais et al., 2011a)) and three time points (2, 4 and 8 days). These concentrations were selected, on the one hand to be able to relate gene effects with known effects at higher levels of biological organization and on the other hand to increase the likelihood of finding effects in gene expression, than would be expected with very low concentrations. Three replicates per condition were used. The total RNA from the organisms in each replicate was extracted using the Trizol extraction method (Invitrogen, Belgium). RNA concentration and purity was determined by spectrophotometry (NanoDrop 1000, Thermo Fisher Scientific), quality was checked by denaturing formaldehyde agarose gel and a pool containing RNA from all the exposure conditions was made using 1 replicate of each. A second pool containing RNA from control organisms (organisms exposed to clean LUFA 2.2 soil) was similarly prepared. For the library enriched for pesticide exposure, enchytraeids were exposed to dimethoate, atrazine, lindane and carbendazim. Exposure was performed in the same way as for the SSH-metals in regard to concentrations (EC_{50} and EC_{90} (Novais et al., 2010)) and duration (2, 4 and 8 days). Similarly, two different RNA pools were obtained: one from organisms exposed to the pesticides and one from control organisms. The exposure concentrations of all compounds are given in table 1.

Table 1. Concentrations of the four metals and four pesticides to which *E. albidus* were exposed for the SSH libraries development. Concentrations of exposure are based on the effect concentrations on reproduction, available on the literature.

	EC_{50} (mg/kg)	**EC_{90} (mg/kg)**	**References**
SSH Metals			
Copper	100	320	(Amorim et al., 2005b)
Cadmium	6	150	(Novais et al., 2011a)
Zinc	40	100	(Novais et al., 2011a)
Nickel	50	120	(Lock and Janssen, 2002)
SSH Pesticides			
Dimethoate	2	25	(Novais et al., 2010)
Atrazine	3	50	(Novais et al., 2010)
Carbendazim	0.5	3	(Novais et al., 2010)
Lindane	40	130	(Novais et al., 2010)

EC_{50} = 50% effect concentration on reproduction; EC_{90} = 90% effect concentration on reproduction.

A schematic representation of the exposures and RNA pools made for both SSH enriched cDNA libraries is shown in Figure 1.

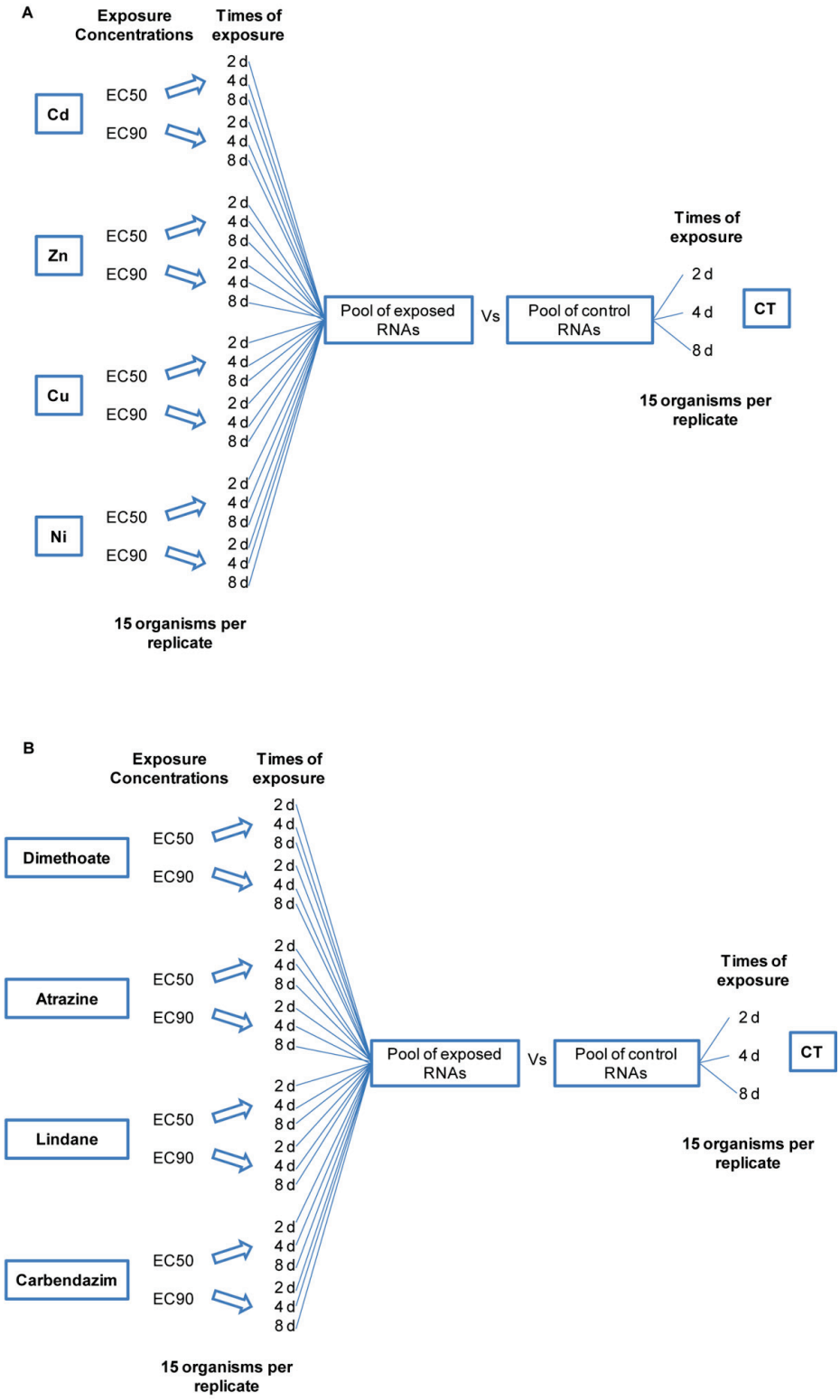

Figure 1. Schematic representation of the exposures and pools of RNA performed for the SSH libraries development: A) Library enriched for metals; B) Library enriched for pesticides. CT = Control; EC50 = 50% effect concentration on reproduction; EC90 = 90% effect concentration on reproduction.

To each RNA pool, 0.1 volumes of 3M sodium acetate and 3 volumes of 96% ethanol were added and the pairs of pools were shipped at room temperature to Evrogen (Moscow, Russia). Amplification of the double stranded cDNAs (using SMART approach (Zhu et al., 2001)) and the subtraction procedures were performed by Evrogen for both libraries. The cDNA was SMART-amplified (19 cycles), starting from 0.5µg of each RNA pool, and used for subtractive hybridization using SSH method in both directions (Diatchenko et al., 1996; Diatchenko et al., 1999). Prior to the library construction, the samples were subjected to the mirror orientation selection (MOS) procedure (Rebrikov et al., 2000) to eliminate false positive clones resulting from the SSH procedures (Evrogen). The treated samples were then handled by us for the libraries construction. Briefly, the subtracted cDNAs were ligated in a TA-vector system (pGEM-T easy vector, Promega). *Escherichia coli* calcium competent cells (JM109, Promega) were transformed through heat shock. The recombinant clones were picked and grown in 96-well plates. Glycerol stocks were made (12.5%) and stored at -80ºC. Clones were amplified with vector-specific primers (T7 and SP6 primers, Promega), and purified by an exosap reaction (Werle et al., 1994) based on exonuclease I and shrimp alkaline phosphatase (Fermentas).

EST sequencing and comparative sequence analysis

From the SSH libraries, a total of 1920 clones were selected (960 from each library). After checking the quality of the PCR inserts on an agarose gel, 67 clones had no inserts or had more than one insert and were excluded. Therefore, the remaining 1853 purified clones were sent to sequence with primers SP6 and T7 (VIB service, Flemish Institute for Biotechnology).

CodonCode Aligner software (www.codoncode.com/aligner) was used to remove vectors and screen for low-quality sequences. From the 1853, 101 sequences were shorter than 50 base pairs (bp) or did not pass the quality control and were thus removed from further analysis. In sum, from the 921 clones sequenced from the metals enriched library, we obtained 875 good quality sequences (95%) and from the 932 clones sequenced from the

pesticides enriched library, we obtained 877 good quality sequences (94%). All good quality sequences were submitted to GenBank dbEST (accession numbers: JK309883-JK310757; JK474167 - JK475043).

Sequences from the first cDNA library developed by Amorim and co-authors (2011) were added for further analysis. In total 2100 ESTs were retrieved from the three libraries and aligned and assembled using Cap3 program (http://www.genome.clemson.edu/cgi-bin/cugi_cap3). This procedure resulted in 1124 unique sequences (clusters): 947 singletons (338 ESTs from the normalized library, 370 ESTs from the metals enriched library and 239 EST from the pesticides enriched library) and 177 contigs. The 45% singletons obtained in this study is inferior to the 80% observed in the EST sequencing project for *Eisenia fetida* (Pirooznia et al., 2007) but similar to the percentages of singletons observed in the EST sequencing projects of other terrestrial invertebrate species: 49% for *Eisenia andrei* (Lee et al., 2005), 52% for *Folsomia candida* (Timmermans et al., 2007) or 53% for *Lumbricus rubelus* (Owen et al., 2008). From the 177 contigs, nearly 85% were assembled from 2 to 5 sequences and more than half were assembled from only 2 sequences (Figure 2).

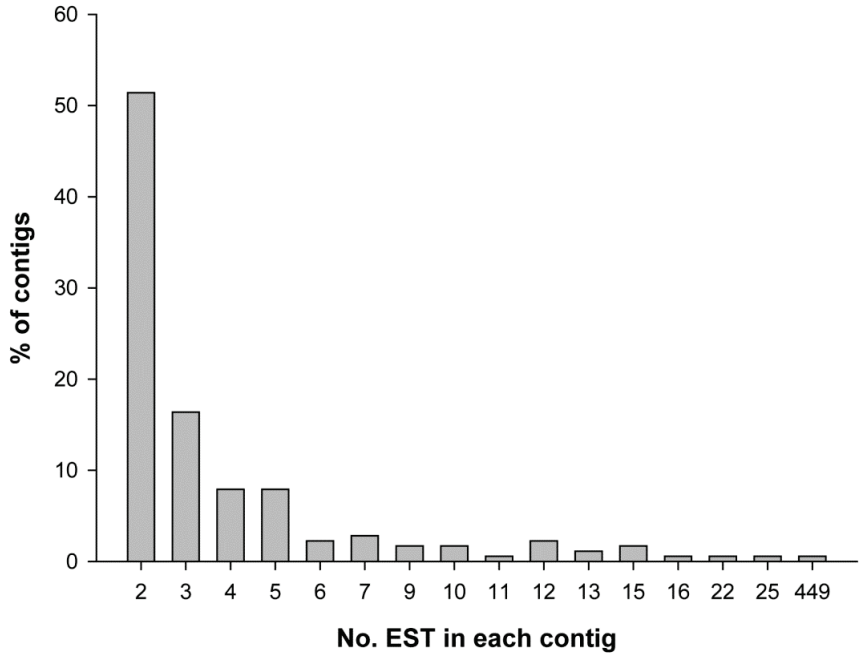

Figure 2. Expressed Sequence Tags distribution in the 177 contigs.

The length of the 177 contigs varied from 69 to 1630 bp with an average of 735 bp. The highest number of sequences in one contig was by far observed on the pesticides enriched library with 449 ESTs, whereas the highest depth among the contigs in the metals enriched library was 24 ESTs and in the normalized library was 4 ESTs. In terms of redundancy [total number of sequences divided by the number of clusters (Timmermans et al., 2007)], the pesticides library was the most redundant (3.22) followed by the metals library (1.80) and the least one, the normalized library (1.02). Overall data had a redundancy of 1.87. Interestingly, also Timmermans et al. (2007) refer a similar difference obtained for the phenantrene library (3.18) in comparison to the cadmium (1.62) and normalized (1.32) ones. The overlaping ESTs from the different cDNA libraries is represented in Figure 3.

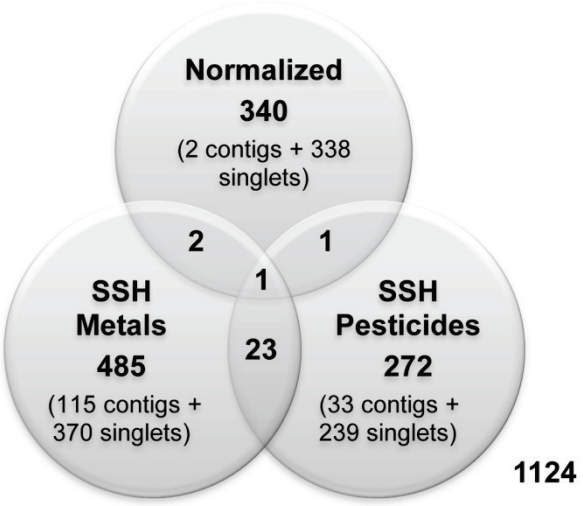

Figure 3. Cluster overlaps between the three different libraries. Normalized: normalized library; SSH Metals: metals enriched library; SSH Pesticides: pesticides enriched library.

Interestingly, very little overlap occurred, with only one contig containing sequences from the three libraries (cluster EAC00169, Table 2). This was also observed in other studies (e.g. (Timmermans et al., 2007)) confirming the relevance of the enrichment with as much varied conditions as possible.

The sequenced unique fragments (singletons and consensus sequences of assembled contigs) were identified based on their similarity to sequences in the National Centre for Biotechnology Information (NCBI) database as determined by the Basic Local Alignment

Search Tool (BLAST) (Altschul et al., 1990). The sequences were submitted to Blast2GO (Conesa et al., 2005) being compared with peptide sequence databases using BLASTX analysis (with minimum). From the 1124 clusters, a total of 459 sequences (41%) matched known proteins in the database with a minimum E-value of 10^{-5}. Among these, 72 sequences (16%) had E-values between 10^{-123} and 10^{-50}.

The most abundant sequenced transcripts identified were actin, myosin, Sarcoplasmic calcium-binding protein, ADP/ATP carrier protein 3, MADS FLC-like protein 2, hemoglobin c and ribosomal protein s7 (Table 2).

Table 2. The most represented sequenced transcripts in *E. albidus* cDNA libraries.

Cluster ID	ESTs	Length	Library	Blast Hit	E-value	GeneBank
EAC00048	449	660	Pest	No significant hit	-	-
EAC00024	25	480	Met+Pest	Actin	7.25E-12	ADJ56346
EAC00129	22	960	Met+Pest	Sarcoplasmic calcium-binding protein	1.01E-09	P04572
EAC00065	16	720	Pest	No significant hit	-	-
EAC00139	15	1620	Met	Actin	1.04E-17	NP_001003349
EAC00074	15	660	Pest	myosin heavy chain	5.51E-07	CAC28360
EAC00083	15	840	Pest	No significant hit	-	-
EAC00094	13	1260	Met	No significant hit	-	-
EAC00138	13	1140	Met	No significant hit	-	-
EAC00108	12	720	Met+Pest	myosin heavy chain	3.11E-06	AAD52842
EAC00109	12	840	Met	No significant hit	-	-
EAC00157	12	720	Met	ADP/ATP carrier protein 3	1.34E-11	NP_001187478
EAC00066	12	720	Pest	No significant hit	-	-
EAC00070	11	540	Pest	MADS FLC-like protein 2	4.33E-05	ACL54966
EAC00035	10	660	Met+Pest	hemoglobin c chain precursor	3.30E-06	CAA09958
EAC00092	10	780	Met	No significant hit	-	-
EAC00143	10	660	Met+Pest	ribosomal protein s7	8.78E-14	AAW50967
EAC00169	9	1020	Met+Pest+Norm	Myosin regulatory light chain	4.12E-12	P80164
EAC00015	9	900	Met	No significant hit	-	-
EAC00082	9	720	Pest	myosin heavy chain isoform 3	3.44E-05	EFA08290

Met: sequences from the SSH library enriched with metal exposures; Pest: sequences from the SSH library enriched with pesticide exposures; Norm: sequences from the normalized library.

As can be seen in table 2, housekeeping genes like actin were highly represented in the SSH libraries but not in the normalized library, indicating that the normalization method was efficient (Sturzenbaum et al., 2003; Timmermans et al., 2007).

From the 459 blast hits, 46 (10%) matched sequences from earthworms, soil organisms phylogenetically close to *E. albidus* (*Eisenia fetida*, *Lumbricus rubellus*, *Lumbricus terrestris*, *Lumbricus variegatus*).

Functional Annotation

Gene ontology terms (GO) were assigned to the predicted proteins by homology blast using the same Blast2GO software (Conesa et al., 2005). A total of 415 sequences had at least one GO term assigned (37% of the 1124 clusters).

The summary of GO terms showing the representation of the higher-level terms (GO-slim), assigned to 5 or more sequences is given in table 3.

Transcription and translation are the most represented biological processes in the developed libraries. As for the molecular functions and cellular components, the most represented are the nucleotide and DNA binding and the cytoskeleton and protein complex, respectively. Most of the biological functions have a higher representation in the metals enriched library, with the exception of reproduction which is not represented in this library and transcription which is mainly represented in the pesticides enriched library.

Molecular pathways were addressed to the clusters using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Ogata et al., 1999) based on their Enzyme Commission numbers (EC). In total, 61 sequences matched enzymes with an EC number (5.5% of the 1124 clusters), belonging to 25 different pathways, all related to metabolism. The metabolisms with more pathways where the enzymes coded for *E. albidus* sequences are involved were the energy and amino acid metabolisms, followed by e.g. the metabolisms of carbohydrates and nucleotides (Table 4).

Table 3. GO-slim terms represented by more than 5 sequences in the totality of cDNA libraries for *E. albidus*.

	Gene Ontology ID	No. Sequences	Library(no. sequences)
Biological process			
Lipid metabolic process	GO:0006629	5	Met(3)+Norm(2)
Cellular amino acid and derivative metabolic process	GO:0006519	5	Met(4)+Pest(1)
Reproduction	GO:0000003	6	Pest(2)+Norm(4)
Carbohydrate metabolic process	GO:0005975	7	Met(4)+Pest(3)
Cell proliferation	GO:0008283	8	Met(5)+Norm(3)
Ion Transport	GO:0006811	8	Met(8)
Cell death	GO:0008219	8	Met(6)+Pest(2)
Signal transduction	GO:0007165	9	Met(3)+Pest(2)+Norm(4)
Protein modification process	GO:0006464	11	Met(4)+Pest(3)+Norm(4)
Growth	GO:0040007	12	Met(5)+Pest(3)+Norm(4)
Cell cycle	GO:0007049	12	Met(6)+Pest(4)+Norm(2)
Response to stress	GO:0006950	13	Met(8)+Pest(1)+Norm(4)
Embryonic development	GO:0009790	14	Met(7)+Pest(3)+Norm(4)
Catabolic process	GO:0009056	18	Met(11)+Pest(5)+Norm(2)
Cytoskeleton organization	GO:0007010	18	Met(14)+Pest(4)
Cell differentiation	GO:0030154	22	Met(17)+Pest(3)+Norm(2)
Generation of precursor metabolites and energy	GO:0006091	23	Met(13)+Pest(6)+Norm(4)
Anatomical structure morphogenesis	GO:0009653	28	Met(21)+Pest(4)+Norm(3)
Translation	GO:0006412	35	Met(20)+Pest(7)+Norm(8)
Transcription	GO:0006350	72	Met(5)+Pest(65)+Norm(2)
Molecular function			
Kinase activity	GO:0016301	5	Met(2)+Pest(2)+Norm(1)
Translation factor activity, nucleic acid binding	GO:0008135	8	Met(3)+Pest(3)+Norm(2)
Electron carrier activity	GO:0009055	8	Met(4)+Pest(1)+Norm(3)
Transcription regulator activity	GO:0030528	8	Met(5)+Pest(2)+Norm(1)
Actin binding	GO:0003779	12	Met(4)+Pest(8)
Peptidase activity	GO:0008233	13	Met(7)+Pest(4)+Norm(2)
Transporter activity	GO:0005215	16	Met(12)+Pest(1)+Norm(3)
Calcium ion binding	GO:0005509	17	Met(9)+Pest(6)+Norm(2)
Motor activity	GO:0003774	18	Met(4)+Pest(13)+Norm(1)
RNA binding	GO:0003723	21	Met(10)+Pest(3)+Norm(8)
Structural molecule activity	GO:0005198	45	Met(31)+Pest(5)+Norm(9)
DNA binding	GO:0003677	68	Met(3)+Pest(64)+Norm(1)
Nucleotide binding	GO:0000166	82	Met(51)+Pest(28)+Norm(3)
Cellular Component			
Nucleolus	GO:0005730	7	Met(4)+Norm(3)
Nucleoplasm	GO:0005654	8	Met(4)+Pest(2)+Norm(2)
Extracellular region	GO:0005576	8	Met(7)+Norm(1)
Plasma membrane	GO:0005886	10	Met(6)+Norm(4)
Mitochondrion	GO:0005739	26	Met(15)+Pest(4)+Norm(7)
Ribosome	GO:0005840	29	Met(18)+Pest(3)+Norm(8)
Cytosol	GO:0005829	34	Met(21)+Pest(6)+Norm(7)
Protein complex	GO:0043234	64	Met(35)+Pest(22)+Norm(7)
Cytoskeleton	GO:0005856	82	Met(50)+Pest(29)+Norm(3)

Met: sequences from the SSH library enriched with metal exposures; Pest: sequences from the SSH library enriched with pesticide exposures; Norm: sequences from the normalized library.

Table 4. KEGG pathways in the totality of cDNA libraries for *E. albidus*.

	No. Sequences involved in the pathway
Amino acid metabolism	**12**
Alanine, aspartate and glutamate metabolism	1
Arginine and proline metabolism	2
Cysteine and methionine metabolism	2
Glycine, serine and threonine metabolism	3
Phenylalanine metabolism	1
Phenylalanine, tyrosine and tryptophan biosynthesis	1
Tyrosine metabolism	2
Energy metabolism	**25**
Carbon fixation in photosynthetic organisms	3
Methane metabolism	4
Oxidative phosphorylation	14
Photosynthesis	4
Carbohydrate metabolism	**3**
Glycolysis / Gluconeogenesis	2
Pentose phosphate pathway	1
Nucleotide metabolism	**3**
Purine metabolism	2
Pyrimidine metabolism	1
Lipid metabolism	**1**
Glycerophospholipid metabolism	1
Glycan Biosynthesis and Metabolism	**2**
N-Glycan biosynthesis	1
Various types of N-glycan biosynthesis	1
Metabolism of Cofactors and Vitamins	**2**
Nicotinate and nicotinamide metabolism	1
Thiamine metabolism	1
Biosynthesis of Other Secondary Metabolites	**3**
Isoquinoline alkaloid biosynthesis	1
Novobiocin biosynthesis	1
Tropane, piperidine and pyridine alkaloid biosynthesis	1
Metabolism of Terpenoids and Polyketides	**1**
Biosynthesis of ansamycins	1
Xenobiotics Biodegradation and Metabolism	**1**
Styrene degradation	1

Development of the database

EnchyBASE development required the integration of diverse bioinformatics software. Four intertwined components were needed to deploy the whole system: a web application server, a database management system, a sequence clustering tool and a local BLAST tool.

The ESTs and associated annotation information led to deployment of EnchyBASE in an Apache Web Server with PostgreSQL for the database backend. PartiGene (Parkinson et al., 2004), the chosen gene sequence-clustering tool, is the key responsible for the adopted solutions. Its web component, wwwPartiGene, requires serving dynamic PHP pages and a connection to a PostgreSQL database. ViroBLAST (Deng et al., 2007) was selected as a local BLAST tool as it provides an eased setup process for executing various distinct BLASTs against local sequence clusters.

Whereas the miscellaneous system components were relatively easy to adapt or implement, the constructed integration pipeline was a more complex task. EnchyBASE deployment workflow involved three key steps: sequence annotation, sequence clustering and annotation of clusters, and BLAST database migration.

The first step involved the annotation of *E. albidus* sequences using the BLAST2GO bioinformatic tool (Conesa et al., 2005). Sequences were clustered using Cap3 program and clusters were also annotated. Generated data was then moved on to PartiGene. This application populates EnchyBASE database with our sequences and annotations. At last, annotated sequences were used to generate a BLASTable database using NCBI BLAST toolkit (Gertz, 2005). The resulting dataset was made available to ViroBLAST for realtime BLAST against *E. albidus* sequence data.

For end-users, the system provides three key features: sequence download, annotation search and BLAST. Researchers are able to download the entire sequence dataset or specific clustered sequences. The search engine allows browsing data through multiple queries. User can search for specific clusters or sequences, common BLAST annotations, ontology annotations and primer features. At last, BLASTing can be performed against

Enchytraeus albidus data. Available BLAST functions are blastn, blastx, tblastn and tblastx. BLAST parameters may be easily configured in EnchyBASE's BLAST interface.

3. Discussion

The information gathered with the development of the three cDNA libraries is stored at enchyBASE, freely web available for scientific community usage. At this moment, the database has information on 2100 ESTs, assembled into 1124 clusters from genes expressed in *E. albidus* when exposed to different stress conditions (metals, pesticides and different soil properties). With this database the users can access the sequences present in each of the libraries, as well as all the information related to each sequence (BLAST homologies and GO terms) when available. This information can be downloaded after simple search queries by Cluster ID, GenBank accession number, BLAST annotation or GO term. Furthermore, designed primers with tested efficiencies for some of the sequences are provided. The database also enables the users to run blasts with their own sequences and look for homologies with the enchytraeids species.

In the present work it was possible to observe that only one gene was shared by the three libraries and, in general the gene overlap between libraries was limited (Figure 3). Also, the exposure to the two different groups of chemicals (metals and pesticides) affected distinct biological functions e.g. reproduction or lipid metabolic processes were only affected by pesticides or metals, respectively (Table 3).

These findings suggest that the exposure to pesticides triggered a different set of genes in comparison to metals exposure. However, the actual expression profiles of *E. albidus*, when exposed to the different chemicals or natural stressors, require confirmation through experiments of gene expression analysis.

The development of the normalized library and enrichment of the existing microarray through this EST generation had two main purposes: 1) soil quality assessment and 2) mechanistic understanding of toxic responses. A new custom Agilent microarray was thus developed, with printed 60-mer oligonucleotides designed from the unique sequences in

the database. All transcription data generated with this microarray will be stored in enchyBASE similarly to what is presently done with the data gathered with the former cDNA microarray, where information on the differentially expressed genes to each stress condition is available. This information, along with the respective differentially expressed gene sequences, can be used by the scientific community in functional genomics studies and quantitative polymerase chain reaction (qPCR) experiments.

In the near future and through hybridizations on this microarray we expect to increase knowledge on the molecular pathways involved in response to stress factors. This information can improve the current understanding of chemicals mode of action on soil invertebrates, which along with data on other organisms can help to develop predictive models of toxic effects. Additionally, generating specific stress signature fingerprints would be of particular interest to classify different types of stressors, levels of toxicity, or chemical groups.

The obtained sequence information can be potentially used to answer questions regarding chemical exposure as e.g. adaptation to chemical stress. It is commonly agreed that soil invertebrates can genetically adapt to metal stress, modifying metal toxicity and gaining resistance to contaminated soils (Posthuma and Van Straalen, 1993; Timmermans et al., 2005; Holmstrup et al., 2011). Some known mechanisms of detoxification like the storage of metal ions in membrane enclosed cellular granules or in metallothionein complexes (Posthuma and Van Straalen, 1993; Schill and Kohler, 2004; Vijver et al., 2004; Timmermans et al., 2005; Holmstrup et al., 2011) have been associated with changes in the energy metabolism due to the need of energy for these detoxification processes (Holmstrup et al., 2011). The use of transcriptomics to determine differential gene expression in metal tolerant populations has recently been successfully applied in the soil arthropod *Orchesella cincta* (Roelofs et al., 2007; Roelofs et al., 2009). Roelofs and co-authors (2009) observed different gene expression patterns between reference and tolerant populations of this species after cadmium exposure, confirming the micro-evolutionary processes occurring in this soil species populations. Mechanisms of adaptation to metal stress in enchytraeids are not known and can be investigated with similar transcription studies using enchyBASE and the newly developed microarray.

These new tools can also be potentially used to answer other ecological questions, e.g. drought tolerance. Maraldo and co-authors (Maraldo et al., 2009; Maraldo and Holmstrup, 2010) found that *E. albidus* is able to adapt to environments with strong fluctuations in humidity, being able to keep its water content stable during moist and relatively dry conditions. It is known that *E. albidus* can even tolerate incredibly low temperature (-20ºC), probably related to the ability to synthesise high concentrations of glucose (Slotsbo et al., 2008). Knowledge of drought and freeze tolerance mechanisms and strategies in enchytraeids can be further studied using transcriptomic tools as it is currently being done for the springtail *Onychiurus arcticus* (Clark et al., 2007).

Also, *E. albidus* is known to be able to avoid unfavourable conditions such as natural stressors like soil properties (e.g. pH, clay content) (Amorim et al., 2008a) or chemicals (Amorim et al., 2005a; Amorim et al., 2008b). Interestingly, not all chemicals are equally avoided, and some are even not perceived despite their high toxicity. The underlying mechanisms of these differences can also be pursued with transcription studies using the information here developed.

Among other potential utility of EnchyBASE is the study of the mechanisms behind chemical mixtures toxicity or combinations of environmental stressors, relevant issues in soil ecotoxicology.

4. Conclusions

Genomic information on *E. albidus* was increased with two SSH library enrichments. This new information was used to develop a new Agilent microarray enhancing the knowledge of molecular responses to stress.

A new web freely available database was also implemented, which gathers all the existing genomic data for *E. albidus*: EST sequences, BLAST homologies, GO terms and KEGG pathways. This information will be extended in the near future for other enchytraeid species. Transcription data from the initial cDNA microarray are also available in the database and will be continuously complemented with further experiments in the newly

developed microarray. EnchyBASE provides the scientific community with information and potential multiple applications, setting a background for ecotoxicology, genomics and molecular ecological studies with enchytraeids.

Availability

EnchyBASE can be accessed from URL: http://bioinformatics.ua.pt/enchybase.

Authors' contributions

SN contributed in the experimental setup, organisms' exposure and all preparations involved in the library development and analysis, having also drafted the manuscript. JA and PL designed and implemented the database, web server configuration and web application programming. TV assisted in setting up the project and in the laboratory work. WDC and AS contributed in the project conceptual phase and DR has advised some steps and critically revised the final version of the manuscript. MA contributed for the experimental setup, constructed the normalized library, supervised the project and helped to draft the final manuscript. All authors have read and approved the final manuscript.

Acknowledgments and Funding

The authors would like to thank Bieke Van Dorst for the practical help provided during the cDNA cloning processes. This study was sponsored by Fundação para a Ciência e Tecnologia (Portugal), through a PhD grant to Sara Novais (SFRH/BD/36253/2007) and through the project references PTDC/BIA-BCM/64745/2006 (REDE) and PTDC/BIA-BEC/103716/2008 (NANOkA).

References

Altschul, S., Gish, W., Miller, W., Meyers, E., Lipman, D., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403- 410.

Amorim, M., Rombke, J., Soares, A., 2005a. Avoidance behaviour of *Enchytraeus albidus*: Effects of Benomyl, Carbendazim, phenmedipham and different soil types. Chemosphere 59, 501-510.

Amorim, M.J.B., Novais, S., Römbke, J., Soares, A.M.V.M., 2008a. Avoidance test with *Enchytraeus albidus* (Enchytraeidae): Effects of different exposure time and soil properties. Environ. Pollut. 155, 112-116.

Amorim, M.J.B., Novais, S., Römbke, J., Soares, A.M.V.M., 2008b. *Enchytraeus albidus* (Enchytraeidae): A test organism in a standardised avoidance test? Effects of different chemical substances. Environ. Int. 34, 363-371.

Amorim, M.J.B., Novais, S.C., Van der Ven, K., Vandenbrouck, T., Soares, A.M.V.M., De Coen, W., 2011. Development of a Microarray for *Enchytraeus albidus* (Oligochaeta): Preliminary Tool with Diverse Applications. Environ. Toxicol. Chem. 30, 1395-1402.

Amorim, M.J.B., Rombke, J., Schallnass, H.J., Soares, A.M.V.M., 2005b. Effect of soil properties and aging on the toxicity of copper for *Enchytraeus albidus*, *Enchytraeus luxuriosus*, and *Folsomia candida*. Environ. Toxicol. Chem. 24, 1875-1885.

Clark, M.S., Thorne, M.A.S., Purac, J., Grubor-Lajsic, G., Kube, M., Reinhardt, R., Worland, M.R., 2007. Surviving extreme polar winters by desiccation: clues from Arctic springtail (Onychiurus arcticus) EST libraries. BMC Genomics 8, 475.

Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674-3676.

Deng, W., Nickle, D.C., Learn, G.H., Maust, B., Mullins, J.I., 2007. ViroBLAST: a stand-alone BLAST web server for flexible queries of multiple databases and user's datasets. Bioinformatics 23, 2334-2336.

Diatchenko, L., Lau, Y.F.C., Campbell, A.P., Chenchik, A., Moqadam, F., Huang, B., Lukyanov, S., Lukyanov, K., Gurskaya, N., Sverdlov, E.D., Siebert, P.D., 1996. Suppression subtractive hybridization: A method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc. Natl. Acad. Sci. U. S. A. 93, 6025-6030.

Diatchenko, L., Lukyanov, S., Lau, Y.-F.C., Siebert, P.D., 1999 Suppression subtractive hybridization: a versatile method for identifying differentially expressed genes. Methods Enzymol. 303, 349-380.

Dodard, S.G., Sunahara, G.I., Kuperman, R.G., Sarrazin, M., Gong, P., Ampleman, G., Thiboutot, S., Hawari, J., 2005. Survival and reproduction of enchytraeid worms, oligochaeta, in different soil types amended with energetic cyclic nitramines. Environ. Toxicol. Chem. 24, 2579-2587.

Gertz, E.M., 2005. BLAST Scoring Parameters.

Gomes, S.I.L., Novais, S.C., Scott-Fordsmand, J.J., de Coen, W., Soares, A.M.V.M., Amorim, M.J.B., 2011a. Effect of Cu-Nanoparticles versus Cu-salt in *Enchytraeus albidus* (Oligochaeta): differential gene expression through microarray analysis. Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology, In press.

Gomes, S.I.L., Novais, S.C., Soares, A.M.V.M., Amorim, M.J.B., 2011b. Effects of soil properties and time of exposure on gene expression of *Enchytraeus albidus* (Oligochaeta). Soil Biol. Biochem. 43, 2078-2084.

Heckmann, L.H., Sibly, R.M., Connon, R., Hooper, H.L., Hutchinson, T.H., Maund, S.J., Hill, C.J., Bouetard, A., Callaghan, A., 2008. Systems biology meets stress ecology: linking molecular and organismal stress responses in Daphnia magna. Genome Biology 6, 25.

Holmstrup, M., Sorensen, J.G., Overgaard, J., Bayley, M., Bindesbol, A.M., Slotsbo, S., Fisker, K.V., Maraldo, K., Waagner, D., Labouriau, R., Asmund, G., 2011. Body metal concentrations and glycogen reserves in earthworms (*Dendrobaena octaedra*) from contaminated and uncontaminated forest soil. Environ. Pollut. 159, 190-197.

ISO, 2004. Soil quality - Effects of pollutants on Enchytraeidae (Enchytraeus sp.) - Determination of effects on reproduction and survival. Guideline no. 16387, ISO (International Organization for Standardization). Geneve, Switzerland.

Jeffery, S., Gardi, C., Jones, A., Montanarella, L., Marmo, L., Miko, L., Ritz, K., Peres, G., Römbke, J., van der Putten, W.H., 2010. European Atlas of Soil Biodiversity. European Commission, Publications Office of the European Union, Luxembourg.

Lee, M.S., Cho, S.J., Tak, E.S., Lee, J.A., Cho, H.J., Park, B.J., Shin, C., Kim, D.K., Park, S.C., 2005. Transcriptome analysis in the midgut of the earthworm (*Eisenia andrei*) using expressed sequence tags. Biochem. Biophys. Res. Commun. 328, 1196-1204.

Lock, K., Janssen, C.R., 2001a. Ecotoxicity of mercury to *Eisenia fetida, Enchytraeus albidus* and *Folsomia candida*. Biol. Fertility Soils 34, 219-221.

Lock, K., Janssen, C.R., 2001b. Effect of clay and organic matter type on the ecotoxicity of zinc and cadmium to the potworm Enchytraeus albidus. Chemosphere 44, 1669-1672.

Lock, K., Janssen, C.R., 2002. Ecotoxicity of nickel to *Eisenia fetida*, *Enchytraeus albidus* and *Folsomia candida*. Chemosphere 46, 197-200.

Løkke, H., van Gestel, C.A.M., 1998. Handbook of soil invertebrate toxicity tests. Wiley & Sons Ltd, Chichester.

Loureiro, S., Amorim, M.J.B., Campos, B., Rodrigues, S.M.G., Soares, A.M.V.M., 2009. Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environ. Pollut. 157, 625-636.

Maraldo, K., Holmstrup, M., 2010. Enchytraeids in a changing climate: A mini-review. Pedobiologia 53, 161-167.

Maraldo, K., Ravn, H., Slotsbo, S., Holmstrup, M., 2009. Responses to acute and chronic desiccation stress in Enchytraeus (Oligochaeta: Enchytraeidae). Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology 179, 113-123.

Nota, B., Bosse, M., Ylstra, B., van Straalen, N.M., Roelofs, D., 2009. Transcriptomics reveals extensive inducible biotransformation in the soil-dwelling invertebrate Folsomia candida exposed to phenanthrene. BMC Genomics 10, 236.

Nota, B., Timmermans, M.J.T.N., Franken, C., Montagne-Wajer, K., Marien, J., De Boer, M.E., De Boer, T.E., Ylstra, B., Van Straalen, N.M., Roelofs, D., 2008. Gene Expression Analysis of Collembola in Cadmium Containing Soil. Environ. Sci. Technol. 42, 8152-8157.

Novais, S.C., Gomes, S.I.L., Gravato, C., Guilhermino, L., de Coen, W., Soares, A.M.V.M., Amorim, M.J.B., 2011a. Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures. Environ. Pollut. 159, 1836-1843.

Novais, S.C., Howcroft, C.F., Carreto, L., Pereira, P.M., Santos, M.A.S., De Coen, W., Soares, A.M.V.M., Amorim, M.J.B., 2011b. Differential gene expression analysis in *Enchytraeus albidus* exposed to natural and chemical stressors at different exposure periods. Ecotoxicology, In press.

Novais, S.C., Soares, A.M.V.M., Amorim, M.J.B., 2010. Can avoidance in *Enchytraeus albidus* be used as a screening parameter for pesticides testing? Chemosphere 79, 233-237.

OECD, 2004. Guidelines for the testing of chemicals. Enchytraeid Reproduction Test. No. 220, OECD (Organization for Economic Cooperation and Development). Paris, France.

OECD, 2010. Guidelines for the testing of chemicals. Bioaccumulation in Terrestrial Oligochaete. No. 317, OECD (Organization for Economic Cooperation and Development). Paris, France.

Ogata, H., Goto, S., Sato, K., Fujibuchi, W., Bono, H., Kanehisa, M., 1999. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 27, 29 - 34.

Owen, J., Hedley, B.A., Svendsen, C., Wren, J., Jonker, M.J., Hankard, P.K., Lister, L.J., Sturzenbaum, S.R., Morgan, A.J., Spurgeon, D.J., Blaxter, M.L., Kille, P., 2008. Transcriptome profiling of developmental and xenobiotic responses in a keystone soil animal, the oligochaete annelid Lumbricus rubellus. BMC Genomics 9, 266.

Parkinson, J., Anthony, A., Wasmuth, J., Schmid, R., Hedley, A., Blaxter, M., 2004. PartiGene-constructing partial genomes. Bioinformatics 20, 1398-1404.

Pirooznia, M., Gong, P., Guan, X., Inouye, L., Yang, K., Perkins, E., Deng, Y., 2007. Cloning, analysis and functional annotation of expressed sequence tags from the Earthworm *Eisenia fetida*. BMC Bioinformatics 8, S7.

Posthuma, L., Van Straalen, N.M., 1993. Heavy-Metal Adaptation in Terrestrial Invertebrates - a Review of Occurrence, Genetics, Physiology and Ecological Consequences. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology 106, 11-38.

Poynton, H.C., Varshavsky, J.R., Chang, B., Cavigiolio, G., Chan, S., Holman, P.S., Loguinov, A.V., Bauer, D.J., Komachi, K., Theil, E.C., Perkins, E.J., Hughes, O., Vulpe, C.D., 2007. *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. Environ. Sci. Technol. 41, 1044-1050.

Rebrikov, D.V., Britanova, O.V., Gurskaya, N.G., Lukyanov, K.A., Tarabykin, V.S., Lukyanov, S.A., 2000. Mirror orientation selection (MOS): a method for eliminating false positive clones from libraries generated by suppression subtractive hybridization. Nucl. Acids Res. 28, e90.

Roelofs, D., Janssens, T.K.S., Timmermans, M.J.T.N., Nota, B., Marien, J., Bochdanovits, Z., Ylstra, B., Van Straalen, N.M., 2009. Adaptive differences in gene expression associated with heavy metal tolerance in the soil arthropod *Orchesella cincta.* Mol. Ecol. 18, 3227-3239.

Roelofs, D., Marien, J., van Straalen, N.M., 2007. Differential gene expression profiles associated with heavy metal tolerance in the soil insect *Orchesella cincta.* Insect Biochem. Mol. Biol. 37, 287-295.

Schill, R.O., Kohler, H.R., 2004. Energy reserves and metal-storage granules in the Hepatopancreas of *Oniscus asellus* and *Porcellio scaber* (Isopoda) from a metal gradient at Avonmouth, UK. Ecotoxicology 13, 787-796.

Shagin, D.A., Rebrikov, D.V., Kozhemyako, V.B., Altshuler, I.M., Shcheglov, A.S., Zhulidov, P.A., Bogdanova, E.A., Staroverov, D.B., Rasskazov, V.A., Lukyanov, S., 2002. A novel method for SNP detection using a new duplex-specific nuclease from crab hepatopancreas. Genome Res. 12, 1935-1942.

Slotsbo, S., Maraldo, K., Malmendal, A., Nielsen, N.C., Holmstrup, M., 2008. Freeze tolerance and accumulation of cryoprotectants in the enchytraeid *Enchytraeus albidus* (Oligochaeta) from Greenland and Europe. Cryobiology 57, 286-291.

Sturzenbaum, S.R., Parkinson, J., Blaxter, M., Morgan, A.J., Kille, P., Georgiev, O., 2003. The earthworm Expressed Sequence Tag project. Pedobiologia 47, 447-451.

Timmermans, M.J., de Boer, M.E., Nota, B., de Boer, T.E., Marien, J., Klein-Lankhorst, R.M., van Straalen, N.M., Roelofs, D., 2007. Collembase: a repository for springtail genomics and soil quality assessment. BMC Genomics 8, 341.

Timmermans, M.J.T.N., Ellers, J., Roelofs, D., Van Straalen, N.M., 2005. Metallothionein mRNA expression and cadmium tolerance in metal-stressed and reference populations of the springtail *Orchesella cincta.* Ecotoxicology 14, 727-739.

Vijver, M.G., Van Gestel, C.A.M., Lanno, R.P., Van Straalen, N.M., Peijnenburg, W.J.G.M., 2004. Internal metal sequestration and its ecotoxicological relevance: A review. Environ. Sci. Technol. 38, 4705-4712.

Werle, E., Schneider, C., Renner, M., Volker, M., Fiehn, W., 1994. Convenient single-step, one tube purification of PCR products for direct sequencing. Nucleic Acids Res. 22, 4354-4355.

Zhu, Y.Y., Machleder, E.M., Chenchik, A., Li, R., Siebert, P.D., 2001. Reverse transcriptase template switching: a SMART approach for full-length cDNA library construction. Biotechniques 30, 892-897.

Chapter V

Transcriptional responses in *Enchytraeus albidus*: comparison between cadmium and zinc exposure and linkage to reproduction effects

V – Transcriptional responses in *Enchytraeus albidus*: comparison between cadmium and zinc exposure and linkage to reproduction effects

Sara C. Novais, Wim De Coen & Mónica J.B. Amorim

Submitted to BMC Genomics (IF = 4.296; 2010)

Abstract

Metal ecotoxicity on soil organisms, e.g. in *Enchytraeus albidus*, has been addressed mainly by assessing effects on survival and reproduction. Very little is known about the molecular underlying mechanisms of responses. The main purpose of this work was to assess and compare the transcriptional responses of *E. albidus* to an essential (zinc) and a non-essential metal (cadmium). Exposure was performed to two concentrations of known effect on reproduction (EC_{50}, EC_{90}) at three time periods (2, 4 and 8 days). Results showed that transcriptional responses were influenced by exposure duration. Independently of that, the mechanisms of response to Cd and Zn were consistently different. Both metals affected pathways related with the regulation of gene expression, calcium homeostasis and cellular respiration. Mechanisms of toxicity that were exclusively associated with Cd exposures were the inhibition of DNA repair and the impairment of ubiquitin-mediated proteolysis. It should be highlighted that not only we could retrieve mechanistic information, but also that genes responded within a short period of exposure. This is an additional advantage of using these molecular endpoints as a complement to the traditional, more time-consuming endpoints.

Keywords: Enchytraeids; gene expression; microarrays; metals

1. Introduction

Metal contamination in soil is a worldwide problem and in the European Union it is even the largest class of soil pollutants (Rodrigues et al., 2009; Nota et al., 2010). The effects of metals on soil organisms have been extensively studied in the past 10 years, mainly through the use of standard guidelines, addressing effects on important life-history traits like survival, reproduction or growth.

Enchytraeids are sensitive to chemical pollution, distributed across soils worldwide and fulfil vital functions in the terrestrial ecosystem, which makes them suitable invertebrate species for soil ecotoxicological assessment. *Enchytraeus albidus* is the commonly used species in the standardized testing guidelines (ISO, 2004; OECD, 2004, 2010), hence information is available on various chemicals and endpoints for this species. For instance, for cadmium (Cd) and zinc (Zn) toxicity data is known on effects on survival and reproduction (Lock and Janssen, 2001b; Lock and Janssen, 2001a, 2003; Novais et al., 2011b), bioaccumulation (Lock and Janssen, 2001c; Egeler et al., 2009), avoidance behaviour (Amorim et al., 2008) and, more recently, on oxidative stress biochemical biomarkers (Novais et al., 2011b). Although much has been studied about Cd and Zn effects on this species, nothing is known in terms of the underlying molecular mechanisms of toxicity at gene level. Because recently a cDNA library was developed (Amorim et al., 2011) and enriched (Novais et al., 2011a), transcriptomic studies became possible, opening to a vast range of new study areas.

Toxicogenomic tools, and in particular microarrays, have been successfully used in the past years bringing added knowledge in regard to the molecular pathways of response to stressors and to elucidate modes of action of chemicals in the terrestrial environment (van Straalen and Roelofs, 2008; Brulle et al., 2010). For *E. albidus* such a tool was developed and effects at gene level have been studied, e.g. as response to exposure to chemicals (phenmedipham, copper salt and copper nanoparticles) or to study the effects of different soil properties and times of exposure (Gomes et al., 2011a; Gomes et al., 2011b; Novais et al., 2011c). The authors have reckoned the need to enrich the gene library [due to limited number of cDNA sequences available and few of the gene fragments could be identified by sequence similarity on public databases] despite the

advances produced by the previous referred studies. The sequence information was enriched with the development of two new cDNA libraries enriched for metal and pesticide exposures, using suppression subtractive hybridization (SSH) method (Novais et al., 2011a).

In this study, the enriched genomic library was used for the first time to assess the effects of Cd and Zn on *E. albidus* gene expression. One of the largest challenges in the ecotoxicogenomics field is to establish links between transcription profiles and effects at the higher individual and population level. For that, gene experiments should be anchored to phenotypic effects. Hence, the organisms used in this study were exposed to concentrations with known effects on reproduction and survival.

The main goal of this work was to compare the transcriptional responses between the two metals to investigate both the existence of a common set of metal stress induced genes as well as the discriminating transcripts for the mechanisms of toxicity. Metals were selected in order to include an essential and a non-essential element – Cd and Zn. Second, different times of exposure were included to assess differences in gene expression along time, selecting 3 time points - 2, 4 and 8 days - to capture the transcriptional changes in the first days of exposure. These short time periods were selected based on previous studies which showed gene expression changes shortly after exposure (e.g. Heckmann et al., 2008; Nota et al., 2008; Amorim et al., 2011; Novais et al., 2011c).

2. Materials and methods

2.1 Test organisms

The enchytraeids *Enchytraeus albidus* (Henle, 1837) were selected for this study. Individuals were maintained in laboratory cultures under controlled conditions with a photoperiod 16:8h light:dark and a temperature of 18ºC. Animals were fed twice a week with finely ground and autoclaved rolled oats.

2.2 Experimental conditions

2.2.1 Test Soil

All exposures were performed in the natural standard soil LUFA 2.2. This soil type is commercially available from the German institution LUFA Speyer. The properties of this soil can be summarised as follows: pH ($CaCl_2$) = 5.5, Organic Matter = 4.4%, texture = 6% clay; 17% silt; 77% sand.

2.2.2 Spiking of the test substances

Two chemicals were individually tested: zinc chloride [$ZnCl_2$, Sigma-Aldrich (Riedel-de Haën), 98%] and cadmium chloride ($CdCl_2$, Fluka, 99%). The chemicals were spiked into pre-moistened batches of soil as aqueous solutions, each test concentration into the whole batch of soil for all replicates. The spiked soil was allowed to equilibrate three days before test start as recommended for metals testing (McLaughlin et al., 2002).

The organisms were exposed to concentrations of each metal in the range of the reproduction EC_{50} and EC_{90} (Zn: 40 and 100 mg/kg; Cd: 6 and 150 mg/kg) as available from Novais et al. (2011b).

2.2.3 Exposure procedures

Fifteen adult worms with well developed clitellum were introduced in the test vessels, each containing 25 g moist soil (40 to 60% of the maximum Water Holding Capacity - WHC). The worms were exposed for 2, 4 and 8 days at 20ºC and a 16:8h photoperiod and fed following the same procedures as in the standard guideline for the Enchytraeid Reproduction Test (ISO, 2004). Three replicates per exposure treatment and 4 replicates

of control conditions were used. At test end, animals of each replicate were carefully removed, rinsed in deionised water, stored in RNA later (Ambion, USA) containing criotubes and frozen in liquid Nitrogen. All the samples were stored at -80ºC till further analysis.

2.3 Probes and microarray design

The designed microarray used 1124 gene clusters, as presented in EnchyBASE (http://bioinformatics.ua.pt/enchybase), obtained from three different genomic libraries enriched for metal and pesticide exposures as described in detail by Novais et al. (2011a). The probes comprised of 60-mer oligonucleotides were designed using the E-array web platform (https://earray.chem.agilent.com/earray) (Agilent Technologies, Palo Alto, CA, USA). A total of 1035 unique probes were obtained and randomly spotted in several replicates on Custom Gene Expression Agilent Microarrays 8 x 15k format, along with replicate control probes from the manufacturer (Agilent Technologies, Palo Alto, CA, USA).

2.4 RNA extraction, labelling and hybridizations

Three biological replicates of each chemical exposure condition and four of control conditions were used, for each time point. Isolation of total RNA from each replicate was performed through the Trizol extraction method (Invitrogen, Belgium). This RNA followed a DNAse treatment (Fermentas, Germany) and further purification steps consisting of phenol/chloroform extractions. The quantity and purity of the isolated RNA were measured spectrophotometrically with nanodrop (NanoDrop ND-1000 Spectrophotometer) and its integrity was checked on a denaturing formaldehyde agarose gel electrophoresis.

A one-colour design was used starting from 500 ng of total RNA which were amplified and labelled with the Agilent Low Input Quick Amp Labelling Kit (Agilent

Technologies, Palo Alto, CA, USA), according to the manufacturer protocol. Positive controls for monitoring the Agilent one colour gene expression microarray workflow were added with the Agilent one-colour RNA Spike-In Kit (Agilent Technologies, Palo Alto, CA, USA). Amplified and labelled cRNA was purified with the RNeasy columns (Qiagen, Valencia, CA, USA).

Hybridization was performed using the Agilent Gene Expression Hybridization Kit (Agilent Technologies, Palo Alto, CA, USA) and each biological replicate was individually hybridized on one array. After the 17h hybridization performed at 65 ºC with a rotation of 10 rpm, microarrays were washed using Agilent Gene Expression Wash Buffer Kit (Agilent Technologies, Palo Alto, CA, USA) and scanned with the default settings of the Agilent DNA microarray scanner G2505B (Agilent Technologies). A total of 48 hybridizations resulted from the present microarray experimental design (two concentrations - EC_{50} and EC_{90}, two metals – Zn and Cd, in triplicate plus four replicates of control conditions, at three time points).

2.5 Analysis of microarray data

Fluorescence intensity data was obtained with Feature Extraction (10.5.1.1) Software (Agilent Technologies). Quality control was done by inspecting the reports on the Agilent Spike-in control probes and by making box plots of each array. Processing of the data and statistical analysis were performed using BRB Array Tools version 4.1 Stable Release (http://linus.nci.nih.gov/BRB-ArrayTools.html). After background subtraction, the replicated spots within each array were averaged and the intensities were $\log_2$ transformed. Data was then normalized using median array as reference. Raw and processed data are available from Gene Expression Omnibus (GEO) at the NCBI website (platform: GPL14928; series: GSE33944).

As one-colour design experiment, individual processed fluorescence expression values were obtained for each gene of each replicate (instead of ratios). This strategy allows the comparison of each stress condition with the respective control as well as between the different exposure conditions. Statistical class comparison was performed between

those different groups of arrays using two-sample t-test with 95% confidence level for the assessment of differentially expressed genes. Following analysis were performed using ratios of expression when comparisons were made with the control group.

Annotation of the differentially expressed genes ($p<0.05$) was performed based on their similarity to sequences in the National Centre for Biotechnology Information (NCBI) database as determined by the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The sequences were submitted to Blast2GO (Conesa et al., 2005) being compared with peptide sequence databases using BLASTX analysis (with minimum 10^{-5} *e*-value). Clustering and principal component analyses (PCA) were performed using MultiExperiment Viewer (MeV, TIGR).

2.6 Quantitative Real-Time PCR confirmations

Total RNA (1µg) of samples from the 2 days of exposure was converted into cDNA through a reverse transcription reaction using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). The 7500 Real-Time PCR System (Applied Biosystems) was used for amplification with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Primer sets were designed for six target genes (EAC00198, EAC00331, EAC00480, EAC01004, EAC01031, EAC01089; see table S2, supporting information) and one endogenous control gene (EAC00302) with the software Oligo ExplorerTM (version 1.1.0). Efficiency and specificity of each primer was determined by observing the obtained standard and melting curves, respectively, for all primer sets. Primer sequences are available in EnchyBASE – http://bioinformatics.ua.pt/enchybase/ (Novais et al., 2011a). cDNA was 4 x diluted and 2 µL were used in a 20 µL PCR reaction containing 2 µL of forward and 2 µL of reverse primers (2 µM), 10 µL of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 4 µL of DEPC-treated water. Quantitative real-time PCR (qPCR) was performed with three biological replicates of each condition (the same used for the microarray experiment), applied in triplicate on a 96-well optical plate (GeneAmp®, Applied Biosystems). Reaction conditions consisted of one initial cycle at 50ºC for 2 min, followed by a denaturation

step at 95ºC for 2 min, 40 cycles at 95ºC for 32 sec and 1 cycle at 60ºC for 1 min. Finally, a dissociation step was made consisting of 15 sec at 95ºC, 1 min at 60ºC, and 15 sec at 95ºC.

A mean normalized expression value was calculated from the obtained Ct values of the test genes with Relative Expression Software Tool (REST-MSC).

3. Results and Discussion

The newly developed Agilent microarray and a one-color design were used for sample hybridizations. Given the large amount of data and endpoints, results and discussion will be presented following a stepwise structure as follows: 1) different exposure periods comparison, 2) Cd exposure, 3) Zn exposure and 4) Cd and Zn comparison.

Exposure period

Class comparison statistical analyses were performed between each chemical condition and the respective control (from 2, 4 or 8 days) and from this analysis resulted a total of 306, 327 and 329 significant differentially expressed genes at 2, 4 and 8 days, respectively. Figure 1 shows the number of significant up and down-regulated transcripts after each metal exposure in the different time points.

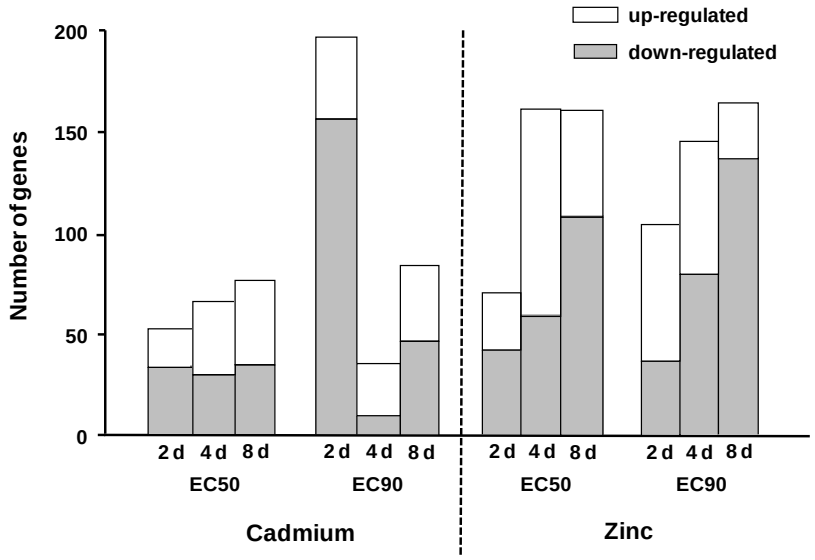

Figure 1: Number of significant up- and down-regulated genes (two sample *t*-tests, $p<0.05$) in *E. albidus* after exposure to Cd and Zn for: 2d – 2 days; 4d – 4 days; 8d – 8 days. Values refer to the comparison with control for each time of exposure. EC50 = 50% effect concentration on reproduction. EC90 = 90% effect concentration on reproduction.

Results show that already after 2 days of exposure alterations on gene expression are visible and that, in general, the number of altered transcripts increased with time of exposure. The exception was verified for Cd EC_{90} exposure, where a higher number was observed at 2 days. These results confirm that, as in previous studies (Amorim et al., 2011; Gomes et al., 2011a; Gomes et al., 2011b; Novais et al., 2011c), gene expression responses in *E. albidus* are observable within short time intervals (2-8 days). This pattern has also been observed in several studies with other invertebrate species (Roelofs et al., 2007; Heckmann et al., 2008; Nota et al., 2008; Ki et al., 2009; Vandenbrouck et al., 2011). For Zn, the two tested concentrations showed a similar pattern of response (fig. 1), with a shift to more down-regulated genes with increasing time of exposure. The minute differentiation between the two Zn concentrations can be depicted in the cluster analysis in figure 2-A.

Overall, we found a clear separation between Cd and Zn in gene expression, despite the observed differences at different times of exposure (see Principal Component Analysis for all samples) (Figure 2-B).

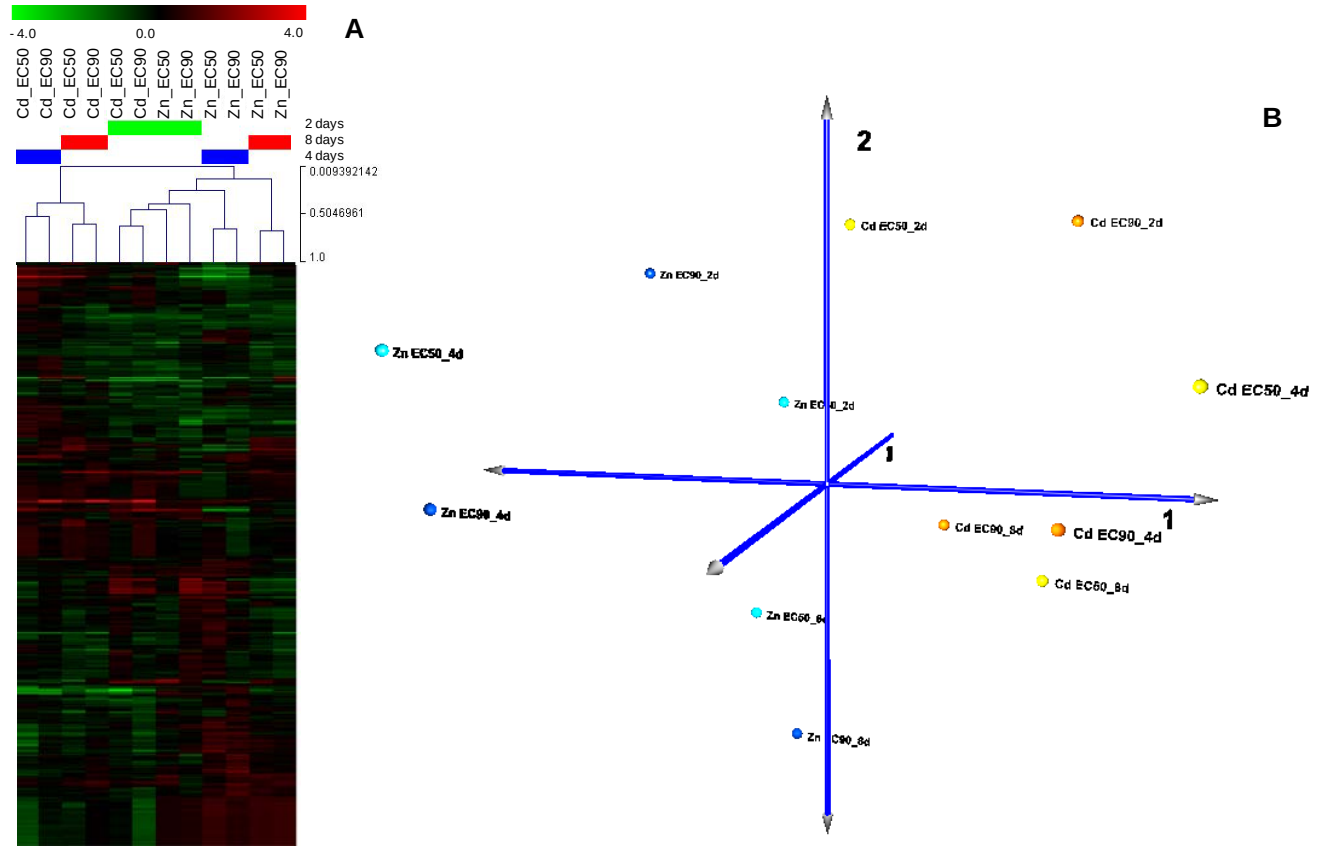

Figure 2: A – Heat map and hierarchical clustering of samples (Pearson's uncentered) from Cd and Zn exposed organisms, based on the differentially expressed genes (two sample *t*-test, $p < 0.05$) when compared with control from each time of exposure. The different colored horizontal bars correspond to the samples from 2, 4 and 8 days of exposure. **B –** Principal Component Analysis (PCA) of the samples based on the differentially expressed genes (two sample *t*-test, $p < 0.05$) when compared with control from each time of exposure. Samples are represented in distinct colors, each representing one concentration of Cd or Zn.

At 4 and 8 days, a clear separation of Cd samples from the rest of the exposure conditions is observed through the cluster analysis (fig. 2-A), indicating different response towards the two metals. Mainly for Cd, differences due to the time of exposure are larger than due to concentration. Time of exposure is indeed an important aspect and several authors have already identified significant differences in gene expression with short time differences (Gracey and Cossins, 2003; Reynders et al., 2006; Soetaert et al., 2007; Nota et al., 2008; Ki et al., 2009; Novais et al., 2011c). The short term gene responses can be transient, with a later stabilization of the physiological state of the stressed organisms. Nevertheless, studies showed that early transcription effects can be associated with ecophysiological stress responses at higher levels of biological organization (e.g. Heckmann at al., 2008). Hence, in the present study the organisms were exposed to concentrations which cause effects on reproduction and within a short time-series range (2 to 8 days). Such attempted to cover the changes in gene expression including a possible activation/deactivation or increase/decrease window frame of expression.

Exposure to cadmium

The statistical analysis performed with Cd samples, from the three time points, resulted in a total of 430 differentially expressed genes in comparison with control. From these transcripts, 156 (36%) match known proteins in public databases and these were the ones discussed here. The biological processes and molecular functions (GO terms) affected by Cd were retrieved from Blast2GO (Conesa et al., 2005) and are represented as pie charts in Figure S1-A (Supporting information).

From the 156 altered transcripts, 52 were uniquely significantly up-regulated and 101 down-regulated after Cd stress. The complete list of altered genes with significant blast homologies, are available in Table S1 (Supporting information).

Cadmium mechanisms of toxicity have been widely studied, e.g. in mammalian cells, yeasts, aquatic invertebrates and, more recently and to a less extent, in terrestrial invertebrates (for reviews see Beyersmann and Hechtenberg, 1997; Beyersmann et al.,

2003; Brulle et al., 2010). These studies showed similar mechanisms in the different species, indicating that this metal induces alterations in the gene expression regulation, oxidative damage, inhibit DNA repair and interfere with metabolism of essential metal ions like Fe, Ca and Zn. For *E. albidus*, gene expression alterations indicated that some of these known mechanisms of toxicity for Cd in other organisms were coincided. The most relevant and significantly affected genes are represented in figure 3.

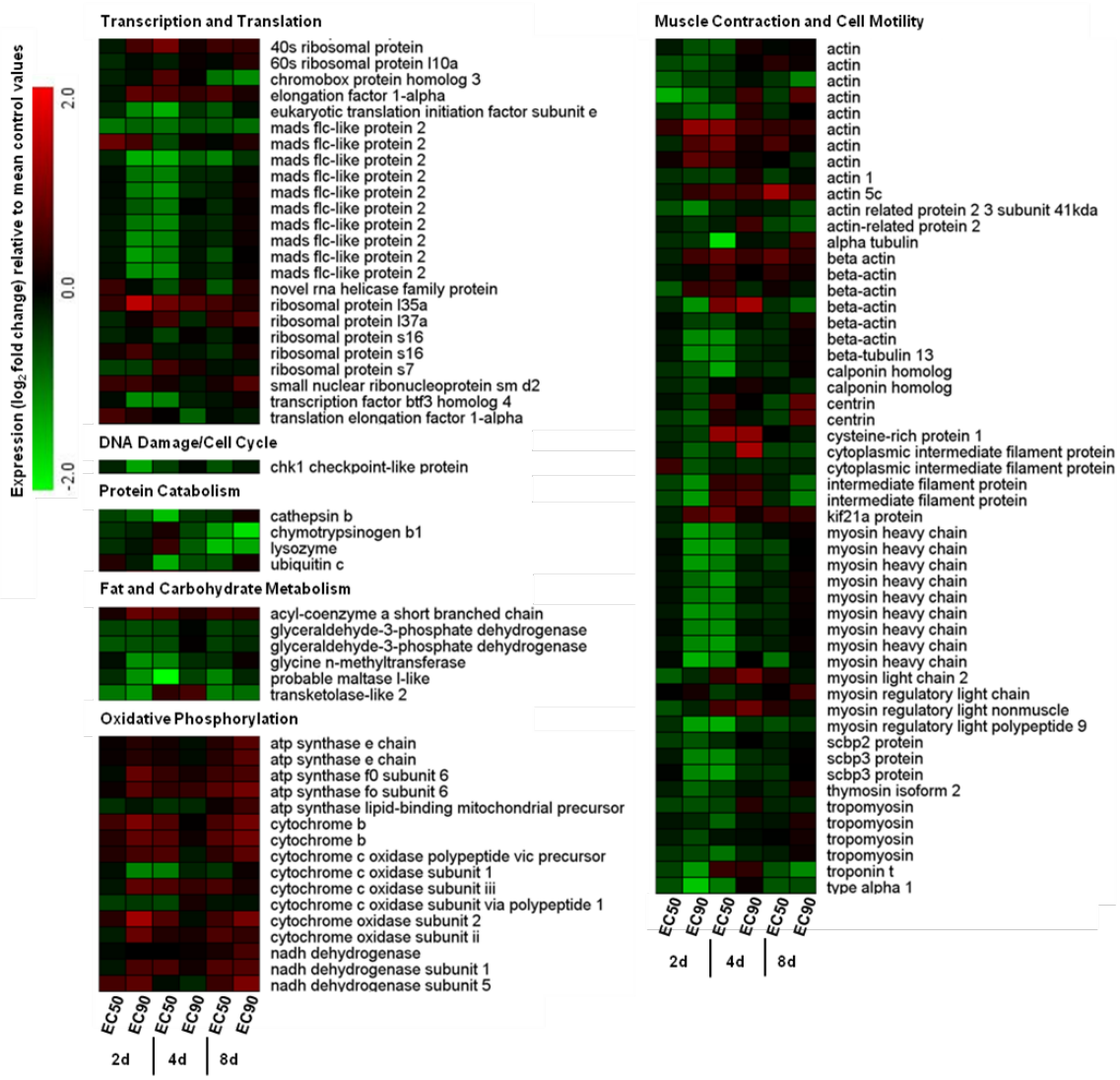

Figure 3: Heat map of a selection of significantly differentially expressed genes (two sample *t*-test, $p < 0.05$) in *E. albidus* exposed to Cd in two concentrations (reproduction EC_{50} and EC_{90}) during three time periods (2, 4 and 8 days). Selected genes are divided into functional groups according to their GO annotation and represent the most relevant functional groups affected by Cd exposure (for all annotated genes see table S1-supporting information).

The influence of Cd on the regulation of gene expression can be seen by the down-regulation of several genes related with transcription (fig. 3), including the transcription factors BTF3 and MADS-box genes. The MADS-box is a family of transcription factors with a conserved sequence motif, found in several eukaryotic organisms (Shore and Sharrocks, 1995). These transcription factors are involved in the regulation of numerous genes with several biological functions. However, some studies refer their importance in the regulation of several muscle-specific genes (Mohun et al., 1991; Buckingham, 1994; Chambers et al., 1994). This is interesting given the fact that in our study several transcripts related with muscle contraction (e.g. actin, myosin, troponin, tropomyosin) were also down-regulated and in the exact same conditions where the MADS transcription factors were repressed (fig. 3). This was confirmed by qPCR analysis where the expression of transcrips coding for a MADS-box transcription factor (EAC01031) and a myosin heavy chain protein (EAC01089) followed the same pattern of down-regulation (table S2, supporting information). This causality relationship has however to be confirmed in further molecular studies.

The effects of Cd on muscle contraction related genes observed for *E. albidus* have also been described for *Daphnia magna* (Connon et al., 2008) and *Lumbricus rubellus* (Owen et al., 2008) and further confirmed with a decrease on these protein levels in rat cells (Sabolic et al., 2001). For *E. albidus*, this could be the result of allocating more energy for detoxification processes and invest less energy for locomotion throughout the soil. This investment to detox Cd is also supported by the up-regulation of several components of the oxidative phosphorylation process, including transcripts related with the electron transfer chain from complex I (NADH dehydrogenase), complex III (cytochrome b) and complex IV (cytochrome c oxidase) and the ATP synthase from which ultimately results energy production (fig. 3). The up-regulation of an ATP synthase coding transcript (EAC00480) was further confirmed by the qPCR analysis (table S2, supporting information).

Cadmium has been shown to affect the mitochondrial metabolism, as observed here, although most studies state an inhibitory effect on complexes II and III with a consequent production of reactive oxygen species (ROS), a well described effect of Cd toxicity (Wang et al., 2004a; Wang et al., 2004b; Owen et al., 2008). In this study the genes involved in the electron transport chain were mainly significantly up-regulated which was also

observed in other studies, e.g. with freshwater and marine bivalves (Achard-Joris et al., 2006), European flounder (Sheader et al., 2006) and Daphnia (Connon et al., 2008). The different expression tendencies of these genes may be species-specific but also dosage-specific, as observed in the work by Owen and co-authors (Owen et al., 2008) where the electron transport coding genes were responding differently to the distinct Cd concentrations and not in a dose-response manner.

Mitochondria is a major source of superoxide and hydrogen peroxide production in cells (Turrens, 1997) and alterations on the normal function of this organelle can increase the generation of ROS. No oxidative stress related genes encoding for antioxidants were triggered in *E. albidus* by Cd exposures but it is important to mention that only some of these genes are present in the library (with known homology). In fact, in a previous study it was observed that these same concentrations of Cd induced significant effects on several antioxidant enzyme activities and substrate levels (glutathione) as well oxidative damage, measured by increased levels of lipid peroxidation (Novais et al., 2011b). DNA damage would also be expected by Cd exposure due to this ROS generation (Sagripanti, 1999). A good indicator that this can be happening in the present exposures is the down-regulation of the cell cycle checkpoint CHK1 (fig. 3), a kinase with conserved functions among several eukaryotes (Chen and Sanchez, 2004). Cell cycle checkpoints like the kinase CHK1 respond to DNA damage by delaying or arresting the cell cycle at specific stages, enabling DNA repair (Chen and Sanchez, 2004; Katsuragi and Sagata, 2004). The down-regulation of this transcript can therefore relate with the inhibition of DNA repair.

Cd is also known to interfere with the ubiquitin-mediated proteolysis (Alam et al., 2003; Owen et al., 2008). Present results in *E. albidus* seem to reinforce such mechanism of toxicity with the down-regulation observed for ubiquitin c and several transcripts related with protein catabolism (fig. 3).

The carbohydrate and fat metabolisms were also affected by Cd, with mainly down-regulated transcripts related with carbohydrate metabolism and with the up-regulation of Acyl-coenzyme A dehydrogenase from fat metabolism (fig. 3). These microarray evidences were further confirmed in a study where cellular energy allocation was measured (Novais et al., 2012). That study revealed a decrease in the carbohydrate and lipid fractions

after 2 days of Cd exposure, as indicated by gene expression analysis, resulting in a global reduction on the net energy budget.

Another observed mechanism of toxicity of Cd is the interference with the metabolism of essential metal ions like Fe, Ca and Zn. This impact was seen, similarly to what was observed for *Lumbricus rubellus* (Owen et al., 2008), with a negative transcriptional influence on several Ca-binding proteins like the sarcoplasmic Ca-binding protein (SCBP3), calponin, myosin or troponin (fig. 3).

The fact that responses of *E. albidus* to Cd exposures were broadly similar to the mechanisms observed for other organisms, points for a cross-species conservation in terms of Cd modes of action.

Exposure to zinc

Exposure to all conditions of Zn resulted in a total of 531 significantly differential expressed genes in comparison with control. From these genes, 189 (36%) match known proteins in public databases and 67 were uniquely significantly up-regulated while 107 were down-regulated due to Zn stress. The complete list of significantly altered genes with blast homologies is available in Table S3 (Supporting information) and their biological and molecular functions (GO terms) represented in Figure S1-B (Supporting information).

Effects of Zn on gene expression of invertebrate ecotoxicological model species are by far less studied than Cd and most of the studies used *Daphnia magna* as a model (Poynton et al., 2007; De Schamphelaere et al., 2008; Poynton et al., 2008; Vandegehuchte et al., 2010a; Vandegehuchte et al., 2010c).

The most relevant and significantly affected genes in *E. albidus* by Zn exposures, with their respective predictive function, are represented in figure 4.

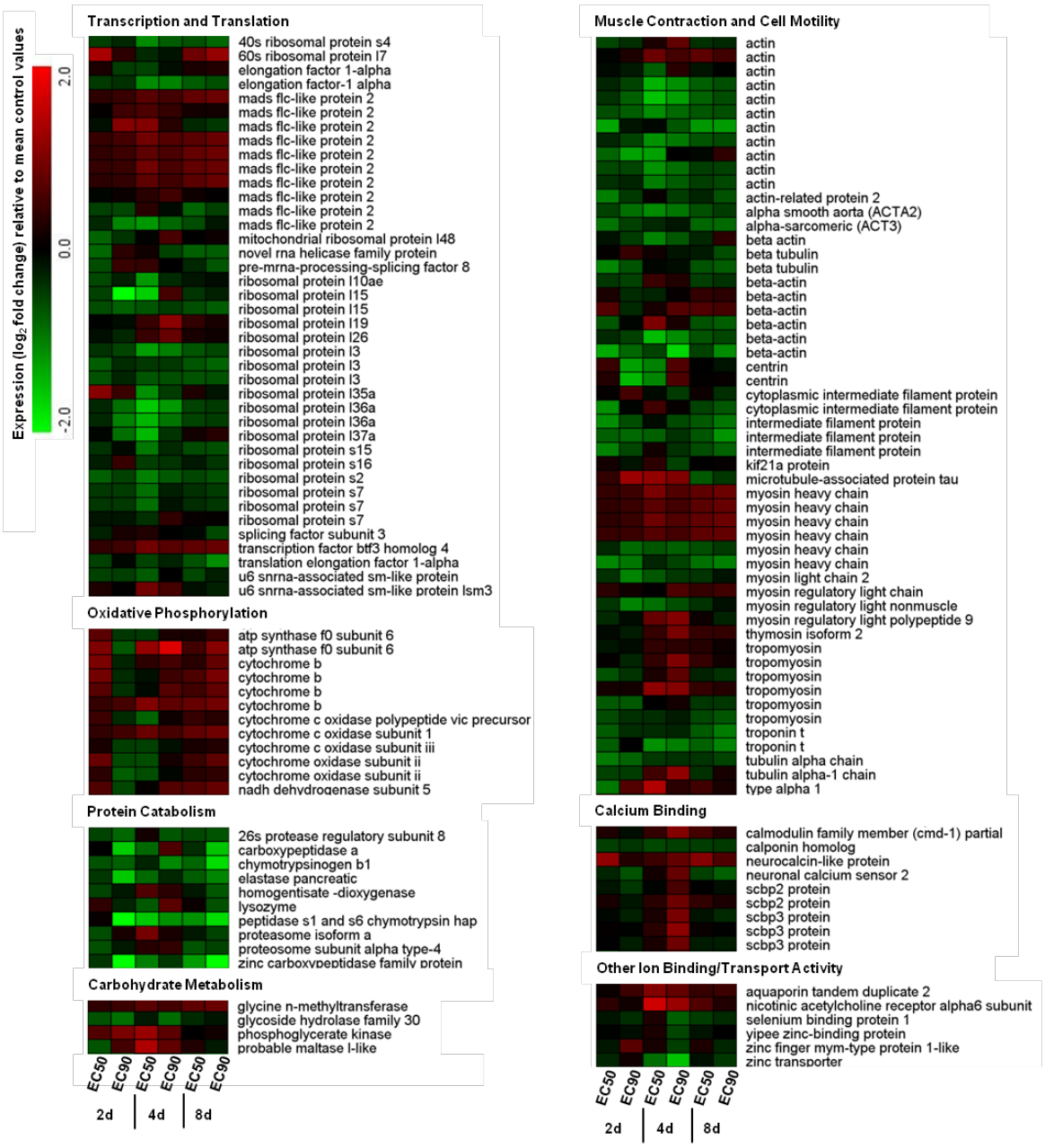

Figure 4: Heat map of a selection of significantly differentially expressed genes (two sample *t*-test, $p < 0.05$) in *E. albidus* exposed to Zn in two concentrations (reproduction EC_{50} and EC_{90}) during three time periods (2, 4 and 8 days). Selected genes are divided into functional groups according to their GO annotation and represent the most relevant functional groups affected by Zn exposure (for all annotated genes see table S3-supporting information).

Known mechanisms of Zn toxicity include effects on mitochondrial respiration, calcium homeostasis, intracellular signaling and metal imbalance [due to competition for protein binding with other essential metals such as copper and selenium] (Gilmour et al., 2006).

In *E. albidus*, several transcripts coding for mitochondrial proteins involved in the electron transport chain were significantly altered by Zn, indicating an impact on mitochondria and ATP-generating pathway (fig. 4). This was also previously observed for copper, another essential metal, in this enchytraeid species (Gomes et al., 2011a; Novais et al., 2011c) and in the earthworm *Lumbricus rubellus* (Bundy et al., 2008). Most of these affected transcripts were up-regulated after 4 days of exposure, which may indicate the need of extra energy production. This hypothesis is corroborated by the down-regulation of several ribosomal proteins (fig. 4), as also documented for *Daphnia magna* (Vandegehuchte et al., 2010b). Ribosomal protein synthesis is an energy costly process and a decrease in this process could be related to an energy saving mechanism to cope with detoxification of xenobiotics (Calow, 1991; Nota et al., 2009; Vandegehuchte et al., 2010b). Both the up-regulation of an ATP synthase (EAC00480) and the down-regulation of a ribosomal protein (EAC00331) after Zn exposure, were confirmed by qPCR analysis (table S2, supporting information).

Interestingly, the same pattern between the expression of MADS-box transcription factors and some muscle contraction related genes, as observed due to Cd stress, occurred for Zn exposed animals. In figure 4 it is possible to observe an up-regulation of most of these transcription factors which seems to correlate with the expression patterns of some myosin heavy chain coding transcripts (confirmed by qPCR – table S2, supporting information).

Zn toxicity is known to interfere with calcium homeostasis in some vertebrates (Gilmour et al., 2006). In *E. albidus*, effects on calcium ion binding transcripts were observed (fig. 4). The fact that some of these transcripts were up-regulated and others down-regulated suggest that the deregulation on calcium homeostasis might occur through different mechanisms.

The repressed expression of a selenium binding protein (fig. 4) suggests that Zn might be also interfering with the metabolism of this other essential element.

The effects of Zn in *E. albidus* strongly correlate with some of the known mechanisms of Zn toxicity as also observed for Cd, again providing evidences of a probable cross-species conservation to metal detoxification.

Comparison between cadmium and zinc exposures

In general, both metals significantly affected a high number of transcripts. It is important to notice that of the total differentially expressed genes due to Cd and Zn only 24% (Cd) and 18% (Zn) were transcripts obtained from the pesticides enriched genomic library. This higher number of affected transcripts from the metals enriched library confirms the effectiveness of the suppressive subtractive hybridization (SSH) method used to enrich the genomic libraries (Novais et al., 2011a).

As discussed before, affected pathways due to Cd and Zn exposures were identified and, in general, within the same reproduction EC a higher number of transcripts were affected by Zn than Cd. Additionally, Zn affected more biological processes and molecular functions than Cd (figure S1, supporting information), which was not surprising since Zn is an essential element required for the maintenance of many physiological functions, therefore a change in the normal levels of Zn would virtually affect all those functions.

A common effect of both metals seems to occur on the mitochondrial electron transport chain related transcripts, with a tendency to an up-regulation of these transcripts with both metal exposures. Another common effect was the interference with calcium metabolism, although it was more evident for Cd as most of the transcripts coding for calcium-binding proteins were down-regulated. Moreover, metabolisms of carbohydrates and proteins were affected by both metals. This tendency for a reduction on the available energy reserves, along with increased cellular respiration, was confirmed in Novais et al. (2012), where the actual content of lipids, carbohydrates and proteins were measured as well as the electron transport system activity.

Effects on transcription and translation processes were also affected by both metals, although apparently by different mechanisms. For example, in Cd exposures most

transcription factors are down-regulated while their expression was enhanced in Zn exposures. One of these transcription factors affected in the opposite way is the basic transcription factor 3 (BTF3). This protein forms a stable complex with RNA polymerase II and is one of the four general transcription factors required for initiation of transcription in eukaryotic cells (Zheng et al., 1990). Our results point to an impairment of general gene expression due to Cd which can be a possible cause for the reduction in reproduction if some vital proteins are not being synthesized. As for Zn, effects on protein synthesis seem to be more evident on the translation phase seen by the down-regulation of several transcripts coding for ribosomal proteins and also an elongation factor.

These differences in the transcriptional responses of *E. albidus* were further confirmed in a subsequent statistical class comparison analysis (two sample *t*-tests, $p<0.05$) between all the samples from the same Cd concentration against the ones from the same Zn concentration. This analysis resulted in 184 differentially expressed genes between the lowest concentration of both metals and 358 genes between the highest concentration. In total, this analysis resulted in 456 transcripts expressed significantly different in Cd and Zn exposures, from which 219 have significant blast hits (table S4 - supporting information). Hierarchical clustering was used on the transcripts with known homologies to group the ones with similar expressions, which could roughly be divided into 2 clusters (figure 5-A).

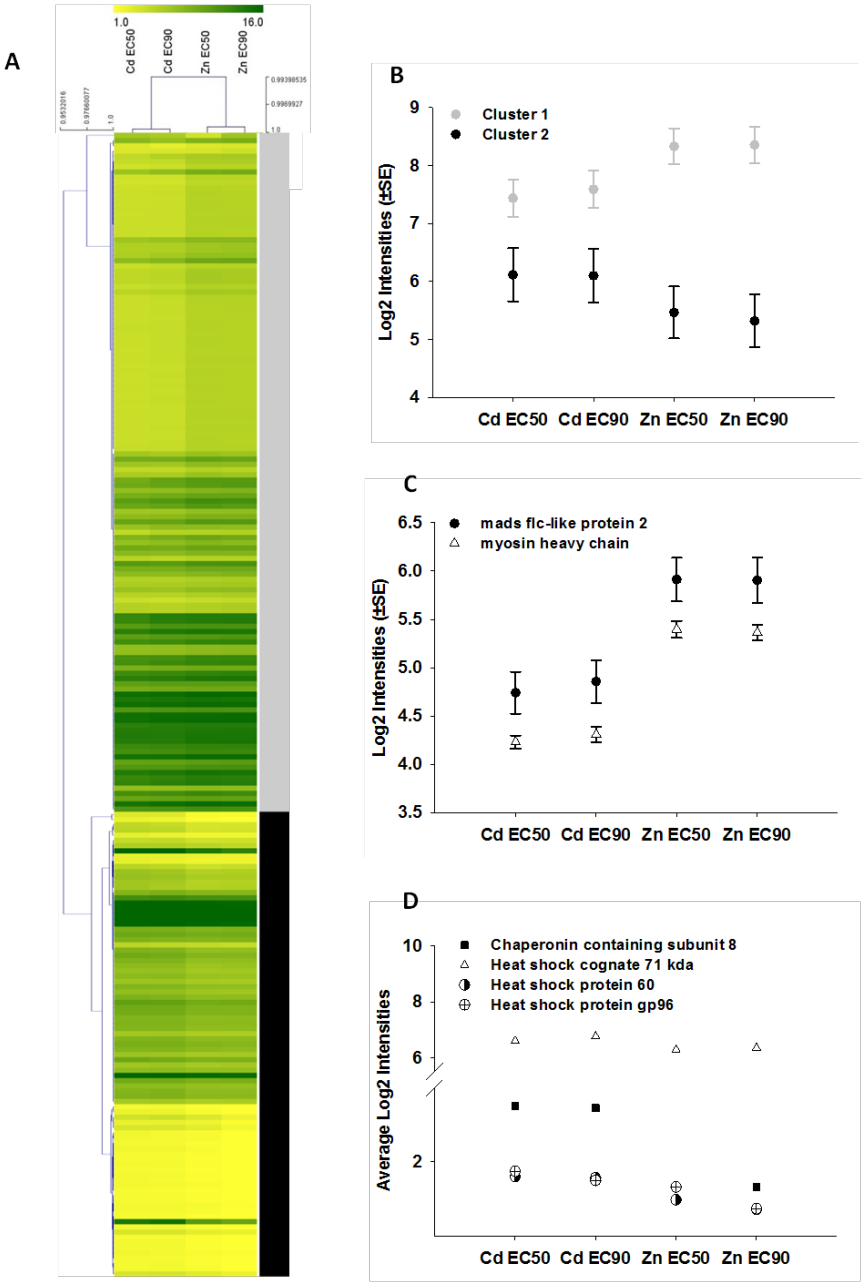

Figure 5: A - Heat map and hierarchical clustering of samples and genes with significant blast homologies (Pearson's uncentered), based on the differentially expressed genes (two sample *t*-test, $p < 0.05$) between samples from each concentration of Cd and Zn. The $\log_2$ intensity values represent the average processed fluorescence expression values of samples from each Cd or Zn concentrations. Two different gene clusters are represented in a grey and a black bars; **B –** Average $\log_2$ intensities of transcripts from cluster 1 (represented in grey) and cluster 2 (represented in black) in the two concentrations of Cd and Zn; **C** - Average $\log_2$ intensities of transcripts coding for mads-box transcription factors and myosin heavy chain in the two concentrations of Cd and Zn; **D -** Average $\log_2$ intensities of transcripts coding for general cellular stress responses in the two concentrations of Cd and Zn.

The first cluster contains transcripts with higher expression values in Zn exposures (represented in grey, figure 5-A and B). This group includes 130 transcripts and a large number of these encode for transcription factors and Ca-binding proteins. In figure 5-C the average intensities of these transcripts coding for MADS-box transcription factors and also myosin heavy chain were plotted and again the parallelism is patent between the expressions of these genes in all metal stress conditions.

On the contrary, the second cluster contains transcripts with higher expression values in Cd exposures (represented in black, figure 5-A and B). There were 89 transcripts included in this second group which has e.g. more ribosomal proteins and elongation factors involved in translation that, as reported before, were more affected by Zn. Another group of transcripts, in this second cluster, code for proteins of general stress response like heat-shock proteins and chaperonins. Expression of these genes (fig. 5-D) shows higher values in Cd exposures, which is a well known effect of Cd toxicity observed in many studies. Interestingly, few or no differences in expression of these transcripts were observed between the two ECs from each metal. In fact, this observation can be extended to most of the transcripts, as can be seen by the small differences in intensity between concentrations in fig. 5-A and 5-B. A possible explanation for this can be the fact that the lowest concentration tested is already causing large effects and the organisms are nearly at their maximum response level, not having further mechanistic options to cope with higher concentrations toxicity. For future studies it would be interesting to test lower concentrations (reproduction EC_{10} or EC_{20}) to confirm this hypothesis.

Quantitative Real-Time PCR

The expression of six genes was confirmed with qPCR to validate the microarray results. Genes were selected based on their differential expression due to Cd or Zn exposures and in order to represent different biological processes. The chosen genes coded for chk1 checkpoint-like protein (EAC00198), cytochrome b (EAC01004), ribosomal protein l15 (EAC00331), mads flc-like protein 2 (EAC01031), myosin heavy chain (EAC01089) and

ATP synthase f0 subunit 6 (EAC00480) and myosin alkali light chain 1 was used as housekeeping (EAC00302).

A significant correlation of 0.755 (Spearman's Rho, p=0.000155) was obtained between microarray and qPCR platforms. All response patterns were confirmed although the magnitude of fold-change responses differed in some of the tested conditions. The direction of expression response (up- or down-regulation) was similar between the two platforms for all 6 genes in the 4 conditions of Cd and Zn exposure (table S2, supporting information).

4. Conclusions

The newly developed microarray for *Enchytraeus albidus* was a useful tool in the detection of molecular pathways affected by metal exposures. Moreover, the SSH method was confirmed to be an effective technique to enrich the genomic library for this species since a large number of significant differentially expressed genes were obtained.

Transcriptional responses were influenced by exposure duration, as often verified in various studies. Nevertheless, independently of exposure duration, the mechanisms of response to Cd and Zn were distinct. Among the commonly affected molecular pathways by both metals are the ones associated with regulation of gene expression, calcium homeostasis and cellular respiration. Mechanisms of toxicity exclusively associated with Cd include the inhibition of DNA repair and the impairment of ubiquitin-mediated proteolysis.

The microarray results showed that transcriptional responses of *E. albidus* strongly correlate with known mechanisms of Cd and Zn responses in other organisms, which could indicate cross-species conserved mechanisms.

The gene expression responses to Cd and Zn were identified within short periods of exposure (2 to 8 days), highlighting the advantages of using these molecular endpoints as a complement to the traditional and more time-consuming endpoints, while bringing knowledge on the mechanisms of toxicity.

Acknowledgments

This study was sponsored by the funding FEDER through COMPETE-Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia, within the research project NANOkA FCOMP-01-0124-FEDER-008944 (Refª. FCT PTDC/BIA-BEC/103716/2008) and through a PhD grant to Sara Novais (SFRH/BD/36253/2007).

References

Achard-Joris, M., Gonzalez, P., Marie, V.r., Baudrimont, M., Bourdineaud, J.-P., 2006. Cytochrome c Oxydase Subunit I Gene is Up-regulated by Cadmium in Freshwater and Marine Bivalves. Biometals 19, 237-244.

Alam, J., Stewart, D., Killeen, E., Naquin, R., Alam, S., 2003. Degradation of transcription factor Nrf2 via the ubiquitin-proteasome pathway and stabilization by cadmium. J. Biol. Chem. 278, 2396-2402.

Altschul, S., Gish, W., Miller, W., Meyers, E., Lipman, D., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403- 410.

Amorim, M.J.B., Novais, S., Römbke, J., Soares, A.M.V.M., 2008. *Enchytraeus albidus* (Enchytraeidae): A test organism in a standardised avoidance test? Effects of different chemical substances. Environ. Int. 34, 363-371.

Amorim, M.J.B., Novais, S.C., Van der Ven, K., Vandenbrouck, T., Soares, A.M.V.M., De Coen, W., 2011. Development of a Microarray for *Enchytraeus albidus* (Oligochaeta): Preliminary Tool with Diverse Applications. Environ. Toxicol. Chem. 30, 1395-1402.

Beyersmann, D., Hechtenberg, S., 1997. Cadmium, gene regulation, and cellular signalling in mammalian cells. Toxicol. Appl. Pharmacol. 144, 247-261.

Beyersmann, D., Waisberg, M., Joseph, P., Hale, B., 2003. Molecular and cellular mechanisms of cadmium carcinogenesis. Toxicology 192, 95-117.

Brulle, F., Morgan, A.J., Cocquerelle, C., Vandenbulcke, F., 2010. Transcriptomic underpinning of toxicant-mediated physiological function alterations in three terrestrial invertebrate taxa: A review. Environ. Pollut. 158, 2793-2808.

Buckingham, M., 1994. Molecular-Biology of Muscle Development. Cell 78, 15-21.

Bundy, J.G., Sidhu, J.K., Rana, F., Spurgeon, D.J., Svendsen, C., Wren, J.F., Sturzenbaum, S.R., Morgan, A.J., Kille, P., 2008. 'Systems toxicology' approach identifies coordinated metabolic responses to copper in a terrestrial non-model invertebrate, the earthworm *Lumbricus rubellus*. BMC Biol. 6:25.

Calow, P., 1991. Physiological Costs of Combating Chemical Toxicants - Ecological Implications. Comparative Biochemistry and Physiology C-Pharmacology Toxicology & Endocrinology 100, 3-6.

Chambers, A.E., Logan, M., Kotecha, S., Towers, N., Sparrow, D., Mohun, T.J., 1994. The Rsrf/Mef2 Protein Sl1 Regulates Cardiac Muscle-Specific Transcription of a Myosin Light-Chain Gene in Xenopus-Embryos. Genes Dev. 8, 1324-1334.

Chen, Y., Sanchez, Y., 2004. Chk1 in the DNA damage response: conserved roles from yeasts to mammals. DNA Repair 3, 1025-1032.

Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674-3676.

Connon, R., Hooper, H.L., Sibly, R.M., Lim, F.L., Heckmann, L.H., Moore, D.J., Watanabe, H., Soetaert, A., Cook, K., Maund, S.J., Hutchinson, T.H., Moggs, J., De Coen, W., Iguchi, T., Callaghan, A., 2008. Linking molecular and population stress responses in *Daphnia magna* exposed to cadmium. Environ. Sci. Technol. 42, 2181-2188.

De Schamphelaere, K.A.C., Vandenbrouck, T., Muyssen, B.T.A., Soetaert, A., Blust, R., De Coen, W., Janssen, C.R., 2008. Integration of molecular with higher-level effects of dietary zinc exposure in *Daphnia magna*. Comparative Biochemistry and Physiology D-Genomics & Proteomics 3, 307-314.

Egeler, P., Gilberg, D., Scheffczyk, A., Moser, T., Römbke, J., 2009. Validation of a Soil Bioaccumulation Test with Terrestrial Oligochaetes by an International Ring Test. Report to the Federal Environmental Agency, R&D No.: 204 67 458: 149 pp. Available for download at: http://www.oecd.org/dataoecd/12/20/42552727.pdf .

Gilmour, P.S., Schladweiler, M.C., Nyska, A., McGee, J.K., Thomas, R., Jaskot, R.H., Schmid, J., Kodavanti, U.P., 2006. Systemic Imbalance of Essential Metals and Cardiac Gene Expression in Rats Following Acute Pulmonary Zinc Exposure. Journal of Toxicology & Environmental Health: Part A 69, 2011-2032.

Gomes, S.I.L., Novais, S.C., Scott-Fordsmand, J.J., de Coen, W., Soares, A.M.V.M., Amorim, M.J.B., 2011a. Effect of Cu-Nanoparticles versus Cu-salt in *Enchytraeus albidus* (Oligochaeta): differential gene expression through microarray analysis. Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology, In press.

Gomes, S.I.L., Novais, S.C., Soares, A.M.V.M., Amorim, M.J.B., 2011b. Effects of soil properties and time of exposure on gene expression of *Enchytraeus albidus* (Oligochaeta). Soil Biol. Biochem. 43, 2078-2084.

Gracey, A.Y., Cossins, A.R., 2003. Application of microarray technology in environmental and comparative physiology. Annu. Rev. Physiol. 65, 231-259.

Heckmann, L.H., Sibly, R.M., Connon, R., Hooper, H.L., Hutchinson, T.H., Maund, S.J., Hill, C.J., Bouetard, A., Callaghan, A., 2008. Systems biology meets stress ecology: linking molecular and organismal stress responses in *Daphnia magna*. Genome Biology 6, 25.

Henle, F.G.J., 1837. Ueber Enchytraeus, eine neue Anneliden-Gattung. Arch. Anat. Physiol. Med., 74-90.

ISO, 2004. Soil quality - Effects of pollutants on Enchytraeidae (Enchytraeus sp.) - Determination of effects on reproduction and survival. Guideline no. 16387, ISO (International Organization for Standardization). Geneve, Switzerland.

Katsuragi, Y., Sagata, N., 2004. Regulation of Chk1 kinase by autoinhibition and ATR-mediated phosphorylation. Mol. Biol. Cell 15, 1680-1689.

Ki, J.S., Raisuddin, S., Lee, K.W., Hwang, D.S., Han, J., Rhee, J.S., Kim, I.C., Park, H.G., Ryu, J.C., Lee, J.S., 2009. Gene expression profiling of copper-induced responses in the intertidal copepod *Tigriopus japonicus* using a 6K oligochip microarray. Aquat. Toxicol. 93, 177-187.

Lock, K., Janssen, C.R., 2001a. Cadmium toxicity for terrestrial invertebrates: Taking soil parameters affecting bioavailability into account. Ecotoxicology 10, 315-322.

Lock, K., Janssen, C.R., 2001b. Effect of clay and organic matter type on the ecotoxicity of zinc and cadmium to the potworm *Enchytraeus albidus.* Chemosphere 44, 1669-1672.

Lock, K., Janssen, C.R., 2001c. Zinc and cadmium body burdens in terrestrial oligochaetes: use and significance in environmental risk assessment. Environ Toxicol Chem 20, 2067-2072.

Lock, K., Janssen, C.R., 2003. Comparative toxicity of a zinc salt, zinc powder and zinc oxide to *Eisenia fetida*, *Enchytraeus albidus* and *Folsomia candida*. Chemosphere 53, 851-856.

McLaughlin, M.J., Hamon, R.E., Parker, D.R., Pierzynski, G.M., Smolders, E., Thornton, I., Welp, G., 2002. Soil Chemistry. In: Fairbrother, A., Glazebrook, P.W., Tarazona, J.V., Straalen, N.M.v. (Eds.). Test Methods to Determine Hazards of Sparingly Soluble Metal Compounds in Soils. Society of Environmental Toxicology and Chemistry (SETAC), pp. 5-85.

Mohun, T.J., Chambers, A.E., Towers, N., Taylor, M.V., 1991. Expression of Genes Encoding the Transcription Factor Srf during Early Development of *Xenopus Laevis* - Identification of a Carg Box-Binding Activity as Srf. EMBO J. 10, 933-940.

Nota, B., Bosse, M., Ylstra, B., van Straalen, N.M., Roelofs, D., 2009. Transcriptomics reveals extensive inducible biotransformation in the soil-dwelling invertebrate *Folsomia candida* exposed to phenanthrene. BMC Genomics 10, 236.

Nota, B., Timmermans, M.J.T.N., Franken, C., Montagne-Wajer, K., Marien, J., De Boer, M.E., De Boer, T.E., Ylstra, B., Van Straalen, N.M., Roelofs, D., 2008. Gene Expression Analysis of Collembola in Cadmium Containing Soil. Environ. Sci. Technol. 42, 8152-8157.

Nota, B., Verweij, R.A., Molenaar, D., Ylstra, B., van Straalen, N.M., Roelofs, D., 2010. Gene Expression Analysis Reveals a Gene Set Discriminatory to Different Metals in Soil. Toxicol. Sci. 115, 34-40.

Novais, S.C., Arrais, J., Lopes, P., Vandenbrouck, T., De Coen, W., Roelofs, D., Soares, A.M.V.M., Amorim, M.J.B., 2011a. *Enchytraeus albidus* microarray (oligonucleotide): enrichment, design, annotation and database (EnchyBASE) Submitted to Plos One.

Novais, S.C., Gomes, S.I.L., Gravato, C., Guilhermino, L., de Coen, W., Soares, A.M.V.M., Amorim, M.J.B., 2011b. Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures. Environ. Pollut. 159, 1836-1843.

Novais, S.C., Howcroft, C.F., Carreto, L., Pereira, P.M., Santos, M.A.S., De Coen, W., Soares, A.M.V.M., Amorim, M.J.B., 2011c. Differential gene expression analysis in *Enchytraeus albidus* exposed to natural and chemical stressors at different exposure periods. Ecotoxicology, in press.

Novais, S.C., Soares, A.M.V.M., De Coen, W., Amorim, M.J.B., 2012. Differences between Cd and Zn exposure in *Enchytraeus albidus* - linking changes in cellular energy allocation to transcriptional, enzymatic and reproductive responses Submitted to Environmental Pollution.

OECD, 2004. Guidelines for the testing of chemicals. Enchytraeid Reproduction Test. No. 220, OECD (Organization for Economic Cooperation and Development). Paris, France.

OECD, 2010. Guidelines for the testing of chemicals. Bioaccumulation in Terrestrial Oligochaete. No. 317, OECD (Organization for Economic Cooperation and Development). Paris, France.

Owen, J., Hedley, B.A., Svendsen, C., Wren, J., Jonker, M.J., Hankard, P.K., Lister, L.J., Sturzenbaum, S.R., Morgan, A.J., Spurgeon, D.J., Blaxter, M.L., Kille, P., 2008. Transcriptome profiling of developmental and xenobiotic responses in a keystone soil animal, the oligochaete annelid *Lumbricus rubellus*. BMC Genomics 9, 266.

Poynton, H.C., Loguinov, A.V., Varshavsky, J.R., Chan, S., Perkins, E.I., Vulpe, C.D., 2008. Gene expression profiling in *Daphnia magna* part I: Concentration-dependent profiles provide support for the No Observed Transcriptional Effect Level. Environ. Sci. Technol. 42, 6250-6256.

Poynton, H.C., Varshavsky, J.R., Chang, B., Cavigiolio, G., Chan, S., Holman, P.S., Loguinov, A.V., Bauer, D.J., Komachi, K., Theil, E.C., Perkins, E.J., Hughes, O., Vulpe, C.D., 2007. *Daphnia magna* ecotoxicogenomics provides mechanistic insights into metal toxicity. Environ. Sci. Technol. 41, 1044-1050.

Reynders, H., van der Ven, K., Moens, L.N., van Remortel, P., De Coen, W.M., Blust, R., 2006. Patterns of gene expression in carp liver after exposure to a mixture of waterborne and dietary cadmium using a custom-made microarray. Aquat. Toxicol. 80, 180-193.

Rodrigues, S.M., Pereira, M.E., da Silva, E.F., Hursthouse, A.S., Duarte, A.C., 2009. A review of regulatory decisions for environmental protection: Part I - Challenges in the implementation of national soil policies. Environ. Int. 35, 202-213.

Roelofs, D., Marien, J., van Straalen, N.M., 2007. Differential gene expression profiles associated with heavy metal tolerance in the soil insect *Orchesella cincta*. Insect Biochem. Mol. Biol. 37, 287-295.

Sabolic, I., Herak-Kramberger, C.M., Brown, D., 2001. Subchronic cadmium treatment affects the abundance and arrangement of cytoskeletal proteins in rat renal proximal tubule cells. Toxicology 165, 205-216.

Sagripanti, J.L., 1999. DNA damage mediated by metal ions with special reference to copper and iron. Metal Ions in Biological Systems, Vol 36 36, 179-209.

Sheader, D.L., Williams, T.D., Lyons, B.P., Chipman, J.K., 2006. Oxidative stress response of European flounder (*Platichthys flesus*) to cadmium determined by a custom cDNA microarray. Mar. Environ. Res. 62, 33-44.

Shore, P., Sharrocks, A.D., 1995. The Mads-Box Family of Transcription Factors. Eur. J. Biochem. 229, 1-13.

Soetaert, A., Vandenbrouck, T., van der Ven, K., Maras, M., van Remortel, P., Blust, R., de Coen, W.M., 2007. Molecular responses during cadmium-induced stress in *Daphnia magna*: Integration of differential gene expression with higher-level effects. Aquat. Toxicol. 83, 212-222.

Turrens, J.F., 1997. Superoxide production by the mitochondrial respiratory chain. Biosci. Rep. 17, 3-8.

van Straalen, N., Roelofs, D., 2008. Genomics technology for assessing soil pollution. J. Biol. 7, 19.

Vandegehuchte, M.B., De Coninck, D., Vandenbrouck, T., De Coen, W.M., Janssen, C.R., 2010a. Gene transcription profiles, global DNA methylation and potential transgenerational epigenetic effects related to Zn exposure history in *Daphnia magna*. Environ. Pollut. 158, 3323-3329.

Vandegehuchte, M.B., Vandenbrouck, T., Coninck, D.D., De Coen, W.M., Janssen, C.R., 2010b. Can metal stress induce transferable changes in gene transcription in *Daphnia magna*? Aquat. Toxicol. 97, 188-195.

Vandegehuchte, M.B., Vandenbrouck, T., De Coninck, D., De Coen, W.M., Janssen, C.R., 2010c. Gene transcription and higher-level effects of multigenerational Zn exposure in *Daphnia magna*. Chemosphere 80, 1014-1020.

Vandenbrouck, T., Dom, N., Novais, S., Soetaert, A., Ferreira, A.L.G., Loureiro, S., Soares, A.M.V.M., De Coen, W., 2011. Nickel response in function of temperature differences: Effects at different levels of biological organization in *Daphnia magna*. Comparative Biochemistry and Physiology Part D: Genomics and Proteomics 6, 271–281.

Wang, Y.D., Fang, J., Leonard, S., Demchuk, E., Rao, K.M.K., 2004a. Cadmium inhibits the activity of Complexes II and III of respiratory chain and induces ROS production in mitochondria. FASEB J. 18, A1196-A1196.

Wang, Y.D., Fang, J., Leonard, S.S., Rao, K.M.K., 2004b. Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic. Biol. Med. 36, 1434-1443.

Zheng, X.M., Black, D., Chambon, P., Egly, J.M., 1990. Sequencing and Expression of Complementary-DNA for the General Transcription Factor-Btf3. Nature 344, 556-559.

Supporting information

Table S1: Significant differentially expressed transcripts (two sample t-test, $p < 0.05$) in response to cadmium when compared with control from each time of exposure. Only annotated genes with significant $\log_2$ Fold Change values (highlighted) in at least one zinc condition are shown.

		log2FC					
		2 days		4 days		8 days	
Cluster ID	**Blast Homology**	**EC_{50}**	**EC_{90}**	**EC_{50}**	**EC_{90}**	**EC_{50}**	**EC_{90}**
Up-regulated							
EAC00276	10 kda heat shock mitochondrial	0.2856495	0.2931403	0.287091	-0.1805057	0.1380042	0.6122871
EAC01014	40s ribosomal protein	-0.2022694	0.5526377	0.9179239	0.187133	0.4924452	0.411132
EAC00855	accessory gland protein	0.1372022	0.1598141	0.3269684	-0.2065228	0.2831796	0.6649803
EAC00253	actin	0.4044879	1.1485748	1.0380947	0.5054614	0.4032349	0.4035445
EAC00432	actin	-0.3165861	0.603572	0.8553309	0.1774073	0.5950475	0.100775
EAC00459	actin	0.1319214	0.7373161	0.4914344	0.1069547	0.0227435	-0.336202
EAC00362	actin 5c	-0.260769	0.3901617	0.5703624	0.5441118	1.2728225	0.5115406
EAC00681	acyl-coenzyme a short branched chain	0.2091049	0.814277	0.6920461	0.297843	0.5610029	0.4323801
EAC00910	adenosine kinase isoform 2t	-0.0288931	0.6898283	0.4686445	-0.0063404	0.523597	0.1478863
EAC00484	aspartate cytoplasmic	0.0086932	-0.0753891	0.6962843	-0.0115131	-0.3297457	-0.4541953
EAC00665	atp synthase e chain	0.0815694	0.2990144	0.1690439	-0.111433	0.2926699	0.6748758
EAC00748	atp synthase e chain	0.0829317	0.2902589	0.1674481	-0.0881172	0.2928084	0.6844293
EAC00480	atp synthase f0 subunit 6	-0.0878597	0.7990514	0.4231698	0.1813676	0.4754755	0.5793513
EAC00781	atp synthase fo subunit 6	0.0978374	0.6702391	0.3891277	0.4098783	0.6731471	0.8962707
EAC00240	atp synthase lipid-binding mitochondrial precursor	-0.3749092	-0.1706175	-0.3871015	-0.3150797	0.1067642	0.4879923

EAC00102	beta actin	-0.2624313	0.5020893	0.7580341	0.4395243	0.7423472	0.3552407
EAC00936	beta-actin	-0.1579917	-0.0806198	0.4378095	-0.0063205	0.3550429	0.1340059
EAC00580	ca189_mouse ame: full=uncharacterized protein c1orf189 homolog	1.748372	1.5966765	-1.1178327	-0.0052187	-0.220288	0.2002157
EAC00206	centrin	-0.1375905	-0.5418238	0.5501363	0.0261327	-0.5545219	0.7263295
EAC00546	centrin	-0.4302076	-0.7402649	0.2618171	-0.1564515	-0.4863024	0.7601684
EAC00684	coiled-coil-helix domain-containing protein mitochondrial	0.0279992	-0.1969315	-0.0505619	-0.0682116	0.0033311	0.5546458
EAC00311	cysteine-rich protein 1	-0.3178308	-0.5048659	1.153681	1.1143004	-0.0122848	-0.240015
EAC00321	cytochrome b	0.502849	0.978023	0.6284185	0.0301058	0.5634051	0.9016902
EAC00364	cytochrome b	0.2885785	0.7576629	0.6605224	0.1207167	0.6655078	0.8938657
EAC00769	cytochrome c oxidase polypeptide vic precursor	0.2711879	0.5464434	0.6122951	-0.1942883	0.3603391	0.7211777
EAC00470	cytochrome c oxidase subunit iii	-0.1693867	0.7391203	0.6182338	0.4241376	0.5791762	0.2130673
EAC00155	cytochrome oxidase subunit 2	0.3209167	1.2607017	0.6229879	-0.1078172	0.493808	0.9298788
EAC00225	cytochrome oxidase subunit ii	-0.2247558	0.8149138	0.2717662	0.1962368	0.5972348	0.393165
EAC00671	dpy-30 like protein	-0.2272858	0.3909735	1.2776081	0.1584427	0.4283688	0.3036782
EAC00229	elongation factor 1-alpha	-0.1685411	0.7527099	0.6008089	0.4231064	0.5961892	0.2171392
EAC00638	endonuclease-reverse transcriptase	1.0072225	0.8796746	0.742077	0.1614447	-0.2040539	-0.3636462
EAC00816	endonuclease-reverse transcriptase	0.2888661	0.2390824	0.8931024	-0.1970952	0.7431889	0.4001026
EAC00481	heat shock protein 60	-0.0346026	0.4167539	0.66083	0.1939834	0.5149628	0.4456108
EAC00316	heat shock protein 8	-0.1274077	-0.2459575	-0.0068307	0.6107241	0.040016	0.0214293
EAC00119	hypothetical protein Bm1_07595 [Brugia malayi]	-0.1711146	0.7532202	2.1889804	2.0081708	0.5976408	0.213029
EAC00710	hypothetical protein TGME49_026570 [Toxoplasma gondii ME49]	-0.0519345	0.6762947	0.8559281	0.2164119	0.5174684	0.413512
EAC00516	kif21a protein	-0.2150797	0.6069869	0.8291787	0.1676806	0.5217635	0.3911339
EAC00283	mflj00348 protein	-0.1788108	0.745311	2.4930718	2.3258577	1.4601474	1.0875379
EAC01023	myosin light chain 2	-0.7218275	-0.3367122	0.4252023	0.9049884	0.2937007	-0.1758533
EAC00259	myosin regulatory light chain	-0.0145017	0.1676174	-0.4677416	-0.1614536	0.0277741	0.4883969
EAC00122	myosin regulatory light nonmuscle	-0.6351983	-0.2784526	0.533233	0.8416293	0.3059599	-0.1458869
EAC00775	nadh dehydrogenase	-0.0690718	0.0107815	-0.0016213	0.0755014	0.2018172	0.5570097
EAC00842	nadh dehydrogenase subunit 1	-0.1650172	0.5750538	0.583607	0.1497796	0.6287499	0.6357191
EAC00216	nadh dehydrogenase subunit 5	0.5473305	0.7160699	-0.0918159	-0.2973069	0.4496152	0.9615015
EAC00677	poly -specific ribonuclease (deadenylation nuclease)	-0.177816	0.6459337	0.7148346	0.3208	0.3387015	0.3202583
EAC00809	protein	-0.1854337	0.6022039	0.7359196	0.182394	0.6645599	0.5706677
EAC00734	ribosomal protein l35a	0.4167743	1.4843713	0.8781143	0.676992	0.552366	0.2729487
EAC00685	ribosomal protein l37a	-0.3095485	0.1219646	0.5335368	-0.3291999	0.3880288	0.6215843
EAC00719	small nuclear ribonucleoprotein sm d2	0.4464422	0.4660342	0.1750915	-0.1768076	0.183681	0.6274905
EAC00994	translation elongation factor 1-alpha	0.5577427	0.2763862	0.0374181	-0.7681494	-0.0852564	-0.2209254
EAC00325	xk-related protein 6	-0.1140787	0.6299645	1.1214038	0.2324823	0.3468012	0.2107886
EAC00880	yipee zinc-binding protein	-0.1704013	0.6290664	0.9147688	0.0951174	0.6198215	0.3029797
Up- and Down-regulated							
EAC00829	elongation factor-1 alpha	1.3152	0.9355731	0.2519989	-0.1768593	0.4933822	-0.9150968
EAC00214	protein	-0.3008675	-0.8447576	1.0650408	0.9881547	-1.0572904	-0.7847345
EAC00431	vesicle-associated protein (vap-1)	-0.1666378	-0.4798186	-0.0820298	0.8472114	0.5676825	0.2036639
Down-regulated							
EAC01052	60s ribosomal protein l10a	-0.3283308	-0.0850627	-0.2279678	0.091507	-0.0626848	0.3063598
EAC00456	a chain low resolution structures of bovine mitochondrial f1-atpase	-0.5664444	-0.8427315	-2.1900607	0.1501264	-0.5316757	0.1832256
EAC00131	actin	-0.1993916	-0.626803	-0.6869619	0.1978788	-0.0547811	0.0631785
EAC00139	actin	-0.5802981	-0.7153508	-0.4250521	0.0375909	0.3152176	0.0974712
EAC00184	actin	-0.779278	-0.4799797	-0.4749575	-0.1141047	-0.4128739	-1.0033942
EAC00273	actin	-1.3545034	-0.9557002	-0.4027367	0.5043395	-0.4678105	0.6734387
EAC00281	actin	-0.3855183	-0.7781483	-0.8676829	0.2935037	-0.3411203	0.0120033
EAC00079	actin 1	-0.372839	-0.4782089	-0.5245411	0.2135173	-0.4866424	-0.0876135
EAC00396	actin related protein 2 3 subunit 41kda	-0.6238931	-1.0633233	-0.3638997	-0.1647429	-0.2492185	-0.5740871
EAC00006	actin-related protein 2	-0.225552	-0.4547455	-0.2351325	0.4942087	-0.5350817	-0.6741177
EAC00354	alpha tubulin	-0.3494105	-0.4172667	-1.7508239	-0.1305211	-0.2530731	0.511486
EAC00199	atp synthase subunit mitochondrial precursor	-0.3065603	-0.2677969	-0.9531083	0.2779225	-0.1600736	0.3800249
EAC00421	b chain structural basis for giant hemoglobin	-0.397605	-0.5825582	-0.5379026	-0.1802038	0.0500744	0.111079
EAC00244	beta-actin	-0.7042753	0.4044782	0.4590063	-0.3126944	0.1400127	-0.1942232
EAC00444	beta-actin	-0.3042853	-1.1503642	0.9384203	1.2889792	-0.3680526	-0.7775321
EAC01012	beta-actin	-0.0674208	-0.5058004	-0.5917983	-0.1138361	-0.3274824	0.2514466
EAC01056	beta-actin	-0.1756398	-1.0764197	-1.1217106	-0.3706789	-0.327369	0.0955453
EAC00899	beta-tubulin 13	-0.1515036	-1.1080827	-1.0664814	-0.3144232	-0.2405508	0.1157146
EAC00305	calponin homolog	-0.3686035	-0.6757325	-1.2992158	-0.301051	-0.4274333	0.0711772
EAC01033	calponin homolog	-0.282203	-0.6061292	-0.0768509	0.2189229	-0.0942436	-0.3279399
EAC00222	cathepsin b	-0.5158296	-0.8133096	-1.5028762	-0.4749262	-0.4169374	0.186727
EAC00198	chk1 checkpoint-like protein	-0.2774957	-1.2907794	-0.4991376	-0.0748765	-0.6375773	-0.2048255
EAC00525	chromobox protein homolog 3	-0.2555733	-0.121993	0.6423547	0.0543309	-0.9075567	-1.0978229
EAC00267	chymotrypsinogen b1	-0.4092122	-0.2945783	0.2057715	-0.5534978	-1.2110816	-1.7583309
EAC00042	cysteine-rich motor neuron 1 protein precursor	-0.2875376	-1.13563	0.3534175	0.3196608	-0.1115258	-1.0162451
EAC00415	cysteine-rich motor neuron 1 protein precursor	-1.1931558	-0.2194226	-2.2162852	-0.7592057	-1.0186752	-0.726294
EAC01004	cytochrome b	-0.1160814	-0.9867982	-0.898857	-0.3344904	-0.3716085	0.0802074
EAC00958	cytochrome c oxidase subunit 1	-0.1123619	-1.014045	-0.9754719	-0.2698764	-0.4062301	0.1090791
EAC00356	cytochrome c oxidase subunit via polypeptide 1	-0.4747913	-0.4458948	-0.5414358	0.191907	-0.1555386	-0.1325309
EAC00371	cytoplasmic intermediate filament protein	-0.2194607	-0.7461824	-0.3940051	1.3065747	-0.4905394	-0.5527574
EAC00429	cytoplasmic intermediate filament protein	0.4683112	-0.6722667	-0.3005877	-0.3417838	-0.3106813	-0.2587522
EAC00863	dual oxidase 2	0.0269837	-0.7093294	-0.8104844	-0.655958	-0.6150563	-0.0513232
EAC01114	egf-like domain-containing protein	-0.0791911	-1.0058674	-0.9777405	-0.2134088	-0.4667017	0.1022925
EAC01079	eukaryotic translation initiation factor subunit e	-0.2989121	-1.0629745	-1.3736996	-0.4122152	-0.6857481	-0.1104336

EAC00117	glyceraldehyde-3-phosphate dehydrogenase	-0.5312217	-0.6056584	-0.5368408	-0.0386978	-0.5033651	-0.3842054
EAC00903	glyceraldehyde-3-phosphate dehydrogenase	-0.6669614	-0.6279247	-0.5888312	0.0354211	-0.5024496	-0.3266847
EAC01005	glycine n-methyltransferase	-0.1105229	-1.1292724	-1.0421085	-0.3562313	-0.3595391	0.0967684
EAC00543	heat shock protein 70	-0.2906626	-0.7065549	-0.6588283	-0.171352	-0.3912982	-0.3661929
EAC01057	heat-responsive protein 12	-0.7138081	-0.7534503	-0.2116106	0.2040666	-0.2603442	-0.2236453
EAC00127	hemoglobin c chain percursor	-1.0684156	-0.4806398	0.3843281	0.3350661	-0.4186867	-0.7796321
EAC00035	hemoglobin c chain precursor	-1.0864527	-0.5739427	0.3639238	0.3012523	-0.3863748	-0.783954
EAC00398	hemoglobin c chain precursor	-0.7735344	-0.0774233	-0.5300751	-0.264342	-0.4216913	-0.3491955
EAC00405	histone h2a	0.8894936	0.599682	-0.6023537	-0.2443599	-0.1370139	0.3801226
EAC00577	homolog precursor	-0.2976362	-1.046384	-0.6305766	-0.3444686	-0.1610997	-0.0291544
EAC00414	hypothetical protein MELLADRAFT_91365	-0.0525518	-0.9684613	-0.3165607	0.2491394	-0.6649535	-0.7084553
EAC00363	ifa-1	-0.5109045	-1.111589	0.3888972	0.4729909	-0.2338302	-0.8357114
EAC00181	intermediate filament protein	-0.5543126	-1.1941931	0.583519	0.7189316	-0.3252427	-0.9941138
EAC00290	lysozyme	-0.3696403	-0.1629693	0.4381017	-0.7255802	-1.4925452	-1.3149264
EAC00895	mads flc-like protein 2	-0.9270105	-0.7726322	-0.8794782	-0.6266681	-0.7000318	-0.8496032
EAC00932	mads flc-like protein 2	0.850774	0.5524634	-0.5856859	0.1224548	-0.054732	0.2688719
EAC01006	mads flc-like protein 2	-0.2906983	-1.3764114	-1.3841374	-0.7695224	-1.0300026	-0.4228475
EAC01031	mads flc-like protein 2	-0.0781323	-0.8959212	-0.9973977	-0.313702	-0.3166548	0.0862672
EAC01044	mads flc-like protein 2	-0.0908749	-0.9606604	-1.1447361	-0.3455853	-0.2395923	0.1694654
EAC01053	mads flc-like protein 2	-0.1183669	-0.6979183	-0.9512447	-0.0249211	-0.343378	0.0771461
EAC01076	mads flc-like protein 2	-0.1755284	-0.9278064	-1.0553124	-0.241961	-0.315417	0.1354747
EAC01077	mads flc-like protein 2	-0.1910121	-1.1253082	-1.0205595	-0.152327	-0.315718	0.1204135
EAC01082	mads flc-like protein 2	-0.1657327	-1.2448461	-0.9720723	-0.1718935	-0.626401	0.0252581
EAC01109	mads flc-like protein 2	-0.1396492	-1.1107386	-1.0943922	-0.1546848	-0.3975932	0.0631466
EAC00777	mgc107908 protein	-1.404067	-1.0332336	-0.0792978	-0.4744209	-1.8562497	-0.6775374
EAC00920	mgc80370 protein	-0.0187283	-1.1090891	-1.0385376	-0.1039864	-0.8020848	-0.0165646
EAC00103	mitochondrial atp synthase gamma subunit	-0.2069862	-0.8975155	-0.2133631	0.0138175	-0.6392558	-0.3149849
EAC00074	myosin heavy chain	-0.3129499	-1.2244097	-0.9297175	-0.3770687	-0.1131037	0.0801977
EAC01025	myosin heavy chain	-0.090882	-1.2337678	-1.1419438	-0.4712243	-0.551774	-0.0154846
EAC01080	myosin heavy chain	-0.141959	-1.212471	-0.9714526	-0.193466	-0.4763563	0.0318136
EAC01089	myosin heavy chain	-0.1520227	-0.81588	-1.1573074	-0.3378987	-0.2390767	0.138848
EAC01091	myosin heavy chain	-0.1188189	-0.9484934	-1.0055035	-0.1458171	-0.3616924	0.1126756
EAC01093	myosin heavy chain	-0.1937251	-1.1421574	-0.9955329	-0.3968946	-0.3386318	0.0754703
EAC01113	myosin heavy chain	-0.0856811	-1.0060695	-1.0905541	-0.1292544	-0.2765968	0.0307461
EAC01119	myosin heavy chain	-0.1922674	-1.0531423	-1.2193483	-0.3431834	-0.2285033	0.0926099
EAC01122	myosin heavy chain	-0.0655096	-1.3889104	-1.046952	-0.0041438	-0.8926768	-0.0641011
EAC00558	myosin regulatory light polypeptide 9	-0.3645916	-1.250089	-1.351102	-0.4040562	-0.6239066	-0.4002983
EAC00636	neutral and basic amino acid transport protein rbat	0.4480184	0.4913772	-0.727246	-0.1966714	-0.4208872	0.4627601
EAC00787	novel rna helicase family protein	0.457086	-0.0735408	-0.6404845	0.263985	-0.6979892	0.3166114
EAC00403	orf2-encoded protein	0.6223601	0.3829151	0.1548936	0.1045589	-0.3161082	-0.8352154
EAC00440	probable maltase l-like	-0.3457188	-1.1393311	-2.0984402	-0.5238478	-1.0364834	-0.3036884
EAC00508	ran-specific gtpase-activating protein	-0.3362861	-0.5028575	0.0784674	0.012978	-0.3901389	-0.542546
EAC00200	ribosomal protein s16	-0.2925494	-0.0849141	-0.5290006	0.0501979	-0.2724518	0.0445483
EAC00298	ribosomal protein s16	0.1989247	0.5258856	-0.1670898	-0.1710065	-0.3730965	0.2422635
EAC00143	ribosomal protein s7	-0.4950995	-0.4861744	0.5327435	0.2227071	-0.1399266	-0.1435836
EAC00159	sb:cb283 protein	-0.2977355	-0.9703197	-1.0508178	-0.1719586	-0.2605334	0.0680897
EAC01124	sb:cb283 protein	-0.1416529	-0.9294478	-1.0785631	-0.3413696	-0.3052014	0.1012772
EAC01038	scbp2 protein	-0.4759002	-0.6665928	-0.3423765	-0.0072153	-0.229296	-0.0778271
EAC00969	scbp3 protein	-0.1831766	-1.0227116	-1.0769759	-0.3664612	-0.2700042	0.026232
EAC01084	scbp3 protein	-0.038619	-1.0400458	-1.2188972	-0.3725391	-0.4081452	-0.0067245
EAC00554	short-chain dehydrogenase	-0.1100406	-0.008239	-0.7577951	0.0416777	-0.3510393	-0.5207581
EAC00503	thymosin isoform 2	-0.2330262	-0.8006442	-0.4483963	-0.1297657	-0.3261708	0.2310881
EAC01097	transcription factor btf3 homolog 4	-0.0843863	-1.0924691	-1.009013	-0.2367738	-0.1476426	0.1395091
EAC00207	transketolase-like 2	-0.9278214	-1.1654736	0.3740424	0.5424904	-0.9831148	-0.8495805
EAC00197	translationally controlled tumer rpotein	-0.5886511	-0.7498591	0.5689824	0.1238746	-0.415975	-0.7191872
EAC00637	transmembrane and coiled-coil domains	-0.1360885	-0.8138851	-0.4369155	0.0828253	-0.2249686	0.2132814
EAC00221	transport protein sec61 subunit beta	-0.2042007	-0.4882046	-0.1272625	0.3184206	-0.0899297	-0.1701461
EAC00115	tropomyosin	-0.5133458	-0.6232963	-0.5411291	0.3079166	-0.2896787	-0.2948305
EAC00224	tropomyosin	-0.1505772	-0.314394	-0.7762297	-0.1628728	-0.1525182	0.2472494
EAC00327	tropomyosin	-0.2195619	-0.5721073	-0.1563693	-0.090554	-0.0228871	0.1694376
EAC01110	tropomyosin	-0.4104629	-0.5484916	-0.8973357	-0.2999067	-0.2259748	0.1051285
EAC00340	troponin t	-0.5039741	-1.1952686	0.4283907	0.3693702	-0.6362883	-1.0816487
EAC00390	type alpha 1	-0.6204306	-1.558842	-0.9496938	0.0814068	-0.674239	-0.5945849
EAC00831	u6 snrna-associated sm-like protein lsm3	0.081968	-0.2520302	-1.1243074	-0.0309201	-0.5829495	-0.2479649
EAC00306	ubiquitin c	0.2857799	-0.205315	-1.3527848	-0.6112372	-0.6445647	0.1871665
EAC00148	universal minicircle sequence binding protein	-0.2601193	-0.7130876	-0.9559824	-0.1079336	-0.6205201	-0.4359317
EAC00835	uromodulin precursor	-0.0486336	-0.0626123	0.8249384	0.077563	-0.4097287	-0.8223153

Table S2. Genes validated with quantitative RT PCR. (-) not tested

Blast Homologyy	**Cluster ID**	**Cd EC50**			**Cd EC90**			**Zn EC50**			**Zn EC90**		
		MA	**qPCR**	**± SE**	**MA**	**qPCR**	**± SE**	**MA**	**qPCR**	**± SE**	**MA**	**qPCR**	**± SE**
chk1 checkpoint-like protein	EAC00198	-0.277	-0.014	0.002	-1.291	-0.113	0.011	-	-	-	-	-	-
cytochrome b	EAC01004	-	-	-	-	-	-	0.420	0.692	0.034	0.553	-0.019	0.033
ribosomal protein l15	EAC00331	-	-	-	-	-	-	-0.511	-0.962	0.082	-2.724	-0.724	0.086
mads flc-like protein 2	EAC01031	-0.078	-1.597	0.058	-0.896	-0.779	0.077	0.440	0.890	0.240	0.547	0.693	0.187
myosin heavy chain	EAC01089	-0.152	-1.234	0.092	-0.816	-0.478	0.097	0.392	-0.109	0.034	0.468	0.285	0.105
atp synthase f0 subunit 6	EAC00480	-0.087	0.556	0.176	0.799	0.787	0.233	0.716	0.586	0.163	-0.437	-0.298	0.053

Table S3: Significant differentially expressed transcripts (two sample t-test, $p < 0.05$) in response to zinc when compared with control from each time of exposure. Only annotated genes with significant log_2 Fold Change values (highlighted) in at least one zinc condition are shown.

		log2FC					
		2 days		4 days		8 days	
Cluster ID	**Blast Homology**	**EC_{50}**	**EC_{90}**	**EC_{50}**	**EC_{90}**	**EC_{50}**	**EC_{90}**
Up-requlated							
EAC00269	60s ribosomal protein l7	1.32771	0.35794	-0.3269	-0.1521	0.8667	1.18423
EAC00254	actin	-0.5451	-0.3652	0.22717	0.77581	-0.38	-0.3214
EAC00483	actin	-0.0308	0.23802	0.85232	0.55368	0.73276	0.51948
EAC00867	aquaporin tandem duplicate 2	0.10044	0.49004	1.01792	0.7843	0.4381	0.47079
EAC00480	atp synthase f0 subunit 6	0.71574	-0.4367	-0.4541	0.21212	0.21041	0.46059
EAC00781	atp synthase f0 subunit 6	1.00204	-0.6842	1.1691	1.74675	0.52487	1.0596
EAC00421	b chain structural basis for giant hemoglobin	0.45559	-0.0645	0.37702	0.24726	0.95207	0.61474
EAC00244	beta-actin	-0.4269	-0.5172	0.46266	0.26709	-0.4793	-0.3919
EAC00498	beta-actin	0.22738	-0.3332	-0.287	0.05632	0.40708	0.33974
EAC00936	beta-actin	0.65896	-0.3259	0.18681	0.67155	0.63997	0.55123
EAC00473	chaperonin containing subunit 2	-0.423	0.48867	0.49642	0.29104	-0.2118	-0.2096
EAC00468	conserved protein	0.49698	-0.2309	0.82406	0.71728	0.96315	0.8509
EAC00193	cytochrome b	0.9789	-0.2889	0.36297	0.5471	0.33328	0.78184
EAC00321	cytochrome b	0.917	-0.3346	-0.1274	0.48109	0.60498	0.95419
EAC00364	*cytochrome b*	0.69515	-0.3839	-0.071	0.67751	0.51289	0.76357
EAC01004	cytochrome b	0.42034	0.55281	1.02187	0.74973	0.80655	0.89906
EAC00958	cytochrome c oxidase subunit 1	0.39762	0.51942	0.82452	0.56365	0.77174	0.8754
EAC00155	cytochrome oxidase subunit ii	0.81008	-0.5849	-0.6086	0.16768	0.47786	0.73742
EAC00429	cytoplasmic intermediate filament protein	0.04206	0.55514	-0.0769	-0.4584	0.11349	-0.3627
EAC00863	dual oxidase 2	-0.3846	0.35411	0.54509	0.12556	0.26409	0.04184
EAC01114	egf-like domain-containing protein	0.39694	0.49972	0.81182	0.59533	0.75215	0.83611
EAC00816	endonuclease-reverse transcriptase	0.19341	0.38194	-0.0335	0.39409	0.74261	-0.26
EAC01005	glycine n-methyltransferase	0.3755	0.47639	0.71028	0.52903	0.75027	0.83348
EAC00405	histone h2a	-0.1663	0.87205	0.51113	0.73378	0.07781	0.14823
EAC00234	integrin alpha-2	-0.4117	0.59203	0.68747	-0.0859	0.59039	0.00608
EAC01115	kazal-type serine protease inhibitor domain-containing protein 1-like	0.04245	0.51874	0.62125	0.40586	0.14404	0.15799
EAC00928	mads flc-like protein 2	0.3611	0.45484	0.70217	0.54518	0.77949	0.85091
EAC00929	mads flc-like protein 2	0.05045	0.49941	0.61558	0.42188	0.17136	0.16277
EAC00932	mads flc-like protein 2	-0.1193	1.06905	1.03661	0.31516	-0.3078	-0.4271
EAC01006	mads flc-like protein 2	0.43969	0.54677	0.89283	0.67028	0.70951	0.82411
EAC01044	mads flc-like protein 2	0.38882	0.48917	0.73719	0.58948	0.80442	0.86076
EAC01053	mads flc-like protein 2	0.41805	0.45515	0.90138	0.5763	0.77759	0.82489
EAC01076	mads flc-like protein 2	0.35131	0.4033	0.73993	0.52116	0.72882	0.83053
EAC01092	mads flc-like protein 2	-0.0407	0.0954	0.32236	0.5193	0.13103	0.02696
EAC00920	mgc80370 protein	0.43078	0.61266	0.83895	0.55553	0.87274	0.95339
EAC01080	myosin heavy chain	0.41566	0.49305	0.96385	0.6492	0.74655	0.84288
EAC01089	myosin heavy chain	0.39168	0.46836	0.72868	0.53001	0.71867	0.81053
EAC01091	myosin heavy chain	0.35387	0.45691	0.86498	0.63789	0.7679	0.84799
EAC01113	myosin heavy chain	0.44659	0.4687	0.68213	0.54213	0.75037	0.78
EAC00259	myosin regulatory light chain	0.3411	0.14478	0.02623	0.6193	0.42513	0.51016
EAC00558	myosin regulatory light polypeptide 9	-0.3267	-0.0877	0.81531	1.03636	0.10578	-0.2687
EAC00216	nadh dehydrogenase subunit 5	0.63306	-0.5496	0.04055	0.65768	0.67876	0.80312
EAC00822	neurocalcin-like protein	1.14481	0.27603	0.46374	0.7812	1.05217	0.62435
EAC00351	neuronal calcium sensor 2	-0.2897	-0.6453	0.15142	0.79636	-0.5154	-0.7577
EAC00805	nicotinic acetylcholine receptor alpha6 subunit	0.35335	0.13371	1.63439	1.21148	0.63777	0.26322
EAC00692	odulin family member (cmd-1) partial	0.24419	-0.1095	0.41054	1.03929	0.55382	0.30549
EAC01086	phosphoglycerate kinase	0.64879	0.97709	1.21378	0.72141	0.01203	0.14451
EAC00619	PREDICTED: hypothetical protein [Homo sapiens]	-0.3197	0.24578	0.62242	-0.1406	-0.0277	-0.1205
EAC00440	probable maltase I-like	-0.6646	0.54784	1.37451	0.77523	0.26177	-0.2038
EAC00927	proteosome subunit alpha type-4	-0.6343	-0.201	0.30844	0.39917	-0.6524	-0.5251
EAC00280	ribosomal protein l19	-0.0173	-0.0965	0.43894	1.11776	0.39602	0.20585
EAC00583	ribosomal protein l26	-0.1165	-0.2996	0.44987	0.81774	0.20023	0.12943
EAC00734	ribosomal protein l35a	1.07424	0.45625	-1.1247	-0.4755	0.24926	-0.1615
EAC00408	scbp2 protein	-0.1621	-0.4386	-0.0328	0.56437	0.00286	-0.1159
EAC01038	scbp2 protein	0.20756	-0.1663	0.32886	0.86136	0.39538	0.21958
EAC00045	scbp3 protein	-0.0489	-0.2513	0.21513	0.94966	-0.1426	-0.3218
EAC00120	scbp3 protein	0.05434	-0.0169	0.33503	1.12039	0.08524	-0.0156
EAC01045	scbp3 protein	-0.0129	-0.2327	0.20706	0.86276	-0.2173	-0.2323
EAC00554	short-chain dehydrogenase	-0.1793	-0.0177	0.31574	-0.0877	0.3533	0.04854
EAC00503	thymosin isoform 2	-0.0688	-0.2183	0.42272	0.94827	0.42464	0.41085
EAC01097	transcription factor btf3 homolog 4	0.39743	0.49977	0.89539	0.69417	0.74917	0.8171
EAC00224	tropomyosin	-0.1668	-0.0938	0.39635	0.34381	0.29509	0.10799
EAC00327	tropomyosin	0.04762	-0.2913	0.39258	1.00515	0.41607	0.13759
EAC00545	tropomyosin	-0.6369	-0.4006	0.21788	0.74502	-0.2718	-0.3857
EAC01110	tropomyosin	0.17409	0.15022	1.11573	1.05462	0.42146	0.30326

EAC00831	u6 snrna-associated sm-like protein lsm3	0.23531	0.02691	0.89708	0.55368	-0.1191	-0.2568
EAC00675	upf0631 protein c17orf108-like	0.23808	0.39499	0.95578	0.05107	0.04401	-0.488
Up- and Down-regulated							
EAC00855	accessory gland protein	0.37799	-0.2978	-0.5326	0.33821	0.40119	0.27303
EAC00419	beta-actin	-0.6694	-0.0406	0.99201	0.19623	-0.6374	-0.7363
EAC00580	uncharacterized protein c1orf189 homolog	0.54212	1.78072	1.21906	1.40314	-0.4966	-0.6011
EAC00471	endonuclease-reverse transcriptase	-0.4554	0.3751	0.75778	-0.1818	-0.4467	-0.6149
EAC00543	heat shock protein 70	-0.9023	-0.7272	1.2368	0.16725	-0.2809	-0.0685
EAC00959	hypothetical protein	-0.2637	0.16488	0.77081	0.42627	-0.1399	-0.441
EAC00777	mgc107908 protein	1.60782	0.15858	-0.5429	0.62212	-0.1258	-0.7315
EAC00589	microtubule-associated protein tau	0.39397	1.23599	1.16481	1.01816	-0.6952	-0.4313
EAC00634	proteasome isoform a	-0.5997	0.20224	0.9279	0.20744	-0.1925	-0.5208
EAC00372	purine nucleoside phosphorylase	-0.6781	0.68523	0.88344	-0.8675	-0.067	0.18253
EAC00491	subfamily member 6	0.08019	1.51406	1.11392	0.79386	-0.5848	-0.5887
EAC00130	tubulin alpha-1 chain	-0.5138	-0.477	0.51951	1.14122	-0.377	0.18802
EAC00390	type alpha 1	-0.9346	0.65427	1.39978	0.3398	0.51637	0.20508
EAC00148	universal minicircle sequence binding protein	-0.4342	-0.1133	0.53463	0.07154	-0.0631	-0.3818
EAC00592	zinc finger mym-type protein 1-like	-0.1779	0.70338	0.19081	-0.4902	0.23107	-0.3035
Down-regulated							
EAC00457	26s protease regulatory subunit 8	-0.5324	-0.802	0.15728	-0.6791	-0.5687	-0.6544
EAC00226	40s ribosomal protein s4	-0.4899	-0.3129	-1.0849	-0.7287	-0.641	-0.8117
EAC00040	actin	-0.0778	-0.1963	-0.7296	0.32782	-0.1573	-0.0227
EAC00090	actin	-0.287	-0.5307	-1.3883	-1.1279	-0.7515	-0.6192
EAC00110	actin	-0.3861	-0.7598	-1.5263	-1.2479	-0.7781	-0.5974
EAC00165	actin	-0.5977	-0.7412	-1.1201	-0.8297	-0.6843	-0.6153
EAC00184	actin	-1.2957	-0.1602	-0.0572	-0.6736	-1.1982	-1.2214
EAC00220	actin	-0.2966	-0.5217	-1.1405	-0.9556	-0.665	-0.5866
EAC00273	actin	-0.7849	-1.2752	-1.2616	0.01578	-0.0522	0.37111
EAC00300	actin	-0.5665	-0.5454	-1.1488	-0.8762	-0.4929	-0.5078
EAC00442	actin	-0.3794	-0.2802	-0.9816	-0.7285	-0.4873	-0.5571
EAC00006	actin-related protein 2	-0.9883	-0.448	0.07237	-0.4661	-0.2779	-0.6489
EAC00910	adenosine kinase isoform 2t	0.04658	-0.0255	-0.0312	-0.9059	-0.2755	-0.1143
EAC00147	alpha smooth aorta	-0.5138	-0.8744	-1.0916	-0.9377	-0.6345	-0.4709
EAC00099	alpha-sarcomeric	-0.9024	-0.5143	-0.3215	-0.1758	-0.8011	-0.8351
EAC00511	atp synthase subunit mitochondrial	-1.111	-0.8196	-0.3777	-0.2727	-0.6178	-0.8001
EAC00326	atp:adp antiporter	-0.7852	-0.8054	-0.8256	-0.6629	-0.1494	-0.2737
EAC00102	beta actin	-0.1372	-0.4762	-1.0401	-0.6854	-0.3051	0.35953
EAC00282	beta tubulin	0.0448	0.43894	0.13547	-0.1645	-0.21	-0.5961
EAC00485	beta tubulin	-1.0534	-0.6559	-0.04	-0.0985	-0.6533	-0.7303
EAC00182	beta-actin	-0.3253	-0.5159	-1.4771	-1.1208	-0.6644	-0.5537
EAC00444	beta-actin	-1.3266	-0.8152	-0.5263	-1.6801	-0.5102	-1.0944
EAC00881	btb poz domain-containing protein	-0.1398	-0.3199	0.15407	-0.8279	-0.084	0.14595
EAC00100	calponin homolog	-0.4981	-0.4092	-0.5679	-0.5002	-0.4839	-0.4483
EAC00286	carboxypeptidase a	0.00033	-1.6077	-0.823	0.61537	-0.3511	-1.5404
EAC00206	centrin	0.45983	-1.2587	-1.0092	0.63688	0.00429	-0.1455
EAC00546	centrin	0.28211	-1.4529	-1.0432	0.60678	0.03237	0.05632
EAC00267	chymotrypsinogen b1	-0.6391	-0.76	-0.2766	-1.0667	-0.8005	-1.7404
EAC00475	collagen alpha-1 chain	-0.0839	0.10309	-0.1597	0.18811	-0.4496	-0.3545
EAC00769	cytochrome c oxidase polypeptide vic precursor	0.48826	-0.3442	-0.7831	0.11915	0.44478	0.31659
EAC00470	cytochrome c oxidase subunit iii	0.20996	-0.4921	-0.5076	-0.1228	0.21037	0.27666
EAC00225	cytochrome oxidase subunit ii	0.3486	-0.7314	-0.6115	-0.0404	0.32655	0.38164
EAC00079	cytoplasmic 2	-0.613	-0.4402	-0.2163	-0.3118	-0.0147	-0.2984
EAC00371	cytoplasmic intermediate filament protein	-1.1318	-0.037	0.52086	0.06216	-0.5196	-0.6888
EAC00111	elastase pancreatic	-0.4258	-1.5776	-0.8306	-0.3125	-0.724	-1.0565
EAC00229	elongation factor 1-alpha	0.21202	-0.4846	-0.5236	-0.1078	0.21264	0.28798
EAC00202	elongation factor-1 alpha	-0.4621	-0.3827	-1.0913	-1.0405	-0.7264	-0.6193
EAC00638	endonuclease-reverse transcriptase	0.54312	0.78513	-0.809	-1.355	-0.1373	-0.2251
EAC00261	extracellular hemoglobin linker l4 subunit precursor	-6.0382	-1.2749	-1.9348	0.71056	-2.2386	-0.8096
EAC00265	glycoside hydrolase family 30	-0.6494	-0.8752	-0.1969	-0.8398	-0.3217	-0.1682
EAC00097	heat shock protein gp96	-0.3281	-0.63	0.17495	-0.7716	-0.2144	-0.2904
EAC01057	heat-responsive protein 12	-0.1141	-0.1091	-1.0762	0.25075	0.27907	-0.1659
EAC00035	hemoglobin c chain precursor	-0.7108	-1.1755	-1.273	-0.9878	-1.0315	-0.7912
EAC00127	hemoglobin c chain precursor	-0.6665	-1.104	-1.2793	-1.0763	-1.0937	-0.716
EAC00398	hemoglobin c chain precursor	-0.1281	-0.4989	-0.0036	0.33073	-0.395	-0.1364
EAC00627	histone	-0.2679	0.69341	0.21802	-0.0118	-0.1419	-0.3845
EAC00532	homogentisate -dioxygenase	-0.5246	-0.1863	0.63031	0.35169	-0.1877	-0.6874
EAC00414	hypothetical protein MELLADRAFT_91365	-0.7003	0.11046	0.64589	-0.0707	-0.2017	-0.7393
EAC00363	ifa-1	-1.0203	-0.5542	0.1482	-0.3715	-0.6039	-0.7728
EAC00181	intermediate filament protein	-1.1151	-0.6093	-0.0875	-0.5729	-0.6972	-0.9455
EAC00999	intermediate filament protein	-0.7455	-0.8169	-0.7531	-0.3901	-0.6726	-1.1748
EAC00397	keratinocyte associated protein 2	-0.6532	-0.797	-0.2922	-0.4356	-0.3267	-0.1377
EAC00516	kif21a protein	0.17223	-0.1443	0.3574	-0.5167	0.01298	0.06108
EAC00324	lysozyme	0.37838	-0.2961	-0.7013	0.71413	0.09146	-0.6321
EAC00895	mads flc-like protein 2	-0.5058	-0.5525	0.33315	-0.0854	-0.7978	-0.5082
EAC01027	mads flc-like protein 2	-0.3164	-1.0444	-1.1969	-0.8086	-0.599	-0.1764
EAC00283	mflj00348 protein	0.20896	-3.3229	-3.6006	-3.3756	1.07571	1.15048

EAC00656	mitochondrial ribosomal protein l48	-0.782	-0.4051	-0.0303	0.57797	-0.0015	0.11328
EAC00088	myosin heavy chain	-0.3084	-0.8344	-0.6766	-0.7574	-0.6234	-0.4582
EAC00108	myosin heavy chain	-1.0605	-0.9359	-0.2922	-0.5312	-0.8808	-1.0117
EAC01023	myosin light chain 2	-0.371	-1.0282	-0.6876	-0.4415	-0.4143	-0.1567
EAC00122	myosin regulatory light nonmuscle	-0.4096	-1.034	-0.8728	-0.626	-0.3881	-0.1184
EAC00787	novel rna helicase family protein	-0.8154	0.34033	0.14381	-0.5172	-0.7532	-0.7254
EAC01024	peptidase s1 and s6 chymotrypsin hap	0.07223	-2.5743	-1.5839	-1.1429	-1.1383	-1.747
EAC00634	pre-mrna-processing-splicing factor 8 protein)	-0.6048	0.4581	0.42613	0.0038	-0.3064	-0.6753
EAC00246	protein	-0.0894	-1.9586	-0.8823	0.37728	-0.7263	-1.4671
EAC00463	protein	0.04509	-0.0721	0.33204	-0.4563	-0.2131	0.24987
EAC00809	protein	-0.0015	-0.1961	0.32574	-0.5667	-0.0468	0.02387
EAC00508	ran-specific gtpase-activating protein	-0.7917	-0.3325	0.16412	0.19184	-0.8918	-0.7509
EAC00549	replication factor a protein 3 domain-containing protein	0.00601	-0.2378	0.23432	-0.5385	-0.3518	-0.2551
EAC00479	ribosomal protein l10ae	-0.5715	-0.2566	-1.2035	-0.331	-0.18	-0.1006
EAC00331	ribosomal protein l15	-0.5112	-2.7242	-1.6311	0.55577	-0.4122	-0.256
EAC00476	ribosomal protein l15	-0.6028	-0.7653	-0.6745	-0.4633	-0.6203	-0.7239
EAC00128	ribosomal protein l3	-0.3866	-0.5385	-1.2413	-1.0015	-0.6678	-0.5484
EAC00208	ribosomal protein l3	-0.7443	-0.3296	-0.4428	-0.4403	-0.6717	-0.7866
EAC00520	ribosomal protein l3	-0.6509	-0.2884	-0.6709	-0.6197	-0.6658	-0.6369
EAC00517	ribosomal protein l36a	-0.3111	-0.8766	-1.5804	-1.2018	-0.5229	-0.4611
EAC00518	ribosomal protein l36a	-0.3159	-1.0273	-1.4214	-0.933	-0.5321	-0.4783
EAC00685	ribosomal protein l37a	-0.0427	-0.8778	-1.4434	-0.4041	0.16104	0.30864
EAC00482	ribosomal protein s15	-0.3901	-0.0737	-0.9411	-0.3264	-0.5961	-0.6271
EAC00298	ribosomal protein s16	-0.2337	0.43247	-0.4935	-0.4019	-0.1981	-0.4204
EAC00287	ribosomal protein s2	-0.7519	-0.5134	-0.9984	-0.7038	-0.5553	-0.6386
EAC00143	ribosomal protein s7	-0.4466	-0.4647	-0.8762	-0.245	-0.3767	-0.472
EAC01078	ribosomal protein s7	-0.426	-0.4419	-0.871	-0.1478	-0.3462	-0.4392
EAC00663	ribosomal protein s7	-0.1627	-0.1694	-0.374	0.38851	0.07469	0.03969
EAC00716	sec14-like protein 2 isoform 1	-0.2155	-0.2432	-1.6846	-0.9262	-0.2407	-0.2692
EAC00373	selenium binding protein 1	-0.1669	-0.3582	0.24153	-0.7376	-0.4732	-0.3372
EAC00404	short-chain collagen c4-partial	-0.2504	-0.6421	0.13399	-0.6899	-0.4782	-0.3568
EAC00847	splicing factor subunit 3	-0.2535	0.19627	0.26664	0.01451	-0.0593	-0.5721
EAC00793	t-cell receptor beta chain ana 11	-0.5377	0.20722	0.15436	0.13948	-0.4488	-0.7469
EAC00859	t-cell receptor beta chain ana 11	-0.2229	0.52818	0.00665	0.03088	-0.8557	-0.9408
EAC01050	tektin 2	0.09566	-0.0732	0.104	-0.7586	-0.776	-0.6465
EAC00207	transketolase-like 2	-1.6266	-1.3227	-0.1244	-0.2981	-0.8376	-1.3352
EAC00994	translation elongation factor 1-alpha	-0.5031	-0.0612	-0.5255	-0.2716	-0.6371	-1.1136
EAC00197	translationally controlled tumor protein	-0.6001	-0.0168	-0.751	-0.3514	-0.5623	-0.7972
EAC00637	transmembrane and coiled-coil domains	-0.3403	-0.8487	0.21256	0.68024	0.03122	0.12044
EAC00115	tropomyosin	-0.7043	-0.6977	-0.0062	0.16559	-0.4298	-0.4263
EAC00173	tropomyosin	-0.5956	-0.3712	-0.3538	-0.2171	-0.5583	-0.6765
EAC00340	troponin t	-0.7185	-0.3558	-0.3383	-0.2881	-0.7091	-0.9882
EAC00496	troponin t	-0.7278	0.01834	-1.1318	-0.9918	-0.7677	-1.0462
EAC00175	tubulin alpha chain	-0.9593	-0.7775	-0.4424	-0.3916	-0.4156	-0.5367
EAC00334	u6 snrna-associated sm-like protein	-0.5119	-0.4827	-0.7143	0.00965	-0.5616	-0.2359
EAC00431	vesicle-associated protein (vap-1)	-0.7193	-0.6699	0.45622	0.48967	0.5287	0.19172
EAC00325	xk-related protein 6	0.06744	-0.1997	0.29499	-0.6272	-0.1422	-0.1263
EAC00880	yipee zinc-binding protein	-0.026	-0.1056	0.22357	-0.5369	-0.1468	0.02195
EAC00284	zinc carboxypeptidase family protein	-0.4092	-2.0155	-0.9909	-0.4322	-1.225	-2.0498
EAC00426	zinc transporter	-0.2936	0.14738	-0.869	-1.5321	-0.1296	-0.4337

Table S4: Significant differentially expressed transcripts (two sample t-test, $p < 0.05$) between samples from each concentration of cadmium and zinc. Only annotated genes with significant log2 intensity values between each concentration of Cd and Zn are shown. The log_2 intensity values represent the average processed fluorescence expression values of samples from each Cd or Zn concentrations.

		Log2 Intensities			
		Cadmium		Zinc	
Cluster ID	**Blast Homology**	**EC_{50}**	**EC_{90}**	**EC_{50}**	**EC_{90}**
EAC00495	20 kda calcium-binding protein (antigen sm20)	10.935125	10.877998	11.558383	11.689961
EAC00457	26s protease regulatory subunit 8	1.8561061	1.6234512	1.4094428	1.0122035
EAC01014	40s ribosomal protein	1.6124403	1.5933747	1.2493156	0.930863
EAC00287	40s ribosomal protein s2-like isoform 2	8.1791242	8.4316404	7.6086605	7.7585597
EAC00226	40s ribosomal protein s4	9.2999676	9.3255233	8.7622193	8.8830224
EAC00001	mitochondrial f1-atpase during controlled dehydration	4.5899983	5.5162654	6.0965137	6.3159542
EAC00024	actin	13.895659	14.220302	14.815704	14.640781
EAC00090	actin	14.759279	15.094821	15.560455	15.411538
EAC00110	actin	8.433444	8.3021771	7.2447129	7.2943792
EAC00131	actin	9.4113314	9.2903467	8.1806168	8.2090708
EAC00139	actin	15.17254	15.364337	15.731653	15.684968

EAC00165	actin	13.878893	13.915508	14.637404	14.566258
EAC00220	actin	10.317944	10.24911	9.2839773	9.3559455
EAC00253	actin	9.3866635	9.3596994	8.4517168	8.4644796
EAC00281	actin	7.8159819	7.8865697	7.0548831	6.9541186
EAC00357	actin	12.744327	13.11822	13.694117	13.474431
EAC00362	actin	10.331403	10.744334	10.732133	10.740354
EAC00382	actin	8.2692617	8.2237277	7.5917181	7.611806
EAC00406	actin	8.3965311	9.0742349	9.6542028	9.4202037
EAC00432	actin	1.5722355	1.587024	1.1929621	0.9691973
EAC01012	actin	1.5857609	1.5017483	1.1627378	0.8918652
EAC01056	actin	14.292889	14.499059	14.803685	14.643908
EAC01081	actin	4.1584139	4.2494693	5.3464804	5.3384031
EAC00396	actin beta	6.8830001	6.9147574	6.1877353	6.165251
EAC00681	actin related protein 2 3 subunit 41kda	4.2514206	4.0630398	4.4011865	4.5479762
EAC00157	acyl-coenzyme a short branched chain	1.6258449	1.6532937	1.2660896	1.0965254
EAC00910	adenine nucleotide translocator	12.874024	13.667319	13.982269	13.936734
EAC00147	adenosine kinase isoform 2t	1.5488975	1.5049061	1.141081	0.8792331
EAC00175	alpha smooth aorta	7.1559167	6.9789388	6.0344293	6.020072
EAC00867	alpha tubulin	7.6630331	7.7586708	7.3232056	7.3603422
EAC00511	aquaporin 1	8.9103886	9.3321335	9.9486754	10.011566
EAC00199	atp h+ mitochondrial f1 alpha subunit cardiac muscle	4.4579372	4.6466473	3.8705283	3.9418776
EAC00326	atp synthase subunit mitochondrial	10.876741	11.480039	11.601674	11.542202
EAC00301	atp:adp antiporter	8.2124547	8.78778	8.1484049	8.154456
EAC00421	b chain structural basis for giant hemoglobin	11.977693	12.55976	13.003678	13.149285
EAC00102	b chain structural basis for giant hemoglobin	4.6834341	4.7613508	5.5734727	5.244419
EAC00523	beta actin	7.7279784	7.7476132	6.821215	7.0480002
EAC00899	beta actin	7.9524444	7.1271555	8.4867219	8.9424915
EAC00377	beta chain	4.2313826	4.2819641	5.3953652	5.3669282
EAC00182	beta tubulin	9.4997127	10.106479	10.828325	11.02324
EAC00183	beta-actin	8.5115115	8.4888979	7.3963043	7.4884103
EAC00881	beta-actin	13.093204	13.460078	13.905333	13.877793
EAC00100	btb poz domain-containing protein	1.5570034	1.5303064	1.2668623	0.9561329
EAC00305	calponin homolog	6.9004333	6.6791053	6.2453417	6.309399
EAC00494	calponin homolog	12.039054	12.435603	13.076547	13.166533
EAC01009	calponin transgelin	4.3484459	4.4760664	5.8195815	5.7950445
EAC00222	carboxypeptidase b	2.2539926	2.4098676	1.9516246	1.266268
EAC00426	cathepsin b	10.698932	11.143643	12.118101	11.967712
EAC00335	catsup protein	8.9447799	8.8342697	8.2850528	8.1096252
EAC00440	ccf-like protein	6.4080119	6.4836399	5.9524346	5.8485364
EAC00233	cg14935 cg14935-pb	5.7982197	6.3028114	7.2823386	7.3315146
EAC00718	chaperonin containing subunit 7	4.7310947	5.3263488	5.3934832	5.4310704
EAC00198	chaperonin containing subunit 8	3.1343821	3.0939994	1.4885507	1.4943004
EAC00492	chk1 checkpoint-like protein	7.6878632	7.635773	8.1715706	8.4220832
EAC01107	chorion b-zip transcription factor	4.8031947	4.4380012	3.5011487	3.5403102
EAC00180	creatine flagellar	8.329641	8.2728754	7.7886554	7.6855201
EAC00042	c-type lectin 11	1.5890587	1.7271671	1.1681042	0.938358
EAC00415	cysteine rich transmembrane bmp regulator 1 (chordin-like)	3.0833573	2.4878344	3.1316479	3.3366961
EAC01004	cysteine-rich repeat-containing protein crim1	9.2104966	10.118228	10.959531	10.99431
EAC00733	cytochrome b	4.1550086	4.2034972	5.3667775	5.3510567
EAC00502	cytochrome c	9.155363	9.4220157	9.5765052	9.4944213
EAC00958	cytochrome c oxidase subunit 1	15.083506	15.240595	15.47797	15.468517
EAC00470	cytochrome c oxidase subunit 1	4.1601159	4.266523	5.3227654	5.3109594
EAC01043	cytochrome c oxidase subunit iii	16.395525	16.511625	16.023756	15.940114
EAC00225	cytochrome oxidase subunit i	12.563175	12.661976	13.086001	13.204576
EAC00300	cytochrome oxidase subunit ii	16.09238	16.345736	15.898835	15.747581
EAC00531	cytoplasmic 1	7.148522	7.261395	6.3935886	6.4865342
EAC00671	cytoplasmic 1-like isoform 1	11.970045	12.104986	12.590745	12.588861
EAC00863	dosage compensation protein dpy-	2.2283615	2.0198293	1.1998957	0.9848969
EAC01114	dual oxidase 2	8.938165	8.9321471	9.5458628	9.5781867
EAC00111	egf-like domain-containing protein	4.173793	4.3093429	5.3353069	5.3253931
EAC00229	elastase pancreatic	5.6945323	5.7008381	5.0655199	4.743427
EAC00202	elongation factor 1-alpha	16.407666	16.529166	16.03186	15.963389
EAC00257	elongation factor-1 alpha	9.1866737	9.2072392	8.3567626	8.43583
EAC00826	eukaryotic peptide chain release factor subunit	5.0913908	4.9282245	3.5850419	3.690817
EAC01079	eukaryotic peptide chain release factor subunit 1	3.7904481	4.0545801	5.1504687	5.1515306
EAC00400	eukaryotic translation initiation factor subunit e	7.3233734	7.5809523	8.2873316	8.4304828
EAC00261	excretory secretory protein juv-p120 precursor	9.37291	9.1534882	9.906553	9.8263493
EAC00354	extracellular hemoglobin linker l4 subunit precursor	6.3832803	5.9231536	3.78222	6.7280846
EAC00265	gl12416-like	12.844923	13.617258	13.988441	14.000943
EAC01005	glucan endo- -beta-glucosidase	1.8497333	1.7020089	1.4225648	1.1841689
EAC00316	glycine n-methyltransferase	4.2274811	4.2686262	5.3435549	5.3445023
EAC00481	heat shock cognate 71 kda	6.6078464	6.7679859	6.2867093	6.3544452
EAC00097	heat shock protein 60	1.7031237	1.674843	1.2319724	1.0398346
EAC00035	heat shock protein gp96	1.8090368	1.6256858	1.4920748	1.0506404
EAC00127	hemoglobin c chain precursor	8.5093955	8.5268153	7.8739175	7.8941886
EAC00398	hemoglobin c chain precursor	8.2239423	8.2831318	7.5783685	7.6261192

EAC00195	hemoglobin c chain precursor	15.08695	15.43173	15.486485	15.560546
EAC00577	hmw glutenin subunit x	16.350568	16.45587	16.020647	15.980841
EAC00308	homolog precursor	7.0417105	6.931479	7.5840382	7.7020445
EAC00283	hypothetical protein Bm1_11025 [Brugia malayi]	14.31559	14.902106	10.982723	9.9616836
EAC00404	hypothetical protein BOS_23218 [Bos taurus]	16.381747	16.509846	14.351628	13.274267
EAC00839	hypothetical protein BRAFLDRAFT_74510 [Branchiostoma floridae]	1.7519956	1.7188132	1.5073528	1.1426385
EAC00710	hypothetical protein NEMVEDRAFT_v1g139821 [Nematostella vectensis]	6.4937118	6.1661406	7.095873	6.9088106
EAC00734	hypothetical protein TGME49_026570 [Toxoplasma gondii ME49]	1.50914	1.5040589	1.1367692	1.0557191
EAC00587	hypotheticial protein	16.25506	16.450746	15.705573	15.579053
EAC00664	immunoglobulin i-set domain containing protein	6.741802	6.5713991	7.7289507	7.5574438
EAC00822	integrin-linked kinase-associated serine threonine phosphatase 2c	5.3408492	5.2475491	5.7141457	5.925844
EAC00397	isoform a	9.1675595	9.1304632	10.166949	9.8405743
EAC00516	keratinocyte associated protein 2	2.5318605	2.2490558	1.9979302	1.9651854
EAC01019	kif21a protein	1.481862	1.4918417	1.2841124	0.9032783
EAC00558	larval visceral protein d	5.0609203	4.8589198	4.6361501	4.4100089
EAC00260	light chain regulatory	7.6756318	7.770684	8.6536146	8.6821534
EAC00889	lombricine kinase	13.418161	14.063908	14.401542	14.330774
EAC00925	mads flc-like protein 2	12.306953	12.590703	13.100847	13.264418
EAC00928	mads flc-like protein 2	4.2692631	4.3235115	5.3438116	5.351542
EAC00929	mads flc-like protein 2	4.2391033	4.2793959	5.2992033	5.3019272
EAC00931	mads flc-like protein 2	7.3076579	7.4361261	7.8814614	7.963682
EAC00943	mads flc-like protein 2	4.8561451	5.0196535	6.274519	6.1392145
EAC00946	mads flc-like protein 2	4.1680406	4.2358551	5.3146475	5.2752965
EAC00951	mads flc-like protein 2	1.8632507	1.8597714	1.4473636	1.0459823
EAC00962	mads flc-like protein 2	4.2708085	4.3541186	5.3653922	5.3401321
EAC00966	mads flc-like protein 2	3.3859604	3.3732079	2.6241144	2.2936868
EAC00967	mads flc-like protein 2	4.2914648	4.3740591	5.3759288	5.3385295
EAC00976	mads flc-like protein 2	4.1876034	4.2365781	5.3682426	5.3595603
EAC00995	mads flc-like protein 2	4.1943265	4.2916791	5.3401492	5.3001348
EAC01003	mads flc-like protein 2	4.3265146	4.4064656	5.3918787	5.3464058
EAC01006	mads flc-like protein 2	1.6620124	1.6379117	1.2308882	1.0732851
EAC01007	mads flc-like protein 2	5.6269728	5.6723251	6.5604609	6.816981
EAC01027	mads flc-like protein 2	4.2021308	4.2726946	5.4002898	5.3695477
EAC01031	mads flc-like protein 2	6.9440308	6.5862896	5.572687	5.6003382
EAC01044	mads flc-like protein 2	4.2228178	4.3124274	5.3675564	5.3672635
EAC01051	mads flc-like protein 2	4.195442	4.3082496	5.3306518	5.3336468
EAC01053	mads flc-like protein 2	12.051154	12.370917	11.933614	11.895022
EAC01058	mads flc-like protein 2	4.1705945	4.4263599	5.3406002	5.260371
EAC01061	mads flc-like protein 2	4.178757	4.292087	5.3535119	5.3287542
EAC01065	mads flc-like protein 2	1.5825041	1.6702946	1.2420555	1.0565591
EAC01066	mads flc-like protein 2	4.1612636	4.3138094	5.3589531	5.3442424
EAC01070	mads flc-like protein 2	7.6536071	8.0906121	9.5772369	9.760462
EAC01075	mads flc-like protein 2	3.9375167	3.9388474	5.1256215	5.1434334
EAC01076	mads flc-like protein 2	4.2174567	4.2869021	5.3788551	5.3640547
EAC01077	mads flc-like protein 2	4.2547471	4.4254021	5.376853	5.355161
EAC01082	mads flc-like protein 2	4.1747585	4.2981145	5.327435	5.2752475
EAC01092	mads flc-like protein 2	4.0158455	4.140087	5.2827599	5.2311398
EAC01094	mads flc-like protein 2	14.010171	13.953116	14.276249	14.352577
EAC01098	mads flc-like protein 2	4.8623585	5.0737827	6.3041466	6.1706405
EAC01109	mads flc-like protein 2	8.0190748	7.8192395	8.3655061	8.2887527
EAC01116	mads flc-like protein 2	4.1358679	4.2789872	5.3214102	5.2686964
EAC01118	mads flc-like protein 2	6.866536	7.430021	8.9958904	9.2251584
EAC01123	mads flc-like protein 2	4.2559212	4.285144	5.3625544	5.320515
EAC00433	mads flc-like protein 2	4.1830518	4.2294781	5.3851924	5.3103921
EAC00777	methyltransferase mb3374	1.622263	1.5686392	1.2802878	1.065251
EAC00920	mgc107908 protein	9.7059354	10.090743	11.132167	10.835531
EAC01115	mgc80370 protein	5.0799752	5.2898788	6.4139155	6.4069546
EAC00456	mgc80370 protein	7.3326188	7.4616824	7.877712	7.9693254
EAC00732	monosaccharide-transporting atpase	1.5919019	1.6047818	1.383499	1.0586446
EAC00302	myosin alkali light chain 1	11.296092	11.375848	11.581616	11.50754
EAC00378	myosin alkali light chain 1	7.3038077	7.4428021	7.766414	7.6366982
EAC00074	myosin heavy chain	3.7965119	3.741342	4.8571578	4.8411997
EAC00088	myosin heavy chain	8.2676297	8.2270256	7.4581434	7.3109345
EAC00108	myosin heavy chain	7.4695637	7.3778241	6.7229798	6.6412215
EAC01025	myosin heavy chain	4.1289017	4.1502761	5.34123	5.3120116
EAC01080	myosin heavy chain	4.1234668	4.1953483	5.3620779	5.3150988
EAC01089	myosin heavy chain	4.2350749	4.4129003	5.364219	5.354177
EAC01091	myosin heavy chain	4.222364	4.3904907	5.3799523	5.3652996
EAC01093	myosin heavy chain	4.198152	4.2195881	5.3473252	5.3414629
EAC01095	myosin heavy chain	4.2746681	4.2354391	5.37078	5.3096883
EAC01096	myosin heavy chain	4.2084412	4.3552963	5.3445056	5.3669455
EAC01105	myosin heavy chain	4.2691646	4.2961276	5.4110863	5.3841831
EAC01113	myosin heavy chain	4.2371063	4.3531911	5.3477464	5.3183246
EAC01119	myosin heavy chain	4.1942409	4.3063753	5.3838311	5.3316577
EAC01122	myosin heavy chain	4.8544101	5.0370711	6.2497357	6.1290302
EAC01023	myosin light chain 2	8.4237028	8.5554853	7.933707	7.8825791

EAC00122	myosin regulatory light chain	8.3866387	8.4577372	7.7618313	7.7258447
EAC00259	myosin regulatory light chain	15.010641	15.326984	15.426287	15.586878
EAC00607	nadh dehydrogenase	8.7776834	9.1220181	9.4273543	9.4640908
EAC00842	nadh dehydrogenase subunit 1	16.162154	16.266558	15.845597	15.654262
EAC00134	nadh dehydrogenase subunit 5	5.1894214	4.8852648	4.1045626	4.31306
EAC00805	nicotinic acetylcholine receptor alpha6 subunit	6.0581503	5.9989394	7.0177721	6.6787391
EAC00513	ovulatory protein-2 precursor	3.2074592	3.3973653	3.6464309	4.057337
EAC01024	peptidase s1 and s6 chymotrypsin hap	3.7867983	4.1257505	3.1090369	2.1710001
EAC01086	phosphoglycerate kinase	6.7120665	7.0044312	8.0549236	8.0443912
EAC00677	poly -specific ribonuclease parn	1.5949703	1.732061	1.3672801	1.0876145
EAC00549	replication factor a protein 3 domain-containing protein	1.5527481	1.545307	1.1789441	0.8723051
EAC00476	ribosomal protein l15	7.6840647	7.6843301	7.2504514	7.2321759
EAC00280	ribosomal protein l19	10.539278	10.914702	11.104329	11.240816
EAC00583	ribosomal protein l26	13.498799	13.807403	13.888087	13.926078
EAC00128	ribosomal protein l3	6.5898804	6.3450032	5.3958408	5.4649127
EAC00517	ribosomal protein l36a	8.6586988	8.4070899	7.6599258	7.6182559
EAC00518	ribosomal protein l36a	9.487761	9.2412919	8.6125791	8.5561861
EAC00482	ribosomal protein s15	8.9579115	8.7317203	8.3115637	8.6115775
EAC00143	ribosomal protein s7	9.6095331	9.5079437	9.0771031	9.2497031
EAC00309	s enesis abnormal family member (syg-2)	6.975711	7.6021851	7.6910714	7.4326828
EAC00159	sb:cb283 protein	11.903196	12.081495	12.615821	12.386281
EAC01124	sb:cb283 protein	4.2026943	4.3213199	5.3532593	5.3133141
EAC01038	scbp2 protein	14.812765	14.911411	15.472556	15.466821
EAC00045	scbp3 protein	5.5036994	5.394308	5.8123872	5.9300177
EAC00120	scbp3 protein	6.5979264	6.57495	7.3396549	7.5440777
EAC00408	scbp3 protein	5.274347	5.0380778	5.4087357	5.475994
EAC00969	scbp3 protein	4.2444969	4.3002355	5.3680356	5.3402769
EAC01045	scbp3 protein	5.9659535	5.8849377	6.2420286	6.3823543
EAC01084	scbp3 protein	4.2472324	4.3293498	5.3589091	5.322608
EAC00373	selenium binding protein 1	1.6811541	1.6122766	1.2637032	0.9189083
EAC00554	short-chain dehydrogenase	9.8206697	10.064522	10.390216	10.208005
EAC00992	stathmin 1 oncoprotein 18 variant 8	5.6118868	5.701605	6.5472689	6.8263604
EAC01121	superfast myosin heavy chain	4.2299295	4.253116	5.3353061	5.3202701
EAC01050	tektin 2	1.8091141	1.6729891	1.3641038	1.0634505
EAC00210	thymosin isoform 2	10.160953	10.275148	10.692853	10.804059
EAC00503	thymosin isoform 2	11.200654	11.303411	11.796037	11.916801
EAC01097	transcription factor btf3 homolog 4	4.2976245	4.3147272	5.3919704	5.3816511
EAC00207	transketolase	2.4728946	2.494338	2.1223553	1.999862
EAC00994	translation elongation factor 1-alpha	2.5535084	2.1459774	1.8283207	1.9013843
EAC00197	translationally controlled tumor protein	8.6907777	8.3876017	8.1981968	8.4475242
EAC00327	tropomyosin	14.083805	14.052336	14.502171	14.500571
EAC00545	tropomyosin	5.4726654	5.3324306	5.6984731	5.9149906
EAC01032	tropomyosin	5.4594666	5.283194	5.701402	5.9070501
EAC01110	tropomyosin	11.653059	11.91656	12.734744	12.667019
EAC00496	troponin t	6.54822	6.0202196	5.4459578	5.6484834
EAC00485	tubulin beta-2b chain-like	6.2910097	6.3710984	5.8643945	5.9517715
EAC00313	type alpha 1	1.6576946	1.6322804	1.2163066	0.9259203
EAC00390	type alpha isoform cra_b	9.8958946	9.9533423	10.971198	11.043734
EAC00831	u6 snrna-associated sm-like protein lsm3	7.2039244	7.5687157	8.0834354	7.8536276
EAC00657	ubiquinol-cytochrome c subunit	2.1705121	2.2322529	1.5219639	1.1217924
EAC00306	ubiquitin c	13.432263	13.792991	14.31251	14.431016
EAC00148	universal minicircle sequence binding protein	11.445313	11.638536	12.069985	11.916321
EAC00325	xk-related protein 6	1.6503965	1.5567662	1.2724285	0.8812791
EAC00880	yippee-like 5	1.578775	1.4664331	1.1409615	0.9172046
EAC00933	zinc carboxypeptidase	3.2561126	3.2914413	2.7599888	1.7910103
EAC00284	zinc carboxypeptidase family protein	3.3811216	3.6142267	2.768231	2.144101

Chapter VI

Differences between Cd and Zn exposure in *Enchytraeus albidus* - linking changes in cellular energy allocation to transcriptional, enzymatic and reproductive responses

VI – Differences between Cd and Zn exposure in *Enchytraeus albidus* - linking changes in cellular energy allocation to transcriptional, enzymatic and reproductive responses

Sara C Novais, Amadeu MVM Soares, Wim De Coen & Mónica JB Amorim

Submitted to Environmental Pollution (IF = 3.395; 2010)

Abstract

Cellular energy allocation (CEA) is a measure of the energy status of an organism, and integrates the available energy (Ea, total carbohydrate, lipid and protein content) and the consumed energy (Ec, mitochondrial electron transport activity - ETS). In this study the effects of Cd and Zn (reproduction EC_{50}s and EC_{90}s) on the total energy budget of *Enchytraeus albidus* (Oligochaeta), were assessed through CEA determination over the periods of time 0-2-4-8 days. Results showed reduction on the energy reserves for both metals after 2 days exposure. Lipids were the first reserves to be used and carbohydrates were reduced exclusively after Cd. ETS activities increased for both tested metals suggesting increased metabolism and higher energy requirements to handle metal detoxification. This was supported by results at transcription level obtained in a combined study. Additionally, the reduction of CEA, plus the oxidative damage on the membrane cells (oxidative stress study), may be related with the decrease on the reproductive output, confirmed at a later stage. No causality can be assured, but the decrease on reproduction of *E. albidus* was partly the consequence of a preceding change in energy allocation occurring after 2 days exposure. As discussed in further detail in the paper, results showed the relevancy of integrating various endpoints to understand mechanisms in an overall perspective.

Keywords: Enchytraeids; soil; energy budget; metals

1. Introduction

Enchytraeus albidus is a model ecotoxicological species, used for Environmental Risk Assessment (ERA) through the determination of effects on survival and reproduction (ISO, 2004; OECD, 2004). Toxicity of cadmium and zinc, persistent contaminants of soils worldwide, has been addressed in this soil dwelling invertebrate organism. The negative impacts of Cd and Zn have been confirmed through various endpoints and levels of biological organization: survival and reproduction (Lock and Janssen, 2001b; Lock and Janssen, 2001a; 2003; Novais et al., 2011), bioaccumulation (Lock and Janssen, 2001c; Egeler et al., 2009), avoidance behaviour (Amorim et al., 2008), oxidative stress and neuromuscular biochemical biomarkers (Novais, et al., 2011) and gene expression (Novais et al., 2012). The information summed so far has contributed for a better understanding of the mechanism of toxicity, i.e. the chain of events that are comprehended.

Recent results addressing gene and oxidative stress markers (Novais, et al., 2011; Novais, et al., 2012), revealed that both Cd and Zn affected the mitochondrial metabolism, among others, and induced oxidative stress. This suggested an imbalance on the general energy metabolism of *E. albidus*. The main goal of the present work was to confirm if and how Cd and Zn interfere with the balance of the net energy budget, essential for basal metabolism, growth and reproduction. Experiments were performed being comparable (concentration, time) to previous transcription studies (Novais, et al., 2012). Cellular Energy Allocation (CEA) was the parameter used to assess the energy status of the organisms and its stress induced effects. This method was proposed by De Coen and Janssen (1997, 2003) and it is based on biochemical measurements on the energy reserves available (Ea) (total carbohydrate, protein, and lipid content) and the energy consumption (Ec) estimated by measuring the electron transport system activity (ETS). The ETS activity is a measure of the oxygen consumption process and the cellular respiration rate (De Coen and Janssen, 2003; Moolman et al., 2007). The integration between those parameters (Ea and Ec) over a certain period of exposure time represents the net energy budget of the organism (De Coen and Janssen, 1997). This endpoint has been considered a sensitive biomarker to measure the energy status of an organism and has proven to be useful in the prediction of long-term

effects, since CEA effects have been successfully linked to higher levels of biological organization (De Coen and Janssen, 2003; Smolders et al., 2004).

2. Materials and methods

2.1 Test organisms

The enchytraeids *Enchytraeus albidus* (Henle, 1837) were selected for this study. Individuals were maintained in laboratory cultures under controlled conditions with a photoperiod 16:8h light:dark and a temperature of 18ºC. Animals were fed twice a week with finely ground and autoclaved rolled oats.

2.2 Test Soil

All exposures were performed in the natural standard soil LUFA 2.2. This soil type is commercially available from the German institution LUFA Speyer. The properties of this soil can be summarised as follows: pH ($CaCl_2$) = 5.5, Organic Matter = 4.4%, texture = 6% clay; 17% silt; 77% sand.

2.3 Spiking of the test substances

Two chemicals were individually tested: zinc chloride [$ZnCl_2$, Sigma-Aldrich (Riedel-de Haën), 98%] and cadmium chloride ($CdCl_2$, Fluka, 99%). The chemicals were spiked into pre-moistened batches of soil as aqueous solutions, each test concentration into the whole batch of soil for all replicates. The spiked soil was allowed to equilibrate three days before test start as recommended for metals testing (McLaughlin et al., 2002).

The organisms were exposed to concentrations of each metal in the range of the reproduction EC_{50} and EC_{90} (Zn: 40 and 100 mg/kg; Cd: 6 and 150 mg/kg) as assessed by Novais et al. (2011).

2.4 Experimental setup

Fifteen adult worms with well developed clitellum were introduced in the test vessels, each containing 25 g moist soil (50% of the maximum Water Holding Capacity - WHC). The worms were exposed for 2, 4 and 8 days at 20ºC and a 16:8h photoperiod and fed following the same procedures as in the standard guideline for the Enchytraeid Reproduction Test (ISO, 2004). The organisms were fed to ensure that a possible depletion of the energy reserves is not caused by starvation. Three replicates per treatment were used. At test end, animals of each replicate were carefully removed, rinsed in deionised water and split into three groups of 5 organisms which were weighted and frozen in liquid Nitrogen. All the samples were stored at -80ºC till further analysis.

2.5 Energy available – Ea

Available energy reserves were measured by determining spectrophotometrically the total protein, carbohydrate and lipid content at each time point and transforming them into energetic equivalents using enthalpy combustion (24 kJ/g proteins, 17.5 kJ/g carbohydrates and 39.5 kJ/g lipids) as described in De Coen and Janssen (1997, 2003). The measure of these energy reserves in *Enchytraeus albidus* was done for the first time by Amorim and co-authors (2011) and more details of the methodology can be found on that publication. In brief, each replicate with 5 organisms was homogenised for protein and carbohydrate measurements. Protein content was determined according to the Bradford method (Bradford, 1976) at 600 nm using bovine serum albumin as standard. Total carbohydrate content was determined with phenol 5% and concentrated H_2SO_4 at 490 nm using glucose as standard. Total lipids were extracted according to the method described by Bligh and

Dyer (1959). Each replicate with five organisms was homogenized and total lipid content was determined by measuring the absorbance at 400 nm using Tripalmitine (Sigma) as standard.

2.5 Energy consumed – Ec

The consumed energy (consumed oxygen rate) was determined based on the measurement of the electron transport system activity (King and Packard, 1975) over the three time periods of exposure (2, 4 and 8 days). Each replicate with five organisms was homogenised and the electron transport activity was measured by adding NADPH solution and INT (*p*-IodoNitroTetrazolium, Sigma) and following the increase in absorbance at 490 nm for 3 minutes. The oxygen consumption rate was then transformed into caloric values using oxyenthalpic equivalents (480 KJ/mol O_2).

2.6 Cellular Energy Allocation – CEA

The total Ea value was calculated by integrating the change in the summed energy reserves fractions over the three time periods (0 to 2 days, 2 to 4 days and 4 to 8 days). The Ec value was similarly calculated, integrating the change in energy consumption over the same exposure periods. The CEA, representing the total net energy budget, was calculated for each time interval as described in De Coen and Janssen (1997), using the following equation (example for the period of exposure from 0 to 48 hours):

$$CEA\ (mJ/mg\ Org) = \frac{\int_0^{48} Ea.dt - \int_0^{48} Ec.dt}{48h}$$

2.7 Statistical Analyses

Significant differences between the control and exposure conditions were determined, for all endpoints, by one way analysis of variance (ANOVA) using the statistical package SigmaPlot version 11.0 (SigmaPlot, 1997). Whenever significant differences were obtained, the post hoc Dunnett's method for multiple comparisons was used ($p < 0.05$).

3. Results and Discussion

Effects of Cd and Zn on the total net energy budget of the organisms were assessed by integrating the available energy (Ea) and the consumed energy (Ec – mitochondrial ETS) over 3 periods of time: 0 to 2 days, 2 to 4 days and 4 to 8 days.

No significant differences were observed in the weight of the organisms with the metal exposures (ANOVA, $p > 0.05$). Consequently, all results were expressed as mJ per mg of organisms (wet weight). The results of the available energy reserves in the different time intervals can be seen in figure 1.

Results presented in figure 1 (A and B) show that in the first 2 days of exposure the relative proportion of each energy reserve is very similar for all conditions. At 4 and 8 days, energy transferences become evident in both metal exposures, with the changes in the relative amount of carbohydrates, lipids and proteins.

The proportion of energy reserves in the control organisms were very similar for all time points and the amount of these measured reserves are in the same range as the ones from the first study in *E. albidus* (Amorim, et al., 2011).

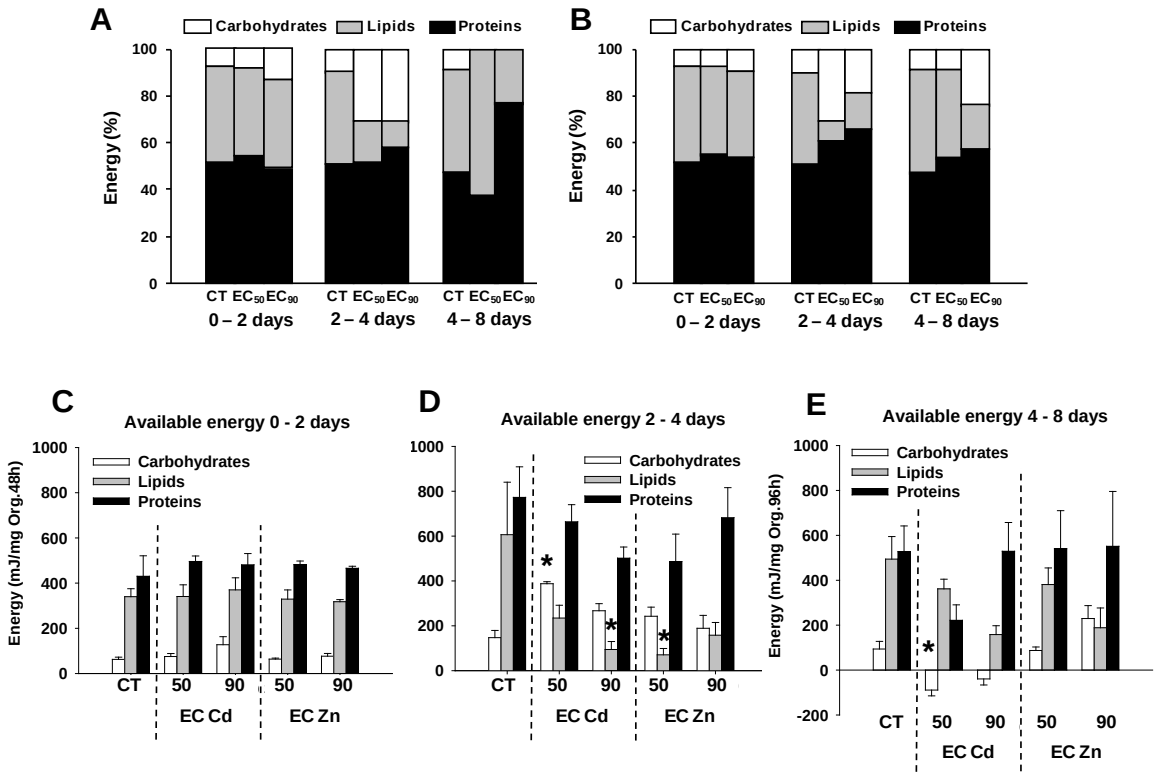

Figure 1: Effects of cadmium and zinc (reproduction EC_{50} and EC_{90}, please see text for further information) on the energy reserves available (carbohydrates, lipids, proteins) in *E. albidus*. **A, B** – Proportion of energy reserves in each condition of Cd and Zn, respectively. **C, D, E –** Content of energy reserves from 0 to 2 days (C), 2 to 4 days (D) and 4 to 8 days (E). Results express average values + standard error. * indicates statistically significant differences between control (CT) and spiked soil (Dunnett's, $p<0.05$).

During the first 2 days of exposure, no significant effects of either Cd or Zn were observable on the available energy reserves (fig. 1 – A). After 2 more days, there was a significant increase on the content of carbohydrates at Cd EC_{50} and a decrease in the lipid fraction at both Cd and Zn EC_{50} and EC_{90} exposures (fig. 1 – B). From 4 to 8 days, a complete depletion on the carbohydrate reserves occurred for Cd exposures alone (fig. 1 – C). The protein content was not significantly affected by any of the metals at any time period.

The use of lipids as a first energy source has also been previously documented for *Daphnia magna* exposed to Cd (De Coen and Janssen, 1997; Soetaert et al., 2007) and in *E. albidus* exposed to Cu (Amorim, et al., 2011). Additionally, this decrease in the lipid fraction can

also be a result of oxidative damage on the membrane cells, which was observed when the same concentrations of Cd and Zn were tested in *E. albidus* (Novais, et al., 2011) and increased levels of lipid peroxidation were observed.

Carbohydrate content was not affected by Zn exposures within the tested time period. Same occurred after Cu exposure (3 weeks), another essential metal, in the study by Amorim et al. (2011). On the contrary, when exposed to Cd, organisms started to increase their reserves as carbohydrates during 2 days, after which, at 4 days, a complete depletion of those reserves occurred. Similarly, this effect on the carbohydrate metabolism has been observed in *Daphnia magna* exposed to Cd (De Coen and Janssen, 2003; Soetaert, et al., 2007) and in earthworms has been observed to affect their glycogen level (Ireland and Richards, 1977). The impact on the carbohydrate reserves in *E. albidus* due to Cd seems to be activated by different mechanisms compared to Zn and Cu, possibly related to being specific for non-essential and essential metals. These results seem to contrast with the ones from a field study (Holmstrup et al., 2011), where no reduction of glycogen levels occurred in the earthworm *Dendrobaena octaedra* in Cd polluted sites, whereas a significant correlation between Zn concentration and glycogen levels was observed for one of the contaminated sites. However, in that same study the correlation between glycogen and Zn contamination was not confirmed for other collecting sites with no clear explanation. These discrepancies between studies in metal effects on glycogen levels are most certainly dose related but can also point for species differences in the energetic strategies and costs for metal regulation.

The energy consumption during the three periods of time was calculated based on the mitochondrial ETS activity and the results are presented in figure 2.

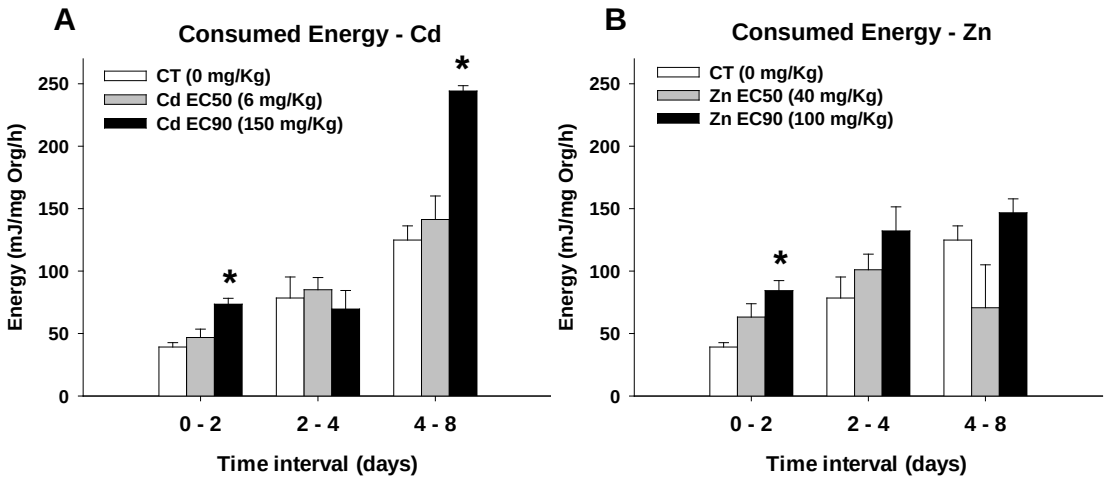

Figure 2: Energy consumption (electron transport activity) in *E. albidus* during 0 to 2 days, 2 to 4 days and 4 to 8 days exposure to two concentrations (reproduction EC_{50} and EC_{90}) of cadmium (**A**) and zinc (**B**). Results express average values + standard error. * indicates statistically significant differences between control (CT) and spiked soil (Dunnett's $p<0.05$).

For both Cd and Zn exposures, the ETS roughly increased in a dose-response related manner and with time (fig. 2), including the first 2 days of exposure where no effects on the energy reserves were yet observed.

De Coen and Janssen (2003) suggested that a reduction on the energy reserves could be the result of either decreased food consumption or increased metabolic activity. Our results showed reduction on the available energy reserves over the 8 days of exposure along with an increased ETS activity, which seems to indicate that the costs for metal detoxification might be the main source for the decreased energy reserves. Nevertheless, one cannot exclude that Cd might also have an effect on the feeding behaviour, even though the organisms had food *ad libitum.* The feeding activity was not addressed in our study, but in other species Cd has shown to cause a reduction in the feeding rate (e.g. Wicklum and Davies, 1996; Pestana et al., 2007).

Interestingly, the increased ETS activity and expenditure of energy, is in accordance with microarray results from a parallel transcription study on this species (Novais, et al., 2012). An up-regulation of genes involved in the mitochondrial oxidative phosphorylation was

induced by Cd and Zn, suggesting the additional energy requirement to handle metal toxicity.

When integrating the results discussed above, on the available and consumed energy at each time point, a reduction in the CEA could be detected for *E. albidus* exposed to Cd and Zn after 2 days of exposure (fig. 3).

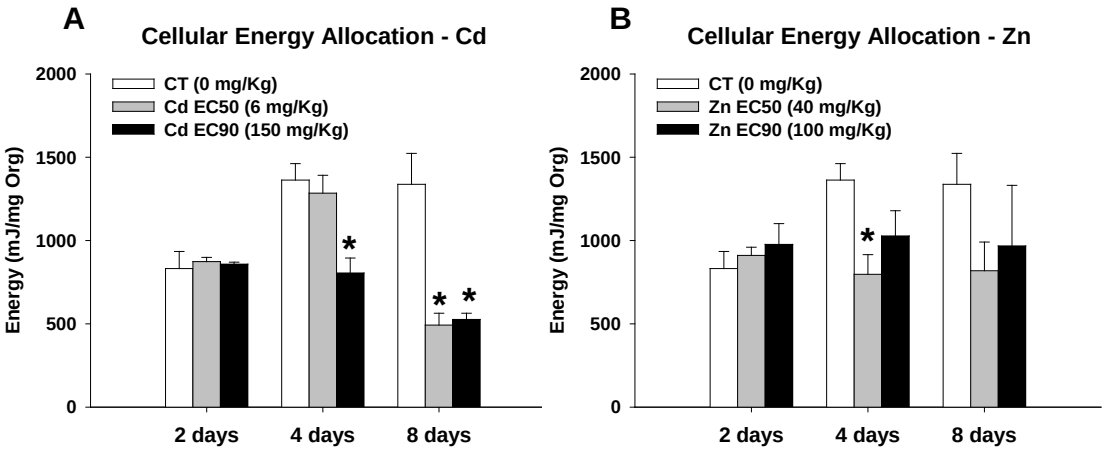

Figure 3: Cellular energy allocation in *E. albidus* over 2, 4 and 8 days of exposure to two concentrations (reproduction EC_{50} and EC_{90}) of cadmium (**A**) and zinc (**B**). Results express average values + standard error. * indicates statistically significant differences between control (CT) and spiked soil (Dunnett's $p<0.05$).

Lipid and carbohydrate contents were the main responsible for the decrease of the CEA in exposures longer than 2 days, along with the increased ETS activity. This decrease in the net energy budget indicated less energy available, e.g. to cope with other physiological functions. This could partly explain the reduction on the reproductive output visible later, after 42 days of exposure (Novais, et al., 2011).

4. Conclusions

Energy metabolism of *E. albidus* was affected by both Cd and Zn, as indicated by the reduced amount of energy reserves and disturbance of the mitochondrial electron transport system (due to increased cellular respiration). These effects resulted in a reduced energy budget which, along with the oxidative damage on the membrane cells, further explains the decrease on the reproductive output.

The extra energy requirements for detoxification were further corroborated by transcriptional evidences, where the induction of genes coding for proteins involved in the mitochondrial electron transport system occurred. The good correlation between the CEA and gene expression responses clearly showed a case of successful confirmation, where microarray gene data was confirmed at other levels, hence, was predictive. Given these effects were occurring at concentrations which affected reproduction (EC_{50}, EC_{90}), we believe there is a relation.

This study highlights not only the usefulness of assessing changes in energy allocation as a stress endpoint but also the usefulness of combining endpoints to better understand and predict the mechanisms of action of chemicals in a broader context.

Acknowledgments

This study was sponsored by the funding FEDER through COMPETE-Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia, within the research project NANOkA FCOMP-01-0124-FEDER-008944 (Refª. FCT PTDC/BIA-BEC/103716/2008) and through a PhD grant to Sara Novais (SFRH/BD/36253/2007).

References

Amorim, M.J.B., Gomes, S.I.L., Soares, A.M.V.M., Scott-Fordsmand, J.J., 2011. Energy basal levels and allocation among lipids, proteins and carbohydrate in *Enchytraeus albidus*: changes related to exposure to Cu-salt and Cu-nanoparticles. Water, Air, Soil Pollut., In press.

Amorim, M.J.B., Novais, S., Römbke, J., Soares, A.M.V.M., 2008. *Enchytraeus albidus* (Enchytraeidae): A test organism in a standardised avoidance test? Effects of different chemical substances. Environ. Int. 34, 363-371.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911-917.

Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248-254.

De Coen, W., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV.Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J. Aquat. Ecosyst. Stress Recovery 6, 43-55.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22, 1632-1641.

Egeler, P., Gilberg, D., Scheffczyk, A., Moser, T., Römbke, J., 2009. Validation of a Soil Bioaccumulation Test with Terrestrial Oligochaetes by an International Ring Test, Report to the Federal Environmental Agency, R&D No.: 204 67 458: 149 pp. Available for download at: http://www.oecd.org/dataoecd/12/20/42552727.pdf.

Henle, F.G.J., 1837. Ueber Enchytraeus, eine neue Anneliden-Gattung. Arch. Anat. Physiol. Med., 74-90.

Holmstrup, M., Sorensen, J.G., Overgaard, J., Bayley, M., Bindesbol, A.M., Slotsbo, S., Fisker, K.V., Maraldo, K., et al., 2011. Body metal concentrations and glycogen reserves in earthworms (*Dendrobaena octaedra*) from contaminated and uncontaminated forest soil. Environ. Pollut. 159, 190-197.

Ireland, M.P., Richards, K.S., 1977. Occurrence and Localization of Heavy-Metals and Glycogen in Earthworms *Lumbricus Rubellus* and *Dendrobaena Rubida* from a Heavy-Metal Site. Histochemistry 51, 153-166.

ISO, 2004. Soil quality - Effects of pollutants on Enchytraeidae (Enchytraeus sp.) - Determination of effects on reproduction and survival, Guideline no. 16387, ISO (International Organization for Standardization). Geneve, Switzerland.

King, F.D., Packard, T.T., 1975. Respiration and the activity of the respiratory electron transport system in marine zooplankton. Limnol. Oceanogr. 20.

Lock, K., Janssen, C.R., 2001a. Cadmium toxicity for terrestrial invertebrates: Taking soil parameters affecting bioavailability into account. Ecotoxicology 10, 315-322.

Lock, K., Janssen, C.R., 2001b. Effect of clay and organic matter type on the ecotoxicity of zinc and cadmium to the potworm *Enchytraeus albidus*. Chemosphere 44, 1669-1672.

Lock, K., Janssen, C.R., 2001c. Zinc and cadmium body burdens in terrestrial oligochaetes: use and significance in environmental risk assessment. Environ Toxicol Chem 20, 2067-2072.

Lock, K., Janssen, C.R., 2003. Comparative toxicity of a zinc salt, zinc powder and zinc oxide to *Eisenia fetida*, *Enchytraeus albidus* and *Folsomia candida*. Chemosphere 53, 851-856.

McLaughlin, M.J., Hamon, R.E., Parker, D.R., Pierzynski, G.M., Smolders, E., Thornton, I., Welp, G., 2002. Soil Chemistry, in: Fairbrother, A., Glazebrook, P.W., Tarazona, J.V., Straalen, N.M.v. (Eds.), Test Methods to Determine Hazards of Sparingly Soluble Metal Compounds in Soils. Society of Environmental Toxicology and Chemistry (SETAC), pp. 5-85.

Novais, S.C., De Coen, W., Amorim, M.J.B., 2012. Transcriptional responses in *Enchytraeus albidus*: comparison between cadmium and zinc exposure and linkage to reproduction effects. Submitted to Toxicological Sciences.

Novais, S.C., Gomes, S.I.L., Gravato, C., Guilhermino, L., de Coen, W., Soares, A.M.V.M., Amorim, M.J.B., 2011. Reproduction and biochemical responses in *Enchytraeus albidus* (Oligochaeta) to zinc or cadmium exposures. Environ. Pollut. 159, 1836-1843.

OECD, 2004. Guidelines for the testing of chemicals. Enchytraeid Reproduction Test, No. 220, OECD (Organization for Economic Cooperation and Development). Paris, France.

Pestana, J.L.T., Re, A., Nogueira, A.J.A., Soares, A.M.V.M., 2007. Effects of Cadmium and Zinc on the feeding behaviour of two freshwater crustaceans: *Atyaephyra desmarestii* (Decapoda) and *Echinogammarus meridionalis* (Amphipoda). Chemosphere 68, 1556-1562.

SigmaPlot, 1997. Statistical Package for the Social Sciences - SigmaPlot for Windows, 11.0 ed, Chicago, IL, USA.

Smolders, R., Bervoets, L., De Coen, W., Blust, R., 2004. Cellular energy allocation in zebra mussels exposed along a pollution gradient: linking cellular effects to higher levels of biological organization. Environ. Pollut. 129, 99-112.

Soetaert, A., Vandenbrouck, T., van der Ven, K., Maras, M., van Remortel, P., Blust, R., de Coen, W.M., 2007. Molecular responses during cadmium-induced stress in *Daphnia magna*: Integration of differential gene expression with higher-level effects. Aquat. Toxicol. 83, 212-222.

Wicklum, D., Davies, R.W., 1996. The effects of chronic cadmium stress on energy acquisition and allocation in a freshwater benthic invertebrate predator. Aquatic Toxicology 35, 237-252.

Chapter VII

Gene expression responses in *Enchytraeus albidus* – exposure to the reproduction EC_{10}, EC_{20}, EC_{50} and EC_{90} of dimethoate, atrazine and carbendazim

VII – Gene expression responses in *Enchytraeus albidus* – exposure to the reproduction EC_{10}, EC_{20}, EC_{50} and EC_{90} of dimethoate, atrazine and carbendazim

Sara C. Novais, Wim De Coen & Mónica J.B. Amorim

Submitted to Toxicological Sciences (IF = 5.093; 2010)

Abstract

Molecular mechanisms of response to pesticides are scarce and information on such responses from soil invertebrates is almost inexistent. *Enchytraeus albidus* is a standard model species for soil toxicology assessment. With the recent microarray development additional information can be retrieved also on the molecular effects. The main goal of this study was to investigate the transcription responses of *E. albidus* when exposed for 2 days to a range of concentrations (reproduction EC_{10}, EC_{20}, EC_{50} and EC_{90}) of three pesticides – an insecticide (dimethoate), an herbicide (atrazine) and a fungicide (carbendazim) – and further identify key biological processes affected by each compound and if dose-related. The Cellular Energy Allocation (CEA) was assessed along with the microarray experiment. Although, results did not reflect the gene expression alterations; we recommend longer exposures (8 days) for this endpoint. Gene effects showed that, in general, higher concentrations affected more transcripts than the lower ones. All three pesticides significantly affected biological processes like translation, regulation of the cell cycle or general response to stress. Intracellular signalling and microtubule-based movement were affected by dimethoate and carbendazim whereas atrazine affected biological processes like lipid and steroid metabolism (also affected by dimethoate) or carbohydrate metabolism (also affected by carbendazim). Response to DNA damage/DNA repair was a process only found to be significantly affected after carbendazim exposures. Mechanisms of action of tested compounds were comparable to the ones in mammals, supporting across species

conserved modes of action. Summing, changes in gene expression were dose-related and significant after 2 days. The present highlighted the advantage of the method as being fast, sensitive, and highly informative for pesticide screening toxicity. It further contributed to (a) understand the effects at reproduction level, while (b) indicating its potential use in risk assessment and as (c) early warning. Finally, *E. albidus* is a model species within the 3R concept - refinement, reduction and replacement of animal testing – and potentially useful to read across species.

1. Introduction

Pesticides are a common source of pollution, being present at a large scale in many European soils. Main source is the extensive use in human activities such as agriculture. Pesticides are designed to affect a certain class of organisms (e.g. insecticides, herbicides, fungicides) but they also affect non-target organisms. The modes of action on how these pesticides affect non-target species are even less known and to understand its risks still constitutes a challenge.

Dimethoate is one of the most used insecticides in agricultural fields and it is known as a cholinesterase inhibitor acting at the cholinergic synapses of insects (IPCS, 1989). This ability to inhibit cholinesterases have been demonstrated for other groups of organisms like freshwater shrimps (Kumar et al., 2010), chironomids (Domingues et al., 2007), fish (Frasco and Guilhermino, 2002) or earthworms (Dell'Omo et al., 1999). Besides these effects related to its mode of action in insects, dimethoate have been described to inhibit steroidogenesis in rats (Walsh et al., 2000). Atrazine also widely applied, is an herbicide which has the function of inhibiting photosynthesis in plants photosystem II. In frogs, fish and rats, atrazine has been described as a possible endocrine disruptor and as an immunotoxin (Bisson and Hontela, 2002; Laws et al., 2003; McMullin et al., 2004; Hecker et al., 2005; Brodkin et al., 2007). Carbendazim is the predominant metabolite of the systemic broad spectrum fungicide benomyl, known for affecting the nucleus division by inhibiting microtubule assembly in fungi (Davidse and Flach, 1977; Davidse, 1986). The

antimitotic action of carbendazim has also been described for mammals (De Brabander et al., 1976).

Toxicity mechanisms of these three pesticides on invertebrates, and in particular on soil invertebrates, are by far less known. Studies on *Enchytraeus albidus* (Oligochaeta), an ecologically relevant and often used test species for soil toxicity testing (ISO, 2004; OECD, 2004), have reported effects of these pesticides on survival, reproduction and avoidance behaviour (Rombke and Moser, 2002; Amorim et al., 2008; Kobeticova et al., 2009; Loureiro et al., 2009; Novais et al., 2010). The mechanisms of such toxicity are still to be understood.

With the cDNA microarray for *E. albidus* (Amorim et al., 2011; Novais et al., 2011c) the first steps were given into the development of a tool which potentially improve the knowledge on the molecular mechanisms of pesticide toxicity. This microarray was enhanced (Agilent oligonucleotide microarray) with the development of two new cDNA libraries enriched for metal and pesticide exposures and all the sequence information was made available in EnchyBASE (Novais et al., 2011a).

The main goal of the present study was to investigate the early transcription responses of *E. albidus* to three pesticides with different mode of action: dimethoate, atrazine and carbendazim (as mentioned above). Ultimately, to also identify key biological processes affected that indicate mechanisms of toxic action for each pesticide. Gene expression studies of pesticides in invertebrates are still scarce and, to the author's knowledge, this is the first transcriptomic study of dimethoate and carbendazim effects in invertebrates. Transcription effects of atrazine have been assessed in a few studies (Reichert and Menzel, 2005; Owen et al., 2008; Svendsen et al., 2008).

In order to establish a link between the transcription responses and effects at higher levels of biological organization, organisms were exposed for 2 days to a range of concentrations with known effects on reproduction – EC_{10}, EC_{20}, EC_{50} and EC_{90} – causing 10, 20, 50 and 90% reduction respectively. Additionally, along with the transcription responses, Cellular Energy Allocation (CEA) was assessed, i.e. the energy budget of the organisms and its stress induced effects. This method was proposed by De Coen and Janssen (1997, 2003) who identified the usefulness of this endpoint in the prediction of long-term effects.

2. Materials and methods

2.1 Maintenance of test species

Enchytraeus albidus (Henle, 1837) were maintained in laboratory cultures under controlled conditions with a photoperiod 16:8h light:dark and a temperature of 18ºC. Animals were fed twice a week with finely ground and autoclaved rolled oats.

2.2 Soil and spiking of the test substances

Spiking of all pesticides was performed in the natural standard soil LUFA 2.2 [pH ($CaCl_2$) = 5.5; Organic Matter = 4.4%, texture = 6% clay; 17% silt; 77% sand]. Three pesticides were used in this study: the insecticide dimethoate [Sigma-Aldrich (Riedel-de Haën), 99.8%], the herbicide atrazine (Sigma-Aldrich, 97.4%) and the fungicide carbendazim (Sigma-Aldrich, 97%). Dimethoate was spiked into the pre-moistened soil as aqueous solution, each test concentration into the whole batch of soil for all replicates. For the non-water soluble chemicals (atrazine and carbendazim) acetone was used as solvent, being this solution homogeneously mixed with the soil and left to evaporate overnight. For these, a solvent control was also performed – control soil spiked with the same amount of acetone. The soils were then moistened to 50% of the soil maximum water holding capacity (WHC).

Four concentrations of each pesticide were tested, based on previous results on reproduction effects (Novais et al., 2010). The concentrations used were in the range of the 95% confidence intervals for the reproduction EC_{10}, EC_{20}, EC_{50} and EC_{90} and are presented in table 1.

Table 1. Concentrations of dimethoate, atrazine and carbendazim used for exposures in *E. albidus*.

	EC_{10} (mg/kg)	**EC_{20} (mg/kg)**	**EC_{50} (mg/kg)**	**EC_{90} (mg/kg)**
Insecticide				
Dimethoate	0.1	0.5	2.0	25
Herbicide				
Atrazine	0.2	0.5	3.0	50
Fungicide				
Carbendazim	0.05	0.1	0.5	3.0

2.3 Exposure conditions

Six replicates per treatment were performed: three for microarray analysis and three for the Cellular Energy Allocation (CEA) measurements. Fifteen adult worms with well developed clitellum were introduced in the test vessels, each containing 25g of moist soil. The worms were exposed for 2 days at 20ºC and a 16:8h photoperiod. In the end of the exposure, animals of each replicate for microarray analysis were carefully removed, rinsed in deionised water, stored in RNA later (Ambion, USA) containing criotubes and frozen in liquid Nitrogen. In the replicates for CEA analysis, the procedure was identical but the organisms were weighted and frozen in liquid Nitrogen without the RNA later. All the samples were stored at -80ºC till further analysis.

2.4 RNA extraction, labelling, hybridizations and microarray analysis

Three biological replicates were used of each pesticide exposure condition and also of both control and solvent control conditions. The procedures followed for the microarray

experiment were the same as described in Novais et al. (2012a). Briefly, isolation of total RNA from each replicate was performed with the Trizol extraction method (Invitrogen, Belgium) followed by a DNAse treatment (Fermentas, Germany) and further purification steps consisting of phenol/chloroform extractions. The quantity and purity of the isolated RNA were measured with nanodrop (NanoDrop ND-1000 Spectrophotometer) and its integrity was checked on a denaturing formaldehyde agarose gel electrophoresis.

A single-colour design was used starting from 500 ng of total RNA which was amplified and labelled with the Agilent Low Input Quick Amp Labelling Kit (Agilent Technologies, Palo Alto, CA, USA). Positive controls were added with the Agilent one-colour RNA Spike-In Kit (Agilent Technologies, Palo Alto, CA, USA). Purification of the amplified and labelled cRNA was performed with the RNeasy columns (Qiagen, Valencia, CA, USA).

The cRNA samples were hybridized on Custom Gene Expression Agilent Microarrays (8 x 15k format) developed for this species (Novais et al., 2011a). Hybridization was performed using the Agilent Gene Expression Hybridization Kit (Agilent Technologies, Palo Alto, CA, USA) and each biological replicate was individually hybridized on one array. After the 17h hybridization at 65 ºC with a rotation of 10 rpm, microarrays were washed using Agilent Gene Expression Wash Buffer Kit (Agilent Technologies, Palo Alto, CA, USA) and scanned with the Agilent DNA microarray scanner G2505B (Agilent Technologies). A total of 42 hybridizations resulted from the present microarray experimental design (four concentrations – EC_{10}, EC_{20}, EC_{50} and EC_{90} – of three pesticides – dimethoate, atrazine and carbendazim – in triplicate, plus three replicates of control and solvent control conditions).

Fluorescence intensity data was obtained with Feature Extraction (10.5.1.1) Software (Agilent Technologies). Quality control was done by inspecting the reports on the Agilent Spike-in control probes and by making box plots of each array. Processing of the data and statistical analysis were performed using BRB Array Tools version 4.1 Stable Release (http://linus.nci.nih.gov/BRB-ArrayTools.html). After background subtraction, the replicated spots within each array were averaged and the intensities were $\log_2$ transformed. Data was then normalized using median array as reference. Raw and processed data are

available from Gene Expression Omnibus (GEO) at the NCBI website (platform: GPL14928; series: GSE33945).

Given that two of the pesticides were dissolved in acetone (atrazine and carbendazim), comparisons of those exposure treatments were made against the solvent control, whereas the dimethoate treatments were compared to the water control. Statistical class comparison was performed between those different groups of arrays using two-sample t-test with 95% confidence level for the assessment of differentially expressed genes and the $\log_2$ ratios of expression between those classes were calculated and used for further analysis.

Annotation of the differentially expressed genes ($p<0.05$) was performed based on their similarity to sequences in the National Centre for Biotechnology Information (NCBI) database as determined by the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The sequences were submitted to Blast2GO (Conesa et al., 2005) being compared with peptide sequence databases using BLASTX analysis (with minimum 10^{-5} *e*-value). GO term enrichment analysis (Alexa et al. 2006) was performed for the differentially expressed genes using the same Blast2GO software. Clustering and principal component analyses (PCA) were performed using MultiExperiment Viewer (MeV, TIGR). The in each time point were separately analysed for

2.5 Cellular Energy Allocation – CEA

CEA was measured as described in Novais et al. (2012b) with slight modifications. The main change was that the 15 organisms of each replicate were not split into three groups of 5 organisms but were rather homogenised as a whole in 1000 µL of deionised water, to measure all the endpoints necessary for the CEA determination from the same exact replicate. The homogenised solution was then divided transferring 300 µL to three different eppendorfs: one for the total protein and carbohydrate content determinations, one other for the total lipid content determination and the last for the measurement of electron transport system activity.

Protein content was determined according to the Bradford method (Bradford, 1976) at 600 nm using bovine serum albumin as standard. Total carbohydrate content was determined with phenol 5% and concentrated H_2SO_4 at 490 nm using glucose as standard. Total lipids were extracted according to the method described by Bligh and Dyer (1959) and determined by measuring the absorbance at 400 nm using Tripalmitine (Sigma) as standard. The available energy reserves content measured in 300 µL was then extrapolated for the total volume of 1000 µL and transformed into energetic equivalents using enthalpy combustion (24 kJ/g proteins, 17.5 kJ/g carbohydrates and 39.5 kJ/g lipids) as described in De Coen and Janssen (1997, 2003).

The consumed energy (consumed oxygen rate) was determined based on the measurement of the electron transport system activity (King and Packard, 1975) from day 0 to 2 days of exposure. The electron transport activity was measured by adding NADPH solution and INT (*p*-IodoNitroTetrazolium, Sigma) and following the increase in absorbance at 490 nm for 3 minutes. The oxygen consumption rate was then transformed into caloric values using oxyenthalpic equivalents (480 KJ/mol O_2).

The CEA was then calculated by integrating the available and consumed energy over the period of 2 days of exposure. Significant differences between the control and dimethoate treatments or between solvent control and atrazine and carbendazim exposure conditions, were determined for all endpoints by one way analysis of variance (ANOVA) with Dunnett's method for multiple comparisons (SigmaPlot, 1997).

3. Results

3.1 Transcription responses to pesticide exposures

Transcription dose-responses were determined for the three pesticides from different classes: the insecticide dimethoate, the herbicide atrazine and the fungicide carbendazim.

Analysis of the statistical class comparison results (two sample *t*-tests, $p<0.05$) between control and each of the dimethoate treatments resulted in a total of 317 significant differentially expressed genes. The comparison between solvent control and atrazine or carbendazim conditions resulted in 161 and 334 significant differentially expressed genes, respectively. From these transcripts, 135 (43%), 51 (32%) and 127 (38%) for dimethoate, atrazine and carbendazim exposures respectively, match known proteins in public databases. The complete list of affected genes with significant blast homologies, is given in Table S1 (supporting information).

The total number of over and under expressed transcripts in each of the pesticide conditions is represented in figure 1.

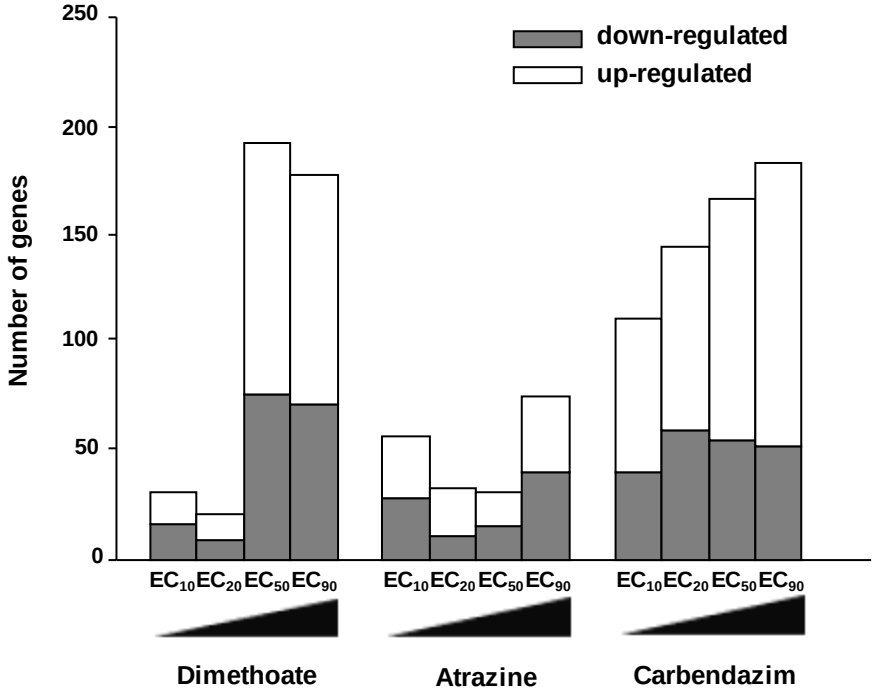

Figure 1. Number of significant up- and down-regulated genes (two sample *t*-tests, $p<0.05$) in *E. albidus* after exposure to dimethoate, atrazine and carbendazim in four different concentrations (reproduction EC_{10}, EC_{20}, EC_{50} and EC_{90}). Values refer to the comparison with control for each time of exposure.

In general, higher concentrations of the tested pesticides affected more transcripts than lower ones. Atrazine affected the lowest number of transcripts in total (fig. 1). In exposure to carbendazim it was possible to observe dose-response relation, increasing the number of differentially expressed genes with increasing concentration. There was also a tendency for higher up-regulation with increasing concentration (fig.1).

The effect of dosage on the transcriptional profiles becomes more clear (fig. 2) from the clustering analysis of samples from each pesticide treatment.

The gene expression was concentration dependent but the pattern between dosages was distinct for the different pesticides. In dimethoate exposures (fig.2 – A), the two lower and two higher concentrations were grouped separately, whereas for atrazine and carbendazim the concentrations that cause 20% and 50% effect on reproduction were more closely related.

Expression profiles show distinct patterns for each pesticide (fig. 2), suggesting that responses occur through different molecular pathways. These different responses are depicted by the different directions in which the same genes are affected (up- or down-regulation) and by the uniquely affected transcripts in each pesticide exposure.

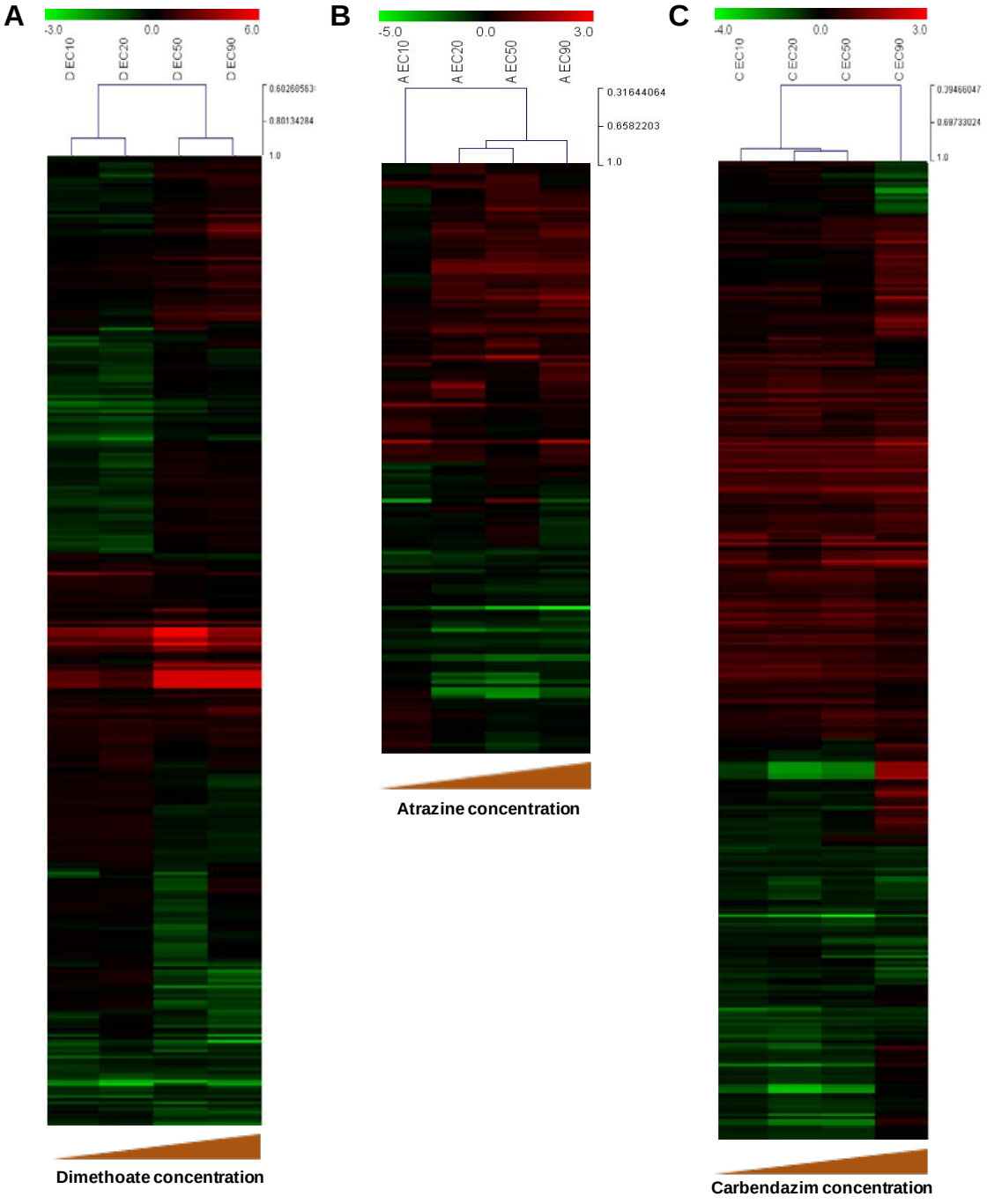

Figure 2. Heat map and hierarchical clustering of samples (Pearson's uncentered) based on the differentially expressed genes (two sample *t*-test, $p < 0.05$) in *E. albidus* when comparing: **A –** dimethoate exposure treatments against control; **B –** atrazine exposure treatments against solvent control; **C –** carbendazim exposure treatments against solvent control.

The number of common and uniquely affected transcripts is represented in the Venn diagram of figure 3.

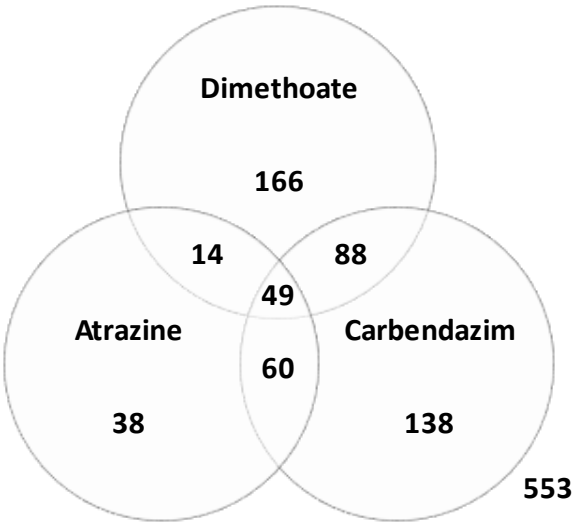

Figure 3. Venn diagram representing the number of differentially expressed genes (two sample *t*-test, $p < 0.05$) shared by the three pesticides.

The number of overlapping genes (fig. 3), as an indicative of a common response, is higher between carbendazim and dimethoate. Atrazine seems to induce a more similar response to carbendazim than to dimethoate and, in fact, only 38 transcripts were exclusively affected by this herbicide. Overall, 49 transcripts are affected by all compounds which may represent general stress responses to stress. From the 49 genes, 11 have known homologies and code for e.g. heat shock protein 90, lombricine kinase, neutral and basic amino acid transport protein or integrin-linked kinase-associated serine threonine phosphatase 2c (ILKAP).

The seven different sets of differentially expressed genes, as presented in the Venn diagram (fig. 3), correspond to 3 lists of uniquely affected transcripts by each pesticide and 4 lists of transcripts shared by two or three of these compounds. Those lists were used to perform an improved gene set enrichment analysis of GO terms (Alexa et al., 2006) and evaluate the biological functions significantly affected in each case (table 2). All differentially expressed genes, with significant blast homologies, present in each of the seven lists used for this analysis can be found in Table S2 (supporting information).

Table 2. Significant enriched GO terms ($p < 0.05$) in the following lists of differentially expressed genes: 1) uniquely affected by dimethoate; 2) uniquely affected by atrazine; 3) uniquely affected by carbendazim; 4) affected by dimethoate and atrazine; 5) affected by dimethoate and carbendazim; 6) affected by atrazine and carbendazim; 7) affected by the three pesticides. Only the biological process results are given.

GO ID	GO term definition	Library annotated ESTs[1]	Significant[2]	p-Value
Genes uniquely affected by dimethoate				
GO:0010035	response to inorganic substance	3	3	0.0039
GO:0045214	sarcomere organization	4	3	0.0139
GO:0050896	response to stimulus	23	8	0.0184
GO:0032989	cellular component morphogenesis	11	5	0.0190
GO:0051592	response to calcium ion	2	2	0.0252
GO:0007163	establishment or maintenance of cell polarity	2	2	0.0252
GO:0016070	RNA metabolic process	20	7	0.0266
Genes uniquely affected by atrazine				
GO:0051641	cellular localization	8	2	0.0199
GO:0031532	actin cytoskeleton reorganization	1	1	0.0293
GO:0007155	cell adhesion	1	1	0.0293
GO:0032507	maintenance of protein location in cell	1	1	0.0293
GO:0070972	protein localization in endoplasmic reticulum	1	1	0.0293
GO:0008360	regulation of cell shape	1	1	0.0293
GO:0006616	SRP-dependent cotranslational protein targeting to membrane	1	1	0.0293
Genes uniquely affected by carbendazim				
GO:0051716	cellular response to stimulus	4	3	0.0064
GO:0048870	cell motility	4	3	0.0064
GO:0006950	response to stress	13	5	0.0135
GO:0006066	alcohol metabolic process	5	3	0.0145
GO:0006259	DNA metabolic process	5	3	0.0145
GO:0051103	DNA ligation involved in DNA repair	2	2	0.0148
GO:0016477	cell migration	2	2	0.0148
GO:0007243	intracellular protein kinase cascade	2	2	0.0148
GO:0043408	regulation of MAPKKK cascade	2	2	0.0148
GO:0023014	signal transmission via phosphorylation event	2	2	0.0148
GO:0006412	translation	36	9	0.0206
GO:0044267	cellular protein metabolic process	56	12	0.0254
GO:0040011	locomotion	6	3	0.0266
GO:0048511	rhythmic process	3	2	0.0408
GO:0042274	ribosomal small subunit biogenesis	3	2	0.0408
GO:0006281	DNA repair	3	2	0.0408
GO:0006974	response to DNA damage stimulus	3	2	0.0408
GO:0010033	response to organic substance	7	3	0.0425

GO:0006414	translational elongation	12	4	0.0469
Genes affected by dimethoate and atrazine				
GO:0008202	steroid metabolic process	2	2	0.0004
GO:0006629	lipid metabolic process	6	2	0.0057
GO:0016070	RNA metabolic process	20	3	0.0062
GO:0008203	cholesterol metabolic process	1	1	0.0213
GO:0016101	diterpenoid metabolic process	1	1	0.0213
GO:0006775	fat-soluble vitamin metabolic process	1	1	0.0213
GO:0001523	retinoid metabolic process	1	1	0.0213
GO:0006721	terpenoid metabolic process	1	1	0.0213
GO:0006396	RNA processing	12	2	0.0237
GO:0006412	translation	36	3	0.0326
GO:0010467	gene expression	99	5	0.0328
Genes affected by dimethoate and carbendazim				
GO:0022607	cellular component assembly	20	5	0.0230
GO:0007018	microtubule-based movement	4	3	0.0024
GO:0043623	cellular protein complex assembly	7	4	0.0015
GO:0023052	signaling	15	4	0.0340
GO:0023034	intracellular signaling pathway	6	3	0.0104
Genes affected by atrazine and carbendazim				
GO:0007040	lysosome organization	1	1	0.0267
GO:0006643	membrane lipid metabolic process	1	1	0.0267
GO:0006665	sphingolipid metabolic process	1	1	0.0267
GO:0007033	vacuole organization	1	1	0.0267
GO:0005975	carbohydrate metabolic process	12	2	0.0367
Genes affected by all 3 pesticides				
GO:0044267	cellular protein metabolic process	56	5	0.0049
GO:0006486	protein amino acid glycosylation	1	1	0.0240
GO:0046209	nitric oxide metabolic process	1	1	0.0240
GO:0042026	protein refolding	1	1	0.0240
GO:0006470	protein amino acid dephosphorylation	1	1	0.0240
GO:0007090	regulation of S phase of mitotic cell cycle	1	1	0.0240
GO:0006464	protein modification process	12	2	0.0299
GO:0034621	cellular macromolecular complex subunit organization	15	2	0.0457
GO:0006986	response to unfolded protein	2	1	0.0475
GO:0006449	regulation of translational termination	2	1	0.0475

[1] The number of annotated ESTs present in the library within the identified GO terms is indicated for comparison; [2] The number of GO term annotated differentially expressed ESTs in each of the seven gene lists.

GO enrichment analysis determined several biological processes (GO terms) as significantly more abundant in our differentially expressed gene lists, than would be expected by chance (Table 2). Based on this analysis, all three pesticides significantly affected ($p < 0.05$) biological processes like translation, regulation of the cell cycle or response to stress with chaperone proteins. Intracellular signalling and microtubule-based movement were biological processes found to be significantly affected after exposure to dimethoate and carbendazim. Atrazine affected other biological processes like lipid, steroid and RNA metabolisms (also affected by dimethoate) and carbohydrate metabolism (also affected by carbendazim). Response to DNA damage/DNA repair was a process only found to be significantly affected after carbendazim exposures. All significant differential transcripts within each GO term are given in Table S3 (supporting information).

Although several genes, and consequently biological processes, are affected by two or even by the three pesticides tested, some of these transcript expressions are negatively correlated, which can be seen in the heat map with the whole gene expression profiles (fig. 4 – A). From this clustering analysis it is possible to observe that effects of atrazine and carbendazim are more closely related than effects of dimethoate. The behaviour of gene expression change across the range of concentrations of each pesticide is represented in figure 4 for some of the significant differentially expressed transcripts involved in the biological processes mentioned above (figure 4 – B).

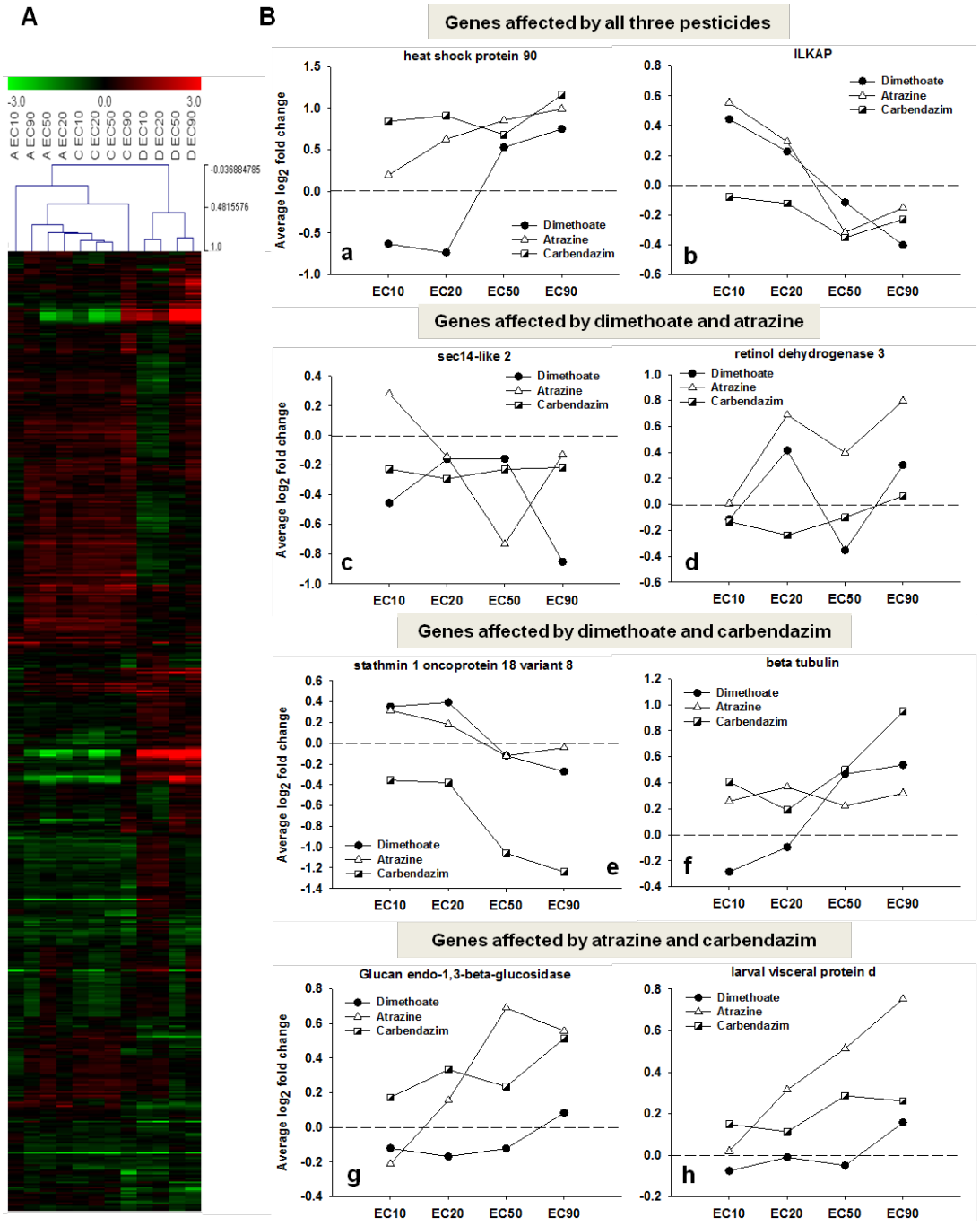

Figure 4. A - Heat map and hierarchical clustering of samples (Pearson's uncentered) based on the differentially expressed genes (two sample *t*-test, $p < 0.05$) in *E. albidus* exposed to four concentrations of dimethoate, atrazine and carbendazim (reproduction EC_{10}, EC_{20}, EC_{50} and EC_{90}). **B –** Expression behavior of some transcripts significantly affected by two or by the three pesticides (dimethoate, atrazine and carbendazim), across a range of concentrations: **a** – heat shock protein 90; **b** – integrin-linked kinase-associated serine threonine phosphatase 2c (ILKAP); **c** – sec 14-like 2; **d** – retinol dehydrogenase 3; **e** – stathmin 1 oncoprotein 18; **f** – beta tubulin; **g** – glucan endo-1,3-beta-glucosidase; **h** – larval visceral protein d.

As it can be seen for some of the differentially expressed genes in figure 4B, these are affected by more than one pesticide, but the expression pattern differs with increasing exposure concentrations (e.g. sec 14-like 2; larval visceral protein d). Moreover, in other cases genes have the same dose-effect relations but the intensities of expression are different (e.g. retinol dehydrogenase 3) or even opposite (e.g. stathmin 1 oncoprotein 18, at EC_{10} and EC_{20}).

3.2 Cellular Energy Allocation – CEA

Effects of dimethoate, atrazine and carbendazim on the total net energy budget of the organisms were assessed simultaneously in a parallel experiment. These effects were determined by integrating the available energy (Ea) and the consumed energy (Ec – mitochondrial ETS) over the 2 days of the exposure (fig. 5).

Effects of dimethoate on the energy reserves were observed by a significant decrease on the carbohydrates fraction at the EC_{50} and EC_{90}, on protein content in all concentrations and a minor increased lipid fraction at the reproduction EC_{20} (fig. 5 – A). Effects of atrazine were observed at the EC_{20} with an increase in both lipid and protein fractions (fig. 5 – B). As for carbendazim, there was an increase in the lipid content at concentrations higher than the reproduction EC_{20} and a decrease in the carbohydrates at the EC_{90} (fig. 5 – C).

The cellular respiration rate (energy consumption) measured by the ETS activity was increased at all dimethoate concentrations and also at the carbendazim reproduction EC_{50} (fig. 5 – D, F). No significant alterations on the ETS activity were observed after atrazine exposures (fig. 5 – E).

Results showed no significant decreases on the cellular energy allocation for none of the tested pesticides (fig. 5 – G, H, I) and for atrazine and carbendazim the energy budget increased in some concentrations.

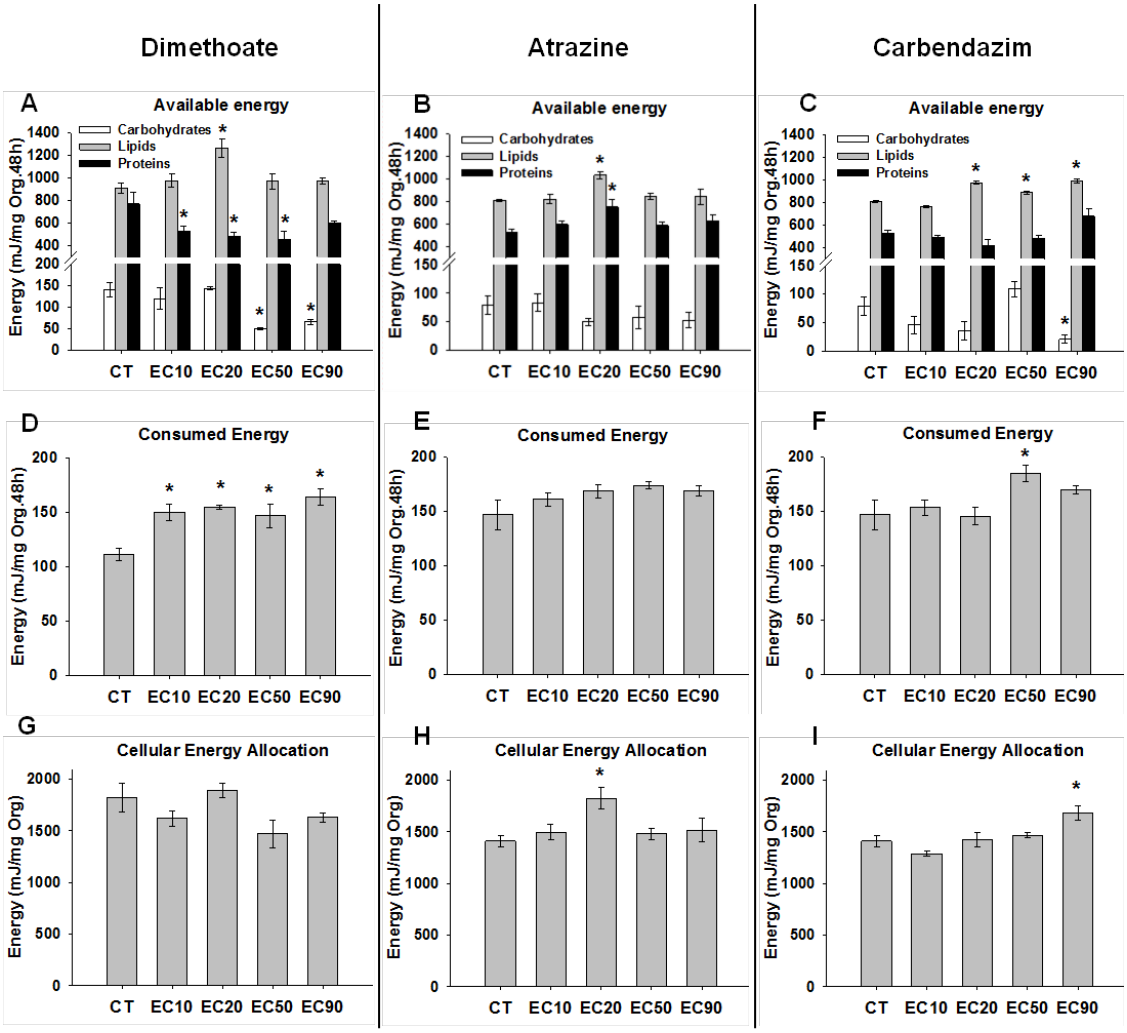

Figure 5. Effects of exposure to four concentrations (reproduction EC_{10}, EC_{20}, EC_{50} and EC_{90}) of dimethoate (left), atrazine (centre) and carbendazim (right) in *Enchytraeus albidus* for 2 days on: **A, B, C** – the energy reserves available (carbohydrates, lipids, proteins); **D, E, F** – the energy consumption (electron transport activity); **G, H, I** – cellular energy allocation. Results express average values ± standard error. * indicates statistically significant differences between control (CT) and treatments (Dunnett's $p<0.05$).

4. Discussion

Overall, and considering all the concentrations, the herbicide atrazine was the compound that induced less gene expression changes. This is not particularly surprising given this is a compound designed to affect mainly plant organisms. Additionally, studies in fish and

human cell lines with atrazine did not reveal significant changes in gene expression (Tchounwou et al., 2001; Sanchez et al., 2011).

In general there was an increase in the number of affected transcripts with increasing concentrations. This tendency was also observed in a study with *E. albidus* exposed to Cd and Zn (Novais et al., 2012a). On the contrary, this was not the pattern when Cu was tested in this species (Gomes et al., 2011) or phenanthrene in *Folsomia candida* (Nota et al., 2009), Although the respective ECx reproduction values were tested for all pesticides, the gene expression correlation between concentrations were different depending on the pesticide (fig. 2). This further corroborates the different mechanisms underlying the reduction on the reproductive rate. It is hard to say with the present test design, but it is possible that gene results are in agreement with the steepness of the reproduction dose-responses as observed for the different chemicals (Novais et al., 2010).

Still, as shown in the Venn diagram (fig. 3), certain gene responses are shared by all three pesticide exposures or by two of them. Due to the fact that *E. albidus* does not have a sequenced genome, many of the significant transcripts have no similarity to known proteins. Hopefully, with future sequencing efforts and with the growing genomic data on invertebrate species, more transcripts will be annotated.

The lists of unique and commonly differentially expressed transcripts (with known homologies) were subjected to a gene set enrichment analysis of GO terms and some different biological processes were found to be significantly affected (table 2).

Dimethoate affected transcripts related with sarcomere organization, maintenance of cell polarity and response to calcium ion. These transcripts code for several actin, calponin, toponin and sarcoplasmic calcium binding proteins which were significantly affected mainly at the EC_{10} and EC_{20} dimethoate concentrations. The clear separation in expression between the lowest and highest concentrations were also observed for several transcripts related with the electron transport system, from complex I (NADH dehydrogenase), complex III (cytochrome b) and complex IV (cytochrome c oxidase) and ATP synthase from which ultimately the energy is produced. All of these transcripts were up-regulated in lower doses and then down-regulated at higher doses, suggesting an inhibition of the electron transport chain and consequent ATP production with increasing concentrations.

Dysfunction of the mitochondrial respiratory chain have been associated with increased peroxide and hydrogen peroxide production in cells (Turrens, 1997), and there is a strong evidence that this is the case in dimethoate exposures shown by the concentration-dependent induction of the transcript coding for the enzymatic antioxidant superoxide dismutase (SOD).

Information on mechanisms of dimethoate toxicity in other organisms is very limited and what is known is that this compound has the ability to 1) inhibit acetylcholinesterase activity in several organisms (Dell'Omo et al., 1999; Kumar et al., 2004; Domingues et al., 2007) and to 2) have an influence on the metabolic pathways controlled by steroid hormones in rats (Walsh et al., 2000; Astiz et al., 2009). The inhibition of acetylcholinesterase activity could not be depicted from the present transcriptomic analysis since this particular transcript is not present in the library for this species. Additional work by the authors (Novais et al., 2011b) confirmed this effect, as cholinesterases activity was inhibited after 8 days exposure. As for the second known mechanism, there are evidences in the present study pointing to an inhibition of steroidogenesis. The under-expression of sec14-like 2 transcript (fig. 4B – c), involved in the positive regulation of cholesterol biosynthesis, suggests that less cholesterol will be synthesized and consequently, less steroids generated. This inhibition of steroidogenesis, along with the overexpression of retinol dehydrogenase involved in the metabolism of vitamin A (fig. 4B – d), are common mechanisms between dimethoate and atrazine toxicity. This disturbance on the steroid and retinol metabolisms, producing imbalanced levels of hormones vital for reproduction, can possibly explain the later reduction in the organisms' reproduction and show some insight on the mechanisms of endocrine disruption of these pesticides.

From the uniquely affected transcripts after atrazine exposure, a gene coding for a histone was significantly up-regulated at the EC_{10}. This protein is involved in biological processes related with cell adhesion or the regulation of cell shape and maintenance of DNA integrity. Its functions have also been linked to positive regulation of growth rate and larval development, and its enhancement was reported in a study where *Caenorhabditis elegans* was exposed to atrazine (Swain et al., 2010). Some studies with atrazine have also reported disruption on the mitochondrial electron flow. Owen et al. (2008) observed

significant up-regulation of several transcripts coding for the oxidative phosphorylation pathway in *Lumbricus rubellus.* The proteomic approach used by Thornton et al. (2010) in *Drosophila* m*elanogaster* exposed organisms also showed significant changes on the mitochondrial protein expressions. In our microarray results, transcripts coding for proteins of the electron transport system are mainly up-regulated after the EC_{20}, confirming the assumption that atrazine affects the normal mitochondrial functioning. Along with carbendazim, atrazine also seems to affect the carbohydrate metabolism by enhancing gluconeogenesis. Both glucan endo-1,3-beta-glucosidase (fig. 4B – g) and larval visceral protein d (probable maltase, fig. 4B – h) transcripts are up-regulated after carbendazim and atrazine exposures and with this last compound the dose-response relation is clearly evident. This tendency for an increased glucose storage has also been described in a study by Zaya et al. (2011) where gene expression coding for glycolysis in *Xenopus laevis* suggested inhibition of this energetic process.

Carbendazim was the only pesticide to induce transcripts encoding for intermediate filament proteins which are involved in DNA ligation during DNA repair. Those transcripts are significantly up-regulated at all carbendazim concentrations suggesting DNA damage and the indication of potential genotoxic effect of this pesticide even at low concentrations. Effects of carbendazim on reproduction have been attributed to its well known function to interfere with the assemble of microtubules (Davidse and Flach, 1977), rather than a mechanism involving endocrine disruption (Sherman et al., 1975; Yamada et al., 2005). Our results seem to be in good agreement with that hypothesis. Stathmin 1 oncoprotein 18 and several tubulin transcripts, that are differentially expressed by this compound, code for proteins directly involved in the regulation of cellular proliferation by assembling/disassembling microtubules. Stathmin 1 (oncoprotein 18) gene encodes for a cytoplasmic tubulin-binding phosphoprotein that acts to sequester tubulin and favour microtubule disassembly (Takahashi et al., 2002). Disturbances in the normal expression of stathmin correlate with a decreased inactivation of tubulin, a constant microtubule and mitotic spindle assembly and a consequent incapacity to regulate cell cycle progression (Zhang et al., 2008). For this reason, disturbances in stathmin 1 expression have been associated with several types of cancer (Brattsand, 2000; Takahashi et al., 2002; Zhang et al., 2008).

In the present study, the microtubule assembly/disassembly process seems to be affected not only by carbendazim but also by dimethoate. However, the gene response pattern is different for each pesticide (fig. 4B – e, f). Responses of stathmin 1 and tubulin in relation to the dose followed the same pattern in both pesticide exposures. Nevertheless, while tubulin was significantly up-regulated in both pesticides (except in the lower concentrations of dimethoate), stathmin 1 response was clearly different between the compounds. In dimethoate EC_{10} and EC_{20} the stathmin 1 expression was significantly up-regulated and only after the EC_{50} its inhibition started to occur, while in carbendazim its expression was severely inhibited and in a dose-response related manner.

Other biological processes were affected by all three pesticides like the response to unfolded proteins with the up-regulation of several heat-shock and chaperonins or the impairment of the normal regulation of cell cycle with the dose-dependent down-regulation of ILKAP gene (fig. 4B – a, b). Several transcripts related to protein catabolism were significantly over expressed in each pesticide exposure and, although they were not the same transcripts they all shared the same putative function.

Regarding the CEA results, in general there was a decrease in the carbohydrate content and an increase in the lipid fraction with the pesticide exposures. Protein content was found to be significantly lower in dimethoate exposures and the energy spent with cellular respiration also increased with this insecticide, probably due to the increased metabolism of carbohydrates and proteins. In general, no expressive effects were seen in the overall net energy budget and it appears that the responses at the protein level are not yet reflecting the gene expression changes. However, it seems that the exposure time used (2 days) might have been too short and effects should be seen with longer periods (4-8 days), as observed in a previous study with metal exposures (Novais et al., 2012a).

5. Conclusions

From the expression profile analysis in *E. albidus* exposed to the different pesticides it was possible to successfully identify molecular pathways that were commonly affected by all compounds, indicative of the presence of general response mechanisms to pesticides. Moreover, some specific key pathways were also depicted for each compound. This provided most interesting and novel information on the mechanisms of pesticide toxicity in invertebrates. Interestingly, some of the known mechanisms of action of these compounds in soil invertebrate were comparable to the ones in mammals, suggesting across species conserved modes of action. This also underlines the usefulness of *E. albidus* as a model species within the 3R concept - refinement, reduction and replacement of animal testing – potentially useful to read across species, including human.

The significant changes in gene expression with the different doses (2 days) can help to explain the distinct effects on reproduction (42 days) and also confirms the potentiality of microarray data as an early warning. The fact that genes responded in a dose-related manner also indicated the possible usefulness in risk assessment. Effects were detected after 2 days of exposure, which highlights the advantages as a fast, sensitive, and highly informative method for pesticide screening toxicity.

Although the results show an increase in the number of differentially expressed genes with increasing concentrations, the lowest concentration tested (reproduction EC_{10}) induced significant gene expression alterations and, in some cases, opposite of what was seen at the higher concentrations. For this reason, when the goal is to obtain the maximum mechanistic information on chemical toxicity, it is important to test a range of concentrations to adequately understand the extent of the results. In the present, CEA results did not reflect the gene expression alterations probably due to the short period of exposure. For future experiments, the authors would recommend an extent in exposure duration till 8 days.

Acknowledgments

This study was sponsored by the funding FEDER through COMPETE-Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia, within the research project NANOkA FCOMP-01-0124-FEDER-008944 (Refª. FCT PTDC/BIA-BEC/103716/2008) and through a PhD grant to Sara Novais (SFRH/BD/36253/2007).

References

Alexa, A., Rahnenfuhrer, J., Lengauer, T., 2006. Improved scoring of functional groups from gene expression data by decorrelating GO graph structure. Bioinformatics 22, 1600-1607.

Altschul, S., Gish, W., Miller, W., Meyers, E., Lipman, D., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403- 410.

Amorim, M.J.B., Novais, S., Römbke, J., Soares, A.M.V.M., 2008. *Enchytraeus albidus* (Enchytraeidae): A test organism in a standardised avoidance test? Effects of different chemical substances. Environ. Int. 34, 363-371.

Amorim, M.J.B., Novais, S.C., Van der Ven, K., Vandenbrouck, T., Soares, A.M.V.M., De Coen, W., 2011. Development of a Microarray for *Enchytraeus albidus* (Oligochaeta): Preliminary Tool with Diverse Applications. Environ. Toxicol. Chem. 30, 1395-1402.

Astiz, M., de Catalfo, G.E.H., de Alaniz, M.J.T., Marra, C.A., 2009. Involvement of Lipids in Dimethoate-Induced Inhibition of Testosterone Biosynthesis in Rat Interstitial Cells. Lipids 44, 703-718.

Bisson, M., Hontela, A., 2002. Cytotoxic and endocrine-disrupting potential of atrazine, diazinon, endosulfan, and mancozeb in adrenocortical steroidogenic cells of rainbow trout exposed in vitro. Toxicol. Appl. Pharmacol. 180, 110-117.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Canadian Journal of Biochemistry and Physiology 37, 911-917.

Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brattsand, G., 2000. Correlation of oncoprotein 18/stathmin expression in human breast cancer with established prognostic factors. Br. J. Cancer 83, 311-318.

Brodkin, M.A., Madhoun, H., Rameswaran, M., Vatnick, I., 2007. Atrazine is an immune disruptor in adult northern leopard frogs (*Rana pipiens*). Environ. Toxicol. Chem. 26, 80-84.

Conesa, A., Gotz, S., Garcia-Gomez, J.M., Terol, J., Talon, M., Robles, M., 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21, 3674-3676.

Davidse, L.C., 1986. Benzimidazole Fungicides - Mechanism of Action and Biological Impact. Annu. Rev. Phytopathol. 24, 43-65.

Davidse, L.C., Flach, W., 1977. Differential Binding of Methyl Benzimidazol-2-Yl Carbamate to Fungal Tubulin as a Mechanism of Resistance to This Antimitotic Agent in Mutant Strains of *Aspergillus Nidulans.* J. Cell Biol. 72, 174-193.

De Brabander, M., Van de Veire, R., Aerts, F., Geuens, S., Hoebeke, J., 1976. A new culture model facilitating rapid quantitative testing of mitotic spindle inhibition in mammalian cells. J. Natl. Cancer Inst. 56, 357-363.

De Coen, W., Janssen, C.R., 1997. The use of biomarkers in *Daphnia magna* toxicity testing. IV.Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. J. Aquat. Ecosyst. Stress Recovery 6, 43-55.

De Coen, W.M., Janssen, C.R., 2003. The missing biomarker link: Relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. Environ. Toxicol. Chem. 22, 1632-1641.

Dell'Omo, G., Turk, A., Shore, R.F., 1999. Secondary poisoning in the common shrew (*Sorex araneus*) fed earthworms exposed to an organophosphate pesticide. Environ. Toxicol. Chem. 18, 237-240.

Domingues, I., Guilhermino, L., Soares, A.M.V.M., Nogueira, A.J.A., 2007. Assessing dimethoate contamination in temperate and tropical climates: Potential use of biomarkers in bioassays with two chironomid species. Chemosphere 69, 145-154.

Frasco, M.F., Guilhermino, L., 2002. Effects of dimethoate and beta-naphthoflavone on selected biomarkers of *Poecilia reticulata*. Fish Physiol. Biochem. 26, 149-156.

Gomes, S.I.L., Novais, S.C., Scott-Fordsmand, J.J., de Coen, W., Soares, A.M.V.M., Amorim, M.J.B., 2011. Effect of Cu-Nanoparticles versus Cu-salt in *Enchytraeus albidus* (Oligochaeta): differential gene expression through microarray analysis. Comparative Biochemistry and Physiology - Part C: Toxicology & Pharmacology, In press.

Hecker, M., Park, J.W., Murphy, M.B., Jones, P.D., Solomon, K.R., Van Der Kraak, G., Carr, J.A., Smith, E.E., du Preez, L., Kendall, R.J., Giesy, J.P., 2005. Effects of atrazine on CYP19 gene expression and aromatase activity in testes and on plasma sex steroid concentrations of male African clawed frogs (*Xenopus laevis)*. Toxicol. Sci. 86, 273-280.

Henle, F.G.J., 1837. Ueber Enchytraeus, eine neue Anneliden-Gattung. Arch. Anat. Physiol. Med., 74-90.

IPCS, 1989. Environmental Health Criteria for Dimethoate, Environmental Health Criteria. World Health Orgnization, Published Under the Joint Sponsorship of the United Nations Environment Programme, the International Labour Organisation and the World Health Organization, Geneva.

ISO, 2004. Soil quality - Effects of pollutants on Enchytraeidae (Enchytraeus sp.) - Determination of effects on reproduction and survival. Guideline no. 16387, ISO (International Organization for Standardization). Geneve, Switzerland.

King, F.D., Packard, T.T., 1975. Respiration and the activity of the respiratory electron transport system in marine zooplankton. Limnol. Oceanogr. 20.

Kobeticova, K., Hofman, J., Holoubek, I., 2009. Avoidance response of *Enchytraeus albidus* in relation to carbendazim ageing. Environ. Pollut. 157, 704-706.

Kumar, A., Doan, H., Barnes, M., Chapman, J.C., Kookana, R.S., 2010. Response and recovery of acetylcholinesterase activity in freshwater shrimp, *Paratya australiensis* (Decapoda: Atyidae) exposed to selected anti-cholinesterase insecticides. Ecotoxicol. Environ. Saf. 73, 1503-1510.

Kumar, A.S., Naruszewicz, I., Wang, P., Leung-Hagesteijn, C., Hannigan, G.E., 2004. ILKAP regulates ILK signaling and inhibits anchorage-independent growth. Oncogene 23, 3454-3461.

Laws, S.C., Ferrell, J.M., Stoker, T.E., Cooper, R.L., 2003. Pubertal development in female Wistar rats following exposure to propazine and atrazine biotransformation by-products, diamino-S-chlorotriazine and hydroxyatrazine. Toxicol. Sci. 76, 190-200.

Loureiro, S., Amorim, M.J.B., Campos, B., Rodrigues, S.M.G., Soares, A.M.V.M., 2009. Assessing joint toxicity of chemicals in *Enchytraeus albidus* (Enchytraeidae) and *Porcellionides pruinosus* (Isopoda) using avoidance behaviour as an endpoint. Environ. Pollut. 157, 625-636.

McMullin, T.S., Andersen, M.E., Nagahara, A., Lund, T.D., Pak, T., Handa, R.J., Hanneman, W.H., 2004. Evidence that atrazine and diaminochlorotriazine inhibit the estrogen/progesterone induced surge of luteinizing hormone in female Sprague-Dawley rats without changing estrogen receptor action. Toxicol. Sci. 79, 278-286.

Nota, B., Bosse, M., Ylstra, B., van Straalen, N.M., Roelofs, D., 2009. Transcriptomics reveals extensive inducible biotransformation in the soil-dwelling invertebrate *Folsomia candida* exposed to phenanthrene. BMC Genomics 10, 236.

Novais, S.C., Arrais, J., Lopes, P., Vandenbrouck, T., De Coen, W., Roelofs, D., Soares, A.M.V.M., Amorim, M.J.B., 2011a. *Enchytraeus albidus* microarray (oligonucleotide): enrichment, design, annotation and database (EnchyBASE) Submitted to Plos One.

Novais, S.C., De Coen, W., Amorim, M.J.B., 2012a. Transcriptional responses in *Enchytraeus albidus*: comparison between cadmium and zinc exposure and linkage to reproduction effects. Submitted to Toxicological Sciences.

Novais, S.C., Gomes, N., Soares, A.M.V.M., De Coen, W., Amorim, M.J.B., 2011b. Reproduction, behaviour and biochemical responses in *Enchytraeus albidus* (Oligochaeta) exposed to different pesticides. *Abstracts*, 21st Annual Meeting of SETAC Europe, Milan, Italy, 15-19 May.

Novais, S.C., Howcroft, C.F., Carreto, L., Pereira, P.M., Santos, M.A.S., De Coen, W., Soares, A.M.V.M., Amorim, M.J.B., 2011c. Differential gene expression analysis in *Enchytraeus albidus* exposed to natural and chemical stressors at different exposure periods. Ecotoxicology, in press.

Novais, S.C., Soares, A.M.V.M., Amorim, M.J.B., 2010. Can avoidance in *Enchytraeus albidus* be used as a screening parameter for pesticides testing? Chemosphere 79, 233-237.

Novais, S.C., Soares, A.M.V.M., De Coen, W., Amorim, M.J.B., 2012b. Differences between Cd and Zn exposure in *Enchytraeus albidus* - linking changes in cellular energy allocation to transcriptional, enzymatic and reproductive responses Submitted to Environmental Pollution.

OECD, 2004. Guidelines for the testing of chemicals. Enchytraeid Reproduction Test. No. 220, OECD (Organization for Economic Cooperation and Development). Paris, France.

Owen, J., Hedley, B.A., Svendsen, C., Wren, J., Jonker, M.J., Hankard, P.K., Lister, L.J., Sturzenbaum, S.R., Morgan, A.J., Spurgeon, D.J., Blaxter, M.L., Kille, P., 2008. Transcriptome profiling of developmental and xenobiotic responses in a keystone soil animal, the oligochaete annelid *Lumbricus rubellus*. BMC Genomics 9, 266.

Reichert, K., Menzel, R., 2005. Expression profiling of five different xenobiotics using a *Caenorhabditis elegans* whole genome microarray. Chemosphere 61, 229-237.

Rombke, J., Moser, T., 2002. Validating the enchytraeid reproduction test: organisation and results of an international ringtest. Chemosphere 46, 1117-1140.

Sanchez, B.C., Carter, B., Hammers, H.R., Sepulveda, M.S., 2011. Transcriptional response of hepatic largemouth bass (*Micropterus salmoides*) mRNA upon exposure to environmental contaminants. J. Appl. Toxicol. 31, 108-116.

Sherman, H., Culik, R., Jackson, R.A., 1975. Reproduction, Teratogenic, and Mutagenic Studies with Benomyl. Toxicol. Appl. Pharmacol. 32, 305-315.

SigmaPlot, 1997. Statistical Package for the Social Sciences - SigmaPlot for Windows. Chicago, IL, USA.

Svendsen, C., Owen, J., Kille, P., Wren, J., Jonker, M.J., Headley, B.A., Morgan, A.J., Blaxter, M., Sturzenbaum, S.R., Hankard, P.K., Lister, L.J., Spurgeon, D.J., 2008. Comparative transcriptomic responses to chronic cadmium, flouranthene, and atrazine expousre in *Lumbricus rubellus*. Environ. Sci. Technol. 42, 4208-4214.

Swain, S., Wren, J.F., Stuerzenbaum, S.R., Kille, P., Morgan, A.J., Jager, T., Jonker, M.J., Hankard, P.K., Svendsen, C., Owen, J., Hedley, B.A., Blaxter, M., Spurgeon, D.J., 2010. Linking toxicant physiological mode of action with induced gene expression changes in *Caenorhabditis elegans*. Bmc Systems Biology 4.

Takahashi, M., Yang, X.M.J., Lavery, T.T., Furge, K.A., Williams, B.O., Tretiakova, M., Montag, A., Vogelzang, N.J., Re, G.G., Garvin, A.J., Soderhall, S., Kagawa, S., Hazel-Martin, D., Nordenskjold, A., Teh, B.T., 2002. Gene expression profiling of favorable histology Wilms tumors and its correlation with clinical features. Cancer Res. 62, 6598-6605.

Tchounwou, P.B., Wilson, B.A., Ishaque, A.B., Schneider, J., 2001. Atrazine potentiation of arsenic trioxide-induced cytotoxicity and gene expression in human liver carcinoma cells (HepG(2)). Mol. Cell. Biochem. 222, 49-59.

Thornton, B.J., Elthon, T.E., Cerny, R.L., Siegfried, B.D., 2010. Proteomic analysis of atrazine exposure in *Drosophila melanogaster* (Diptera: Drosophilidae). Chemosphere 81, 235-241.

Turrens, J.F., 1997. Superoxide production by the mitochondrial respiratory chain. Biosci. Rep. 17, 3-8.

Walsh, L.P., Webster, D.R., Stocco, D.M., 2000. Dimethoate inhibits steroidogenesis by disrupting transcription of the steroidogenic acute regulatory (StAR) gene. J. Endocrinol. 167, 253-263.

Yamada, T., Sumida, K., Saito, K., Ueda, S., Yabushita, S., Sukata, T., Kawamura, S., 2005. Functional genomics may allow accurate categorization of the benzimidazole fungicide benomyl: lack of ability to act via steroid-receptor-mediated mechanisms. Toxicol. Appl. Pharmacol. 205, 11-30.

Zaya, R.M., Amini, Z., Whitaker, A.S., Ide, C.F., 2011. Exposure to atrazine affects the expression of key genes in metabolic pathways integral to energy homeostasis in *Xenopus laevis* tadpoles. Aquat. Toxicol. 104, 254-262.

Zhang, Y., Xiong, J.J., Wang, J.L., Shi, X.P., Bao, G.D., Zhang, Y., Zhu, Z.Y., 2008. Regulation of melanocyte apoptosis by Stathmin 1 expression. Bmb Reports 41, 765-770.

Supporting information

Table S1: Significant differentially expressed transcripts (two sample t-test, $p < 0.05$) in response to four concentrations of dimethoate, atrazine and carbendazim (reproduction EC_{10}, EC_{20}, EC_{50} and EC_{90}). Only annotated genes with significant log2 Fold Change values in at least one condition are shown.

		log2FC											
		Dimethoate				Atrazine				Carbendazim			
Cluster ID	Blast Homology	EC_{10}	EC_{20}	EC_{50}	EC_{90}	EC_{10}	EC_{20}	EC_{50}	EC_{90}	EC_{10}	EC_{20}	EC_{50}	EC_{90}
EAC00276	10 kda heat shock mitochondrial	0.381	0.542	0.021	0.266	-0.190	-0.098	-0.183	-0.317	-0.260	-0.198	-0.018	-0.088
EAC00457	26s protease regulatory subunit 8	-0.192	-0.177	-0.022	0.499	-0.110	0.533	0.181	0.295	0.404	0.332	0.103	0.705
EAC00568	29-kda galactose-binding lectin	0.714	0.741	-0.407	-0.340	-0.182	0.120	0.046	-0.043	0.085	0.115	0.040	-0.546
EAC01014	40s ribosomal protein	-0.011	-0.289	-0.846	0.273	-0.060	0.378	0.464	-0.581	-0.139	-0.424	-0.417	0.353
EAC00287	40s ribosomal protein s2-like isoform 2	-0.140	-0.142	-0.035	0.034	0.038	-0.346	-0.006	-0.247	-0.132	-0.084	-0.133	-0.375
EAC00226	40s ribosomal protein s4	-0.129	-0.161	-0.162	-0.144	-0.160	-0.205	0.222	-0.096	0.239	0.260	0.258	0.158
EAC00855	accessory gland protein	0.391	0.468	-0.270	-0.299	-0.357	-0.526	-0.347	-0.485	-0.273	-0.265	-0.457	-0.564
EAC00001	actin	-0.568	-0.701	0.337	0.046	0.256	0.351	0.210	0.194	0.184	0.054	0.199	0.152
EAC00024	actin	-0.498	-0.617	0.308	0.047	0.207	0.217	0.131	0.157	0.097	0.142	0.184	0.118
EAC00090	actin	-0.401	-0.320	-0.019	-0.075	-0.185	-0.292	0.177	-0.069	-0.098	0.127	-0.105	-0.285
EAC00105	actin	-0.468	-0.552	0.155	-0.082	0.072	-0.005	0.024	0.042	-0.107	0.083	0.059	0.105
EAC00110	actin	-0.292	-0.333	-0.122	-0.080	-0.354	-0.427	0.248	-0.046	-0.037	0.236	0.022	-0.118
EAC00139	actin	-0.313	0.241	-0.437	-0.717	0.019	0.343	-0.377	0.454	0.257	-0.151	-0.077	-1.293
EAC00223	actin	0.225	0.207	0.355	0.207	0.198	-0.060	-0.358	-0.166	-0.130	-0.108	-0.228	-0.169
EAC00281	actin	-0.361	-0.302	0.325	0.228	0.121	0.356	0.543	0.399	0.679	0.601	0.709	0.546
EAC00357	actin	0.309	0.588	0.448	0.169	-0.014	-0.091	-0.045	-0.232	0.036	-0.130	-0.046	-0.074
EAC00406	actin	0.176	0.083	-0.352	0.200	0.021	-0.037	0.479	-0.597	-0.398	-0.592	-0.568	0.199
EAC00444	actin	-0.727	-1.023	0.044	-0.158	0.010	0.320	0.665	0.235	0.847	0.668	0.842	0.850
EAC01012	actin	-0.132	-0.227	0.267	0.196	0.082	0.052	0.250	0.130	0.172	0.168	0.136	0.237
EAC00079	actin 1	-0.329	-0.564	0.069	-0.052	-0.111	0.091	0.397	0.239	0.291	0.179	0.256	0.446
EAC01081	actin beta	-0.418	-0.480	-0.120	0.356	-0.097	0.077	0.488	0.193	0.401	0.331	0.378	0.293
EAC00396	actin related protein 2 3 subunit 41kda	0.229	0.476	0.721	1.062	0.039	0.813	0.459	0.820	0.607	0.413	0.303	0.942
EAC00787	activating signal cointegrator 1 complex subunit 3	-0.977	-0.821	-0.072	-0.259	0.012	0.072	-0.014	-0.390	-0.472	-0.353	-0.363	-0.312
EAC00681	acyl-coenzyme a short branched chain	0.128	0.154	-0.735	0.677	-0.256	0.510	0.294	-0.467	0.089	-0.252	-0.355	0.360
EAC00910	adenosine kinase isoform 2t	0.316	0.073	-0.506	0.480	-0.127	-0.079	0.369	-0.715	-0.313	-0.701	-0.455	0.107
EAC00579	adp-ribosylation factor-like protein 2-binding protein	-0.020	0.134	-0.349	-0.523	0.227	0.122	-0.035	0.009	-0.117	0.100	-0.339	-0.585
EAC00099	alpha 1	-0.719	-0.910	-0.143	-0.133	0.072	0.310	0.754	0.410	0.451	0.397	0.670	0.392
EAC00350	alpha isoform a	-0.328	-0.644	0.061	0.291	0.255	0.191	0.544	0.289	0.340	0.388	0.363	0.550
EAC00867	aquaporin 1	-0.545	-0.242	0.604	0.499	-0.364	0.042	-0.241	-0.607	-0.444	-0.442	-0.133	1.151
EAC00511	atp h+ mitochondrial f1 alpha subunit cardiac muscle	-0.461	-0.169	-0.150	-0.075	0.047	0.419	0.790	0.395	0.602	0.516	0.498	0.275
EAC00665	atp synthase e chain	0.380	0.475	-0.172	-0.599	-0.455	-0.526	-0.451	-0.687	-0.547	-0.553	-0.539	-0.842
EAC00748	atp synthase e chain	0.390	0.484	-0.174	-0.589	-0.457	-0.492	-0.414	-0.667	-0.531	-0.546	-0.542	-0.826
EAC00240	atp synthase lipid-binding mitochondrial precursor	0.494	0.633	-0.073	-0.227	-0.210	-0.228	0.056	-0.296	-0.056	-0.054	-0.131	-0.311
EAC00421	b chain structural basis for giant hemoglobin	-0.010	-0.070	0.544	0.857	-0.019	0.217	0.009	-0.111	-0.112	-0.374	-0.141	0.501
EAC00254	beta actin	0.348	0.415	0.005	0.534	-0.244	0.567	0.355	0.805	0.496	0.717	0.392	0.411
EAC00523	beta actin	-1.690	-2.477	-0.933	-1.931	-0.675	-0.037	0.067	0.730	-0.834	-0.679	-0.548	0.249
EAC00377	beta tubulin	-0.284	-0.095	0.466	0.537	0.258	0.368	0.222	0.319	0.405	0.191	0.499	0.948
EAC00936	beta-actin	0.806	0.650	0.232	0.031	-0.187	-0.043	-0.145	-0.291	-0.236	-0.185	-0.118	0.025
EAC00009	betaine-homocysteine methyltransferase	-0.324	0.187	-0.138	0.509	-0.630	-0.385	0.123	-0.174	-0.151	-0.191	-0.383	-0.265
EAC00486	betaine-homocysteine methyltransferase 2	-0.262	-0.029	0.155	0.782	0.039	-0.035	-0.088	-0.097	-0.124	-0.310	-0.523	-0.128
EAC00881	btb poz domain-containing protein	-0.621	-0.440	-1.221	-1.299	0.059	-0.093	0.326	-0.509	-0.353	-0.300	0.487	-0.689
EAC00580	uncharacterized protein c1orf189 homolog	-0.275	0.061	-0.741	-0.513	-0.138	0.337	0.034	0.233	0.023	0.246	-0.545	-1.270
EAC00697	calcium-dependent protein kinase	0.269	0.562	-0.681	-1.056	-0.200	-0.003	0.092	0.029	0.026	0.120	0.117	-0.841
EAC00100	calponin homolog	-0.043	0.066	-0.075	0.061	-0.110	0.029	0.493	0.365	0.285	0.324	0.311	0.123
EAC00305	calponin homolog	0.327	0.613	0.258	0.066	0.196	0.029	-0.006	-0.073	0.047	-0.181	-0.057	-0.224
EAC00494	calponin transgelin	0.006	-0.100	1.209	1.358	0.459	-0.277	-0.875	-0.241	-0.291	-0.684	-0.609	1.123
EAC00904	calponin-like protein	0.091	0.343	0.079	0.027	-0.020	0.122	0.565	0.354	0.401	0.403	0.370	0.085
EAC01033	calponin-like protein	0.010	0.265	0.033	0.013	-0.016	0.167	0.537	0.329	0.401	0.420	0.433	0.326
EAC00157	carrier protein	0.130	0.389	0.262	-0.073	-0.138	-0.038	0.034	-0.079	0.076	-0.007	0.080	0.047
EAC00222	cathepsin b	0.250	-0.026	0.494	0.436	-0.016	-0.080	-0.143	-0.018	-0.065	0.070	0.117	0.814
EAC00440	cg14935 cg14935-pb	-0.581	-0.599	0.506	0.620	0.401	0.392	-0.298	0.187	0.236	0.086	0.018	0.268
EAC00233	chaperonin containing subunit 7	0.581	0.494	0.098	0.230	0.079	0.326	-0.080	-0.130	0.119	-0.195	-0.036	-0.164
EAC00718	chaperonin containing subunit 8	-0.339	0.204	-0.923	-0.785	0.150	0.307	0.606	-0.174	-0.062	0.285	0.724	-0.071
EAC00178	cholinergic beta polypeptide 4	0.290	0.350	0.532	0.808	-0.251	0.122	0.103	0.601	-0.237	-0.489	-0.116	0.061
EAC00525	chromobox protein homolog 3	0.359	0.094	-0.009	0.241	-0.072	0.560	0.474	0.522	0.617	0.690	0.518	0.354
EAC00267	chymotrypsinogen b1	-0.036	0.092	0.572	2.071	-0.289	0.115	0.178	-0.450	-0.243	-0.575	-0.508	0.650
EAC00366	cmf receptor cmfr1	-0.390	-0.294	0.241	0.929	-0.274	0.410	0.793	0.790	0.281	0.417	0.688	0.714
EAC00684	coiled-coil-helix domain-containing protein mitochondrial	0.176	0.145	0.188	-0.008	-0.143	-0.176	-0.111	-0.273	-0.172	-0.230	-0.328	-0.195
EAC01016	conserved plasmodium membrane protein	-0.488	-0.183	-0.311	0.125	-0.089	0.284	0.825	0.592	-0.034	0.833	0.539	0.732
EAC00468	conserved protein	0.277	0.383	0.268	-0.016	-0.015	0.062	0.288	0.057	0.207	0.290	0.181	0.456

EAC00180	c-type lectin 11	0.383	0.165	-0.566	0.627	-0.270	0.229	0.377	-0.743	-0.194	-0.502	-0.527	0.189
EAC00042	cysteine rich transmembrane bmp regulator 1	-0.439	-0.384	0.465	-0.356	0.176	0.651	0.764	0.523	1.047	0.962	1.116	0.795
EAC00472	cysteine-rich motor neuron 1 protein precursor	-0.454	-0.530	0.330	-0.248	0.004	0.429	0.680	0.332	0.869	0.776	0.933	0.536
EAC00311	cysteine-rich protein 1	0.227	0.760	0.093	0.420	0.004	0.419	1.094	0.470	1.270	1.018	1.448	1.725
EAC00415	cysteine-rich repeat-containing protein crim1	0.255	-0.231	2.209	1.421	-0.206	-1.057	-1.591	-0.452	-0.706	-1.815	-2.018	0.165
EAC00193	cytochrome b	0.113	0.694	-0.172	-0.161	-0.308	-0.477	-0.040	-0.328	-0.219	-0.388	-0.088	-0.267
EAC00356	cytochrome c oxidase subunit via polypeptide 1	-0.164	-0.097	-0.113	-0.083	-0.121	0.375	0.893	0.692	0.682	0.636	0.786	0.754
EAC00068	cytochrome c oxidase subunit via polypeptide	-0.171	-0.002	-0.209	-0.392	-0.007	-0.006	0.024	-0.100	-0.159	-0.125	-0.158	-0.238
EAC00106	cytochrome oxidase subunit i	0.346	0.467	-0.156	0.116	-0.658	0.065	0.589	0.592	0.203	0.433	0.449	-0.048
EAC00307	cytochrome oxidase subunit i	0.305	0.457	-0.048	-0.082	-0.521	-0.047	0.595	0.211	0.142	0.333	0.314	-0.033
EAC01043	cytochrome oxidase subunit i	0.526	0.742	-0.207	-0.268	-0.187	-0.532	-0.567	-0.593	-0.463	-0.590	-0.785	-0.583
EAC00182	cytoplasmic 1	-0.380	-0.436	-0.100	0.003	-0.356	-0.362	0.068	-0.130	-0.203	0.064	-0.100	-0.218
EAC00407	cytoplasmic 2-like isoform 1	-0.204	-0.501	0.318	-0.079	-0.024	-0.185	-0.467	-0.464	-0.447	-0.487	-0.549	-0.280
EAC00183	cytoplasmic actin	-0.437	-0.474	0.264	0.381	0.181	0.278	0.326	0.295	0.201	0.214	0.309	0.246
EAC00455	cytoplasmic actin	-0.279	-0.525	0.197	-0.137	-0.067	-0.229	-0.427	-0.438	-0.511	-0.502	-0.569	-0.290
EAC00371	cytoplasmic intermediate filament protein	-1.016	-0.589	0.066	0.789	0.579	0.470	0.526	0.108	0.718	0.694	0.656	1.023
EAC00950	dimethyladenosine transferase	-0.090	-0.455	-0.376	-0.245	0.348	0.342	-0.002	-0.103	0.188	0.230	0.226	1.133
EAC00212	dna replication licensing factor mcm7	-0.415	-0.315	-0.071	0.073	0.135	0.409	0.636	0.484	0.512	0.614	0.261	-0.090
EAC00095	elongation factor 1-alpha	-0.378	-0.327	0.097	-0.078	-0.080	-0.176	-0.176	-0.141	-0.278	-0.053	-0.115	-0.064
EAC00816	endonuclease-reverse transcriptase	-0.221	-0.508	-0.877	-0.620	-0.637	-1.056	-0.198	-0.761	-0.471	-1.045	-0.379	-0.791
EAC00826	eukaryotic peptide chain release factor subunit 1	-0.363	-0.047	0.723	1.233	0.671	0.448	0.016	0.710	0.368	0.106	0.245	1.293
EAC01079	eukaryotic translation initiation factor subunit e	0.234	0.219	0.375	0.396	0.396	0.082	-0.342	-0.081	-0.125	-0.286	-0.443	0.147
EAC00400	excretory secretory protein juv-p120 precursor	0.202	0.286	0.529	0.828	0.179	-0.065	-0.332	0.163	0.447	-0.264	-0.207	0.215
EAC00261	extracellular hemoglobin linker l4 subunit precursor	0.234	-0.270	0.498	-0.237	-0.187	-1.662	-1.967	-0.360	-1.082	-1.346	-0.460	0.138
EAC00011	tyrosine-protein kinase transforming protein fgr	-0.135	-0.293	-0.197	0.100	-0.177	-0.490	-0.358	-0.489	-0.492	-0.389	-0.335	-0.437
EAC00354	gl12416-like	-0.402	-0.252	0.182	0.371	-0.056	0.248	0.422	0.393	0.231	0.282	0.472	0.648
EAC00265	glucan endo- -beta-glucosidase	-0.120	-0.168	-0.122	0.085	-0.210	0.157	0.690	0.557	0.173	0.334	0.236	0.514
EAC00484	aspartate aminotransferase 1	-0.082	-0.121	-0.122	0.494	-0.159	1.070	0.858	0.649	1.169	1.015	0.866	0.780
EAC00903	glyceraldehyde-3-phosphate dehydrogenase	-0.070	-0.187	0.200	-0.117	0.010	0.182	0.360	0.322	0.491	0.168	0.448	0.467
EAC00316	heat shock cognate 71 kda	-0.527	-0.486	0.087	0.077	-0.248	0.073	0.437	0.257	0.177	0.248	0.359	0.229
EAC00481	heat shock protein 60	-0.039	0.074	-0.664	0.128	-0.291	-0.184	0.241	-0.719	-0.282	-0.535	-0.284	-0.074
EAC00488	heat shock protein 90	-0.630	-0.733	0.530	0.752	0.198	0.627	0.855	0.992	0.844	0.910	0.681	1.160
EAC00097	heat shock protein gp96	-0.039	0.193	0.181	1.398	-0.035	0.665	0.590	0.043	0.182	-0.032	0.069	0.772
EAC00627	histone	-0.112	-0.048	0.092	0.002	0.368	0.023	-0.233	-0.182	-0.168	-0.181	-0.261	-0.168
EAC00577	homolog precursor	0.529	0.493	-0.183	-0.274	0.069	-0.183	-0.403	-0.266	-0.218	-0.478	-0.418	-0.263
EAC00404	hypothetical protein BRAFLDRAFT_74510	0.564	0.571	-0.119	0.368	-0.493	-0.117	0.072	-0.415	-0.115	-0.249	-0.533	0.384
EAC00710	hypothetical protein TGME49_026570	-0.106	-0.239	-0.951	0.220	0.134	0.123	0.502	-0.528	-0.097	-0.617	-0.471	0.236
EAC00379	hypothetical tyrosinase-like protein in chromosome	-0.895	-0.375	-0.300	0.348	0.314	0.326	0.917	0.039	0.479	1.089	1.036	0.580
EAC00664	ILKAP	0.443	0.227	-0.115	-0.402	0.555	0.292	-0.316	-0.151	-0.078	-0.122	-0.351	-0.228
EAC00181	intermediate filament protein	-0.747	-0.635	-0.054	-0.071	0.200	0.521	0.795	0.564	0.789	0.747	0.816	0.828
EAC00363	intermediate filament protein	-0.628	-0.746	-0.061	-0.129	0.183	0.454	0.800	0.571	0.658	0.648	0.784	0.713
EAC00999	intermediate filament protein	-0.607	-0.407	-0.074	-0.448	0.040	0.566	0.552	0.762	0.848	0.750	0.687	0.429
EAC00822	isoform a	0.683	0.957	0.103	-0.173	-0.297	-0.347	-0.239	-0.239	-0.659	-0.476	-0.177	-1.079
EAC00397	keratinocyte associated protein 2	0.192	0.154	0.531	0.933	-0.182	0.362	0.470	0.562	0.540	0.248	0.720	0.848
EAC00030	kiaa0550 protein	-1.271	-1.015	-0.621	-0.330	0.218	0.413	1.096	0.176	0.912	1.055	1.044	0.425
EAC01019	larval visceral protein d	-0.075	-0.010	-0.049	0.157	0.019	0.316	0.514	0.752	0.149	0.113	0.286	0.261
EAC00558	light chain regulatory	0.432	0.285	0.359	0.670	0.182	-0.246	-0.609	-0.501	-0.557	-0.661	-0.744	0.230
EAC00260	lombricine kinase	-0.396	-0.247	0.186	0.410	-0.142	0.176	0.467	0.406	0.386	0.463	0.514	0.692
EAC00290	lysozyme	0.039	0.121	0.037	0.970	-0.351	0.881	0.244	-0.197	0.590	0.720	0.279	1.209
EAC00897	mads flc-like protein 2	-0.082	0.088	0.033	0.391	-0.378	0.659	0.561	1.086	0.247	0.125	0.497	0.725
EAC00946	mads flc-like protein 2	0.346	0.026	0.616	0.649	-0.526	-0.539	-0.446	0.208	-1.011	-0.675	-0.573	-0.541
EAC00966	mads flc-like protein 2	0.051	0.151	0.095	-0.145	0.448	0.119	-0.069	0.250	0.064	-0.119	-0.009	-0.154
EAC00971	mads flc-like protein 2	-0.894	-0.089	-0.750	-1.759	-0.718	0.283	-0.148	-0.167	0.358	0.500	0.164	-0.910
EAC01006	mads flc-like protein 2	0.383	0.355	-0.132	-0.261	0.277	0.153	-0.181	-0.092	-0.255	-0.404	-1.092	-1.260
EAC01039	mads flc-like protein 2	-0.185	0.292	-0.638	-0.440	-0.664	0.286	1.168	0.582	0.851	0.983	1.000	-0.141
EAC01049	mads flc-like protein 2	2.076	1.417	5.112	5.081	0.426	-1.583	-2.147	-0.109	-0.909	-2.326	-1.874	1.676
EAC01059	mads flc-like protein 2	-0.372	-0.820	0.586	0.257	0.336	0.349	-0.119	0.166	0.183	-0.090	0.139	0.188
EAC01066	mads flc-like protein 2	2.021	1.365	4.991	4.914	0.536	-1.470	-2.140	-0.061	-0.716	-2.232	-1.791	1.800
EAC01087	mads flc-like protein 2	-0.863	-0.429	0.178	0.221	-0.063	1.126	1.238	1.160	1.380	1.369	1.484	2.191
EAC01092	mads flc-like protein 2	0.169	0.047	0.037	-0.072	0.382	0.126	0.056	0.094	-0.093	-0.016	-0.024	-0.089
EAC01098	mads flc-like protein 2	-0.578	-0.650	-0.019	-0.112	0.445	0.437	0.104	0.273	0.236	0.421	0.480	1.512
EAC01106	mads flc-like protein 2	0.733	0.599	0.571	0.719	-0.271	0.507	0.161	0.342	-0.199	-0.049	-0.001	0.449
EAC01116	mads flc-like protein 2	2.202	1.432	4.964	5.152	0.548	-1.477	-1.994	0.133	-0.905	-2.054	-1.750	1.663
EAC00433	methyltransferase mb3374	0.261	0.440	-0.071	0.150	-0.426	-0.178	0.388	-0.474	-0.561	-0.728	-0.454	0.324
EAC00428	mgc108470 protein	0.154	-0.459	0.867	0.614	-0.064	0.094	0.071	0.753	0.226	0.070	0.001	0.239
EAC00103	mitochondrial atp synthase gamma subunit	-0.403	-0.196	-0.074	0.187	-0.053	0.510	0.807	0.611	0.729	0.675	0.623	0.452
EAC00294	mitochondrial atp synthase subunit 9 precursor-like protein	0.403	0.554	-0.173	-0.351	-0.157	-0.210	-0.057	-0.372	-0.171	-0.227	-0.211	-0.319
EAC00204	mitochondrial nadh:ubiquinone oxidoreductase	0.161	0.162	-0.364	-0.075	-0.289	0.624	0.805	0.561	0.734	0.817	0.549	0.449
EAC00388	mitochondrial nadh:ubiquinone oxidoreductase esss	0.069	0.043	0.067	-0.171	-0.077	-0.229	-0.356	-0.265	-0.321	-0.319	-0.464	-0.425
EAC00732	monosaccharide-transporting atpase	0.101	0.196	-0.227	0.775	-0.095	0.469	0.264	0.625	0.152	0.384	0.009	0.654
EAC00332	muscle actin	0.165	0.557	-0.340	-0.435	-0.612	-0.732	-0.520	-0.810	-0.835	-0.904	-1.100	-0.710
EAC00378	myosin alkali light chain 1	0.343	0.237	-0.204	0.087	-0.162	-0.109	-0.034	-0.017	-0.309	-1.199	-0.265	-0.843
EAC00541	myosin alkali light chain 1	-0.403	-0.406	0.122	0.421	-0.037	0.204	0.434	0.410	0.521	0.686	0.529	0.432
EAC00002	myosin heavy chain	-0.208	-0.586	0.284	0.273	0.300	0.386	0.510	0.473	0.597	0.592	0.745	0.662
EAC00082	myosin heavy chain	0.156	0.074	0.117	0.645	-0.176	0.547	0.301	0.671	0.232	0.256	0.143	1.054
EAC00088	myosin heavy chain	-0.245	-0.418	-0.068	-0.035	-0.439	-0.312	0.315	0.165	0.018	0.220	0.197	-0.041

EAC00108	myosin heavy chain	-0.408	-0.655	-0.008	-0.088	-0.182	0.109	0.729	0.285	0.563	0.650	0.552	0.247
EAC00167	myosin heavy chain	-0.325	-0.678	0.165	-0.019	0.174	0.091	0.134	0.158	0.000	0.219	0.184	0.258
EAC01023	myosin light chain 2	0.321	0.480	0.036	0.157	-0.434	-0.020	0.649	0.333	0.349	0.468	0.541	0.348
EAC00259	myosin regulatory light chain	0.528	0.734	0.265	-0.052	-0.191	-0.397	-0.587	-0.564	-0.563	-0.663	-0.537	-0.670
EAC00616	nadh dehydrogenase	0.333	0.366	-0.029	-0.209	-0.021	-0.209	-0.223	-0.314	-0.106	-0.085	-0.332	-0.274
EAC00842	nadh dehydrogenase subunit 1	0.338	0.356	-0.416	-0.261	-0.485	-0.473	-0.283	-0.435	-0.436	-0.295	-0.187	-0.441
EAC00478	nadh dehydrogenase subunit 4	0.362	0.497	-0.237	-0.222	-0.426	-0.485	-0.397	-0.455	-0.456	-0.212	-0.204	-0.427
EAC00504	NADH dehydrogenase, putative	0.360	0.515	-0.290	-0.129	-0.256	0.196	0.277	0.178	0.288	0.338	0.286	-0.023
EAC00775	nadh:ubiquinone ndufc2	0.647	0.719	0.050	-0.341	-0.260	-0.264	-0.260	-0.391	-0.330	-0.244	-0.236	-0.571
EAC00636	neutral and basic amino acid transport protein rbat	-0.171	-0.246	0.324	0.046	0.485	0.243	-0.005	0.369	0.441	0.225	0.013	0.142
EAC00513	ovulatory protein-2 precursor	-0.041	-0.038	0.210	0.293	-0.456	-0.291	0.510	0.360	0.495	0.622	0.659	1.483
EAC00257	peptidase inhibitor 16	-0.648	-0.732	0.050	-0.078	0.284	0.291	0.233	0.041	0.062	0.049	0.207	-2.214
EAC00179	plastin 3 (t isoform)	0.186	0.479	0.379	0.929	-0.222	0.018	0.292	0.038	-0.236	-0.394	0.073	0.597
EAC00795	pls_halla ame: full=perlustrin	-0.057	-0.091	-0.934	0.769	-0.068	0.279	0.343	-0.357	-0.163	-0.587	-0.433	0.016
EAC00677	poly -specific ribonuclease parn	-0.119	0.138	-0.471	0.090	-0.025	0.070	0.223	-0.085	-0.049	-0.161	0.004	0.213
EAC00559	predicted protein [Nematostella vectensis]	-0.042	-0.229	1.129	-0.211	-0.166	-0.563	-1.015	-0.146	-0.180	-0.241	-0.017	0.458
EAC00619	PREDICTED: hypothetical protein [Homo sapiens]	-0.383	-0.202	-0.121	-0.240	-0.047	0.034	-0.118	0.040	0.123	0.163	0.026	-0.220
EAC00471	PREDICTED: predicted protein [Hydra magnipapillata]	-0.505	-0.688	-0.049	0.071	0.389	0.437	0.395	0.439	0.316	0.599	0.372	0.290
EAC00712	prostaglandin d2 hematopoietic-like	0.452	0.561	-0.222	-0.246	0.083	0.059	0.080	-0.134	-0.027	-0.082	0.024	-0.128
EAC00632	proteasome isoform a	-0.514	-0.779	0.161	0.562	0.429	0.696	0.167	0.341	0.288	0.323	-0.021	0.826
EAC00214	protein	-0.029	0.049	-0.155	0.590	0.089	1.264	0.664	0.989	1.097	0.975	0.634	0.792
EAC00246	protein	0.076	-0.571	0.016	-0.439	0.854	0.092	0.183	1.193	0.571	0.581	0.534	0.629
EAC00463	protein	0.029	-0.018	-0.717	-0.148	-0.257	-0.015	0.349	-0.666	-0.512	-0.532	-0.470	0.301
EAC00809	protein	0.411	0.218	-0.654	0.071	-0.213	-0.192	0.248	-0.625	-0.180	-0.491	-0.520	-0.101
EAC00372	purine nucleoside phosphorylase	-0.734	-0.204	-0.944	-0.510	1.037	0.877	0.439	0.811	0.018	-0.440	0.270	-0.097
EAC00508	ran binding protein 1	-0.227	-0.281	-0.071	0.535	0.116	0.788	0.631	0.673	0.621	0.695	0.725	0.600
EAC00549	replication factor a protein 3 domain-containing protein	0.139	0.107	-0.724	0.679	-0.407	0.342	-0.015	-0.846	-0.284	-0.792	-0.720	-0.214
EAC00387	retinol dehydrogenase 3	-0.115	0.415	-0.353	0.303	0.007	0.689	0.398	0.797	-0.133	-0.237	-0.100	0.065
EAC01052	ribosomal protein l10a	0.521	0.558	0.169	-0.085	-0.158	-0.283	-0.328	-0.513	-0.382	-0.499	-0.449	-0.469
EAC00479	ribosomal protein l10ae	0.273	0.253	-0.294	-0.228	-0.161	-0.266	-0.195	-0.659	-0.149	-0.325	-0.256	-0.196
EAC00476	ribosomal protein l15	-0.168	-0.073	-0.079	0.111	-0.248	0.095	0.766	0.350	0.590	0.800	0.578	0.517
EAC00280	ribosomal protein l19	0.621	0.716	0.454	0.377	-0.044	-0.076	-0.138	-0.219	-0.017	-0.186	-0.122	0.087
EAC00583	ribosomal protein l26	0.222	0.264	0.199	0.025	-0.232	-0.242	-0.154	-0.247	-0.174	-0.215	-0.150	-0.242
EAC00128	ribosomal protein l3	-0.456	-0.441	-0.403	-0.225	-0.743	-0.541	0.049	-0.160	-0.211	0.067	-0.159	-0.364
EAC00208	ribosomal protein l3	-0.459	-0.538	-0.197	-0.053	-0.269	0.183	0.720	0.304	0.503	0.617	0.607	0.430
EAC00520	ribosomal protein l3	-0.515	-0.537	-0.157	0.030	-0.265	-0.002	0.538	0.146	0.350	0.432	0.477	0.270
EAC00517	ribosomal protein l36a	0.171	0.149	-0.402	-0.111	-0.302	-0.118	0.521	0.146	0.358	0.485	0.485	0.378
EAC00518	ribosomal protein l36a	0.161	0.122	-0.488	-0.183	-0.303	-0.119	0.458	0.124	0.431	0.494	0.447	0.382
EAC00685	ribosomal protein l37a	0.385	0.360	-0.117	-0.398	-0.326	-0.556	-0.457	-0.650	-0.485	-0.536	-0.592	-0.588
EAC00482	ribosomal protein s15	0.235	0.275	-0.197	0.100	-0.338	-0.255	0.128	-0.100	0.132	0.220	0.280	0.149
EAC00200	ribosomal protein s16	-0.308	-0.175	0.177	-0.120	-0.003	0.056	0.008	0.011	0.041	0.037	-0.038	-0.135
EAC00298	ribosomal protein s16	-0.301	-0.389	0.073	-0.262	0.024	-0.426	-0.620	-0.619	-0.572	-0.485	-0.577	-0.599
EAC00143	ribosomal protein s7	0.292	0.230	-0.119	-0.101	-0.132	-0.182	0.073	-0.187	0.165	0.152	0.233	0.111
EAC01078	ribosomal protein s7	0.333	0.281	-0.053	-0.020	-0.121	-0.043	0.270	-0.054	0.364	0.316	0.284	0.219
EAC00663	ribosomal protein s7	0.479	0.550	0.152	-0.093	-0.219	-0.300	-0.202	-0.391	-0.444	-0.479	-0.441	-0.461
EAC00854	rrna-processing protein fcf1 homolog	0.449	0.674	0.185	0.613	-0.168	0.004	-0.032	-0.274	-0.196	-0.103	-0.184	-0.200
EAC00309	s enesis abnormal family member (syg-2)	0.062	-0.091	0.413	-0.344	0.511	0.022	0.432	0.152	0.239	0.384	0.507	0.680
EAC01038	scbp2 protein	0.413	0.431	0.087	-0.219	-0.186	-0.310	-0.337	-0.284	-0.385	-0.221	-0.255	-0.268
EAC00045	scbp3 protein	0.265	0.299	0.048	-0.086	-0.438	0.029	0.492	0.322	0.627	0.556	0.486	0.353
EAC00408	scbp3 protein	0.287	0.310	0.018	0.093	-0.601	0.144	0.566	0.286	0.391	0.518	0.625	0.163
EAC01045	scbp3 protein	0.225	0.367	-0.007	-0.015	-0.372	0.124	0.537	0.357	0.602	0.558	0.569	0.407
EAC00067	sarcoplasmic calcium-binding protein short=scp	0.223	0.294	-0.067	-0.076	-0.535	-0.008	0.625	0.313	0.432	0.551	0.583	0.311
EAC00006	sea urchin arp2	0.121	-0.042	0.320	0.769	0.004	0.205	-0.168	-0.359	-0.103	-0.103	-0.113	0.677
EAC00716	sec14-like 2 (cerevisiae) isoform cra_b	-0.454	-0.159	-0.157	-0.850	0.281	-0.142	-0.732	-0.130	-0.226	-0.291	-0.228	-0.215
EAC01022	short chain dehydrogenase	-0.103	-0.331	0.446	0.070	-0.032	0.073	-0.298	-0.009	-0.060	-0.044	0.188	0.931
EAC00554	short-chain dehydrogenase	-0.196	-0.617	0.346	-0.183	-0.071	-0.034	-0.213	-0.131	0.056	0.110	0.130	0.902
EAC00992	stathmin 1 oncoprotein 18 variant 8	0.352	0.394	-0.119	-0.272	0.317	0.184	-0.122	-0.043	-0.356	-0.380	-1.060	-1.239
EAC00491	subfamily member 6	-0.259	-0.157	-0.706	-0.563	0.403	0.235	0.043	0.297	-0.001	0.362	-0.088	-0.529
EAC00791	superoxide dismutase	-0.094	-0.063	0.840	2.229	0.593	0.161	-0.166	-0.107	-0.051	-0.175	-0.032	0.393
EAC00793	t-cell receptor beta chain ana 11	-0.478	-0.692	-0.197	-0.045	0.005	-0.050	0.046	-0.138	-0.103	0.002	0.041	0.030
EAC00473	t-complex protein 1 subunit beta	0.102	0.194	0.614	0.481	-0.127	-0.214	-0.175	-0.265	-0.524	-0.792	-0.145	-0.270
EAC01050	tektin 2	-0.656	-0.263	-0.570	0.109	-0.162	1.189	0.583	1.146	0.676	0.626	0.344	0.374
EAC00210	thymosin isoform 2	0.680	0.698	-0.127	-0.229	-0.170	-0.390	-0.679	-0.687	-0.593	-0.466	-0.585	-0.237
EAC00207	transketolase	-0.334	-0.224	0.220	1.098	-0.364	0.711	0.463	0.316	0.932	0.644	0.671	1.163
EAC00994	translation elongation factor 1-alpha	-0.638	-0.174	-0.511	0.088	-0.951	-0.202	0.224	0.363	-0.220	-0.071	0.113	0.176
EAC00391	translationally controlled tumor protein	0.386	0.241	0.202	0.026	-0.005	0.034	-0.150	-0.092	-0.189	-0.197	-0.061	-0.140
EAC00221	transport protein sec61 subunit beta	-0.295	-0.311	-0.107	0.198	0.152	0.319	0.087	0.628	0.180	0.054	0.206	0.340
EAC00675	tripartite motif-containing 59	-0.181	-0.230	-0.078	-0.237	0.271	0.596	0.463	0.455	0.416	0.626	0.596	0.508
EAC00327	tropomyosin	0.454	0.538	0.188	0.022	0.027	-0.195	-0.166	-0.203	-0.118	-0.208	-0.177	-0.311
EAC00533	tropomyosin	0.085	0.090	0.072	-0.129	-0.026	-0.013	-0.141	-0.243	-0.131	-0.248	-0.185	-0.347
EAC00545	tropomyosin	0.161	0.275	0.028	-0.034	-0.054	0.340	0.712	0.484	0.630	0.611	0.752	0.457
EAC01032	tropomyosin	0.061	0.195	-0.070	-0.132	-0.091	0.222	0.579	0.434	0.595	0.494	0.641	0.245
EAC01110	tropomyosin	0.215	0.321	0.307	0.166	0.213	0.294	0.219	0.256	0.266	0.230	0.357	0.309
EAC00340	troponin t	-0.435	-0.443	-0.261	-0.300	-0.137	0.265	0.771	0.415	0.491	0.440	0.577	0.401
EAC00496	troponin t	-0.588	-0.898	-0.619	-0.617	-0.047	-0.067	0.368	0.145	0.353	0.325	0.163	-0.024
EAC00175	tubulin alpha chain	-0.448	-0.307	-0.258	0.164	-0.010	0.435	0.653	0.105	0.578	0.758	0.510	1.058

EAC00485	tubulin beta-2b chain-like	-0.585	-0.495	-0.188	-0.064	-0.146	0.123	0.668	0.374	0.600	0.605	0.494	0.408
EAC00234	type alpha 1	-0.375	-0.772	0.505	0.214	0.166	0.194	-0.256	0.032	0.016	-0.226	0.028	0.019
EAC00390	type alpha isoform cra_b	-0.293	-0.519	0.626	0.618	0.477	0.628	0.491	0.534	0.750	0.351	0.881	1.017
EAC00628	type alpha partial	0.452	0.628	0.171	-0.243	0.089	-0.137	-0.468	-0.687	-0.444	-0.761	-0.521	-0.793
EAC00334	u6 snrna-associated sm-like protein	0.509	0.955	-0.227	0.633	-0.599	0.290	0.319	0.333	0.126	0.125	0.079	0.156
EAC00831	u6 snrna-associated sm-like protein lsm3	-0.135	0.112	0.050	0.261	0.109	0.512	0.302	0.322	0.380	0.365	0.174	0.375
EAC00657	ubiquinol-cytochrome c subunit	0.253	0.144	-0.245	0.926	-0.316	0.338	0.313	0.733	-0.506	-0.613	0.122	0.166
EAC00306	ubiquitin c	-0.314	-0.231	0.138	0.225	0.399	0.268	-0.178	0.125	0.066	-0.096	0.095	0.607
EAC00835	uromodulin precursor	-0.543	-0.631	0.031	0.398	-0.269	0.359	-0.373	0.095	0.185	-0.038	0.348	-0.201
EAC00550	venom c-type lectin mannose binding isoform 2	-0.049	0.545	-1.131	-0.417	-0.345	-0.515	-0.649	-1.253	-0.372	-0.873	-0.267	-1.460
EAC00431	vesicle-associated protein (vap-1)	-0.260	-0.594	-0.018	-0.109	-0.143	0.168	0.380	0.353	-0.235	-0.363	0.493	-0.245
EAC00880	yippee-like 5	0.249	0.131	-0.673	0.035	-0.092	-0.343	0.259	-0.737	-0.452	-0.705	-0.678	0.064
EAC00284	zinc carboxypeptidase family protein	0.291	-0.028	-0.057	0.061	0.152	0.004	0.210	0.649	0.366	0.161	0.313	0.088

Table S2: Significant differentially expressed transcripts present in the following lists of differentially expressed genes: 1) uniquely affected by dimethoate; 2) uniquely affected by atrazine; 3) uniquely affected by carbendazim; 4) affected by dimethoate and atrazine; 5) affected by dimethoate and carbendazim; 6) affected by atrazine and carbendazim; 7) affected by the three pesticides. Only annotated genes with significant log2 Fold Change values in each of the lists are shown.

		log2FC											
		Dimethoate				Atrazine				Carbendazim			
Cluster ID	**Blast Homology**	**EC_{10}**	**EC_{20}**	**EC_{50}**	**EC_{90}**	**EC_{10}**	**EC_{20}**	**EC_{50}**	**EC_{90}**	**EC_{10}**	**EC_{20}**	**EC_{50}**	**EC_{90}**
Genes uniquely affected by dimethoate													
EAC00276	10 kda heat shock mitochondrial	0.381	0.542	0.021	0.266								
EAC00568	29-kda galactose-binding lectin	0.714	0.741	-0.407	-0.340								
EAC00855	accessory gland protein	0.391	0.468	-0.270	-0.299								
EAC00001	actin	-0.568	-0.701	0.337	0.046								
EAC00024	actin	-0.498	-0.617	0.308	0.047								
EAC00090	actin	-0.401	-0.320	-0.019	-0.075								
EAC00105	actin	-0.468	-0.552	0.155	-0.082								
EAC00110	actin	-0.292	-0.333	-0.122	-0.080								
EAC00139	actin	-0.313	0.241	-0.437	-0.717								
EAC00223	actin	0.225	0.207	0.355	0.207								
EAC00357	actin	0.309	0.588	0.448	0.169								
EAC01012	actin	-0.132	-0.227	0.267	0.196								
EAC01081	actin beta	-0.418	-0.480	-0.120	0.356								
EAC00396	actin related protein 2 3 subunit 41kda	0.229	0.476	0.721	1.062								
EAC00787	activating signal cointegrator 1 complex subunit 3	-0.977	-0.821	-0.072	-0.259								
EAC00681	acyl-coenzyme a short branched chain	0.128	0.154	-0.735	0.677								
EAC00665	atp synthase e chain	0.380	0.475	-0.172	-0.599								
EAC00748	atp synthase e chain	0.390	0.484	-0.174	-0.589								
EAC00523	beta actin	-1.690	-2.477	-0.933	-1.931								
EAC00936	beta-actin	0.806	0.650	0.232	0.031								
EAC00486	betaine-homocysteine methyltransferase 2	-0.262	-0.029	0.155	0.782								
EAC00881	btb poz domain-containing protein	-0.621	-0.440	-1.221	-1.299								
EAC00305	calponin homolog	0.327	0.613	0.258	0.066								
EAC00494	calponin transgelin	0.006	-0.100	1.209	1.358								
EAC00157	carrier protein	0.130	0.389	0.262	-0.073								
EAC00440	cg14935 cg14935-pb	-0.581	-0.599	0.506	0.620								
EAC00718	chaperonin containing subunit 8	-0.339	0.204	-0.923	-0.785								
EAC00267	chymotrypsinogen b1	-0.036	0.092	0.572	2.071								
EAC00366	cmf receptor cmfr1	-0.390	-0.294	0.241	0.929								
EAC00684	coiled-coil-helix domain-containing protein mitochondrial	0.176	0.145	0.188	-0.008								
EAC00193	cytochrome b	0.113	0.694	-0.172	-0.161								
EAC00068	cytochrome c oxidase subunit via polypeptide isoform cra_a	-0.171	-0.002	-0.209	-0.392								
EAC01043	cytochrome oxidase subunit i	0.526	0.742	-0.207	-0.268								
EAC00182	cytoplasmic 1	-0.380	-0.436	-0.100	0.003								
EAC00183	cytoplasmic actin	-0.437	-0.474	0.264	0.381								
EAC00095	elongation factor 1-alpha	-0.378	-0.327	0.097	-0.078								
EAC01079	eukaryotic translation initiation factor subunit e	0.234	0.219	0.375	0.396								
EAC00400	excretory secretory protein juv-p120 precursor	0.202	0.286	0.529	0.828								
EAC00011	tyrosine-protein kinase transforming protein fgr	-0.135	-0.293	-0.197	0.100								
EAC00097	heat shock protein gp96	-0.039	0.193	0.181	1.398								
EAC00577	homolog precursor	0.529	0.493	-0.183	-0.274								
EAC00379	hypothetical tyrosinase-like protein in chromosome	-0.895	-0.375	-0.300	0.348								

EAC00030	kiaa0550 protein	-1.271	-1.015	-0.621	-0.330								
EAC00946	mads flc-like protein 2	0.346	0.026	0.616	0.649								
EAC00971	mads flc-like protein 2	-0.894	-0.089	-0.750	-1.759								
EAC01059	mads flc-like protein 2	-0.372	-0.820	0.586	0.257								
EAC00428	mgc108470 protein	0.154	-0.459	0.867	0.614								
EAC00294	mitochondrial atp synthase subunit 9 precursor-like protein	0.403	0.554	-0.173	-0.351								
EAC00088	myosin heavy chain	-0.245	-0.418	-0.068	-0.035								
EAC00259	myosin regulatory light chain	0.528	0.734	0.265	-0.052								
EAC00842	nadh dehydrogenase subunit 1	0.338	0.356	-0.416	-0.261								
EAC00478	nadh dehydrogenase subunit 4	0.362	0.497	-0.237	-0.222								
EAC00775	nadh:ubiquinone ndufc2	0.647	0.719	0.050	-0.341								
EAC00795	pls_halla ame: full=perlustrin	-0.057	-0.091	-0.934	0.769								
EAC00677	poly -specific ribonuclease parn	-0.119	0.138	-0.471	0.090								
EAC00559	predicted protein [Nematostella vectensis]	-0.042	-0.229	1.129	-0.211								
EAC00619	PREDICTED: hypothetical protein [Homo sapiens]	-0.383	-0.202	-0.121	-0.240								
EAC00712	prostaglandin d2 hematopoietic-like	0.452	0.561	-0.222	-0.246								
EAC00372	purine nucleoside phosphorylase	-0.734	-0.204	-0.944	-0.510								
EAC00508	ran binding protein 1	-0.227	-0.281	-0.071	0.535								
EAC01052	ribosomal protein l10a	0.521	0.558	0.169	-0.085								
EAC00280	ribosomal protein l19	0.621	0.716	0.454	0.377								
EAC00583	ribosomal protein l26	0.222	0.264	0.199	0.025								
EAC00128	ribosomal protein l3	-0.456	-0.441	-0.403	-0.225								
EAC00685	ribosomal protein l37a	0.385	0.360	-0.117	-0.398								
EAC00854	rrna-processing protein fcf1 homolog	0.449	0.674	0.185	0.613								
EAC01038	scbp2 protein	0.413	0.431	0.087	-0.219								
EAC00491	subfamily member 6	-0.259	-0.157	-0.706	-0.563								
EAC00791	superoxide dismutase	-0.094	-0.063	0.840	2.229								
EAC00793	t-cell receptor beta chain ana 11	-0.478	-0.692	-0.197	-0.045								
EAC00210	thymosin isoform 2	0.680	0.698	-0.127	-0.229								
EAC00391	translationally controlled tumor protein	0.386	0.241	0.202	0.026								
EAC00327	tropomyosin	0.454	0.538	0.188	0.022								
EAC00496	troponin t	-0.588	-0.898	-0.619	-0.617								
EAC00234	type alpha 1	-0.375	-0.772	0.505	0.214								
EAC00334	u6 snrna-associated sm-like protein	0.509	0.955	-0.227	0.633								
EAC00835	uromodulin precursor	-0.543	-0.631	0.031	0.398								
Genes uniquely affected by atrazine													
EAC00287	40s ribosomal protein s2-like isoform 2					0.038	-0.346	-0.006	-0.247				
EAC00269	60s ribosomal protein l7					0.152	0.237	0.324	0.491				
EAC00350	alpha isoform a					0.255	0.191	0.544	0.289				
EAC01016	conserved plasmodium membrane protein					-0.089	0.284	0.825	0.592				
EAC00627	histone					0.368	0.023	-0.233	-0.182				
EAC00404	hypothetical protein BRAFLDRAFT_74510					-0.493	-0.117	0.072	-0.415				
EAC00897	mads flc-like protein 2					-0.378	0.659	0.561	1.086				
EAC00966	mads flc-like protein 2					0.448	0.119	-0.069	0.250				
EAC01092	mads flc-like protein 2					0.382	0.126	0.056	0.094				
EAC00082	myosin heavy chain					-0.176	0.547	0.301	0.671				
EAC00246	protein					0.854	0.092	0.183	1.193				
EAC00221	transport protein sec61 subunit beta					0.152	0.319	0.087	0.628				
EAC00284	zinc carboxypeptidase family protein					0.152	0.004	0.210	0.649				
Genes uniquely affected by carbendazim													
EAC01014	40s ribosomal protein									-0.139	-0.424	-0.417	0.353
EAC00226	40s ribosomal protein s4									0.239	0.260	0.258	0.158
EAC00406	actin									-0.398	-0.592	-0.568	0.199
EAC00444	actin									0.847	0.668	0.842	0.850
EAC00079	actin 1									0.291	0.179	0.256	0.446
EAC00511	atp h+ mitochondrial f1 alpha subunit cardiac muscle									0.602	0.516	0.498	0.275
EAC00254	beta actin									0.496	0.717	0.392	0.411
EAC00580	uncharacterized protein c1orf189 homolog									0.023	0.246	-0.545	-1.270
EAC00100	calponin homolog									0.285	0.324	0.311	0.123
EAC00904	calponin-like protein									0.401	0.403	0.370	0.085
EAC01033	calponin-like protein									0.401	0.420	0.433	0.326
EAC00178	cholinergic beta polypeptide 4									-0.237	-0.489	-0.116	0.061
EAC00525	chromobox protein homolog 3									0.617	0.690	0.518	0.354
EAC00468	conserved protein									0.207	0.290	0.181	0.456
EAC00180	c-type lectin 11									-0.194	-0.502	-0.527	0.189
EAC00042	cysteine rich transmembrane bmp regulator 1									1.047	0.962	1.116	0.795
EAC00472	cysteine-rich motor neuron 1 protein precursor									0.869	0.776	0.933	0.536
EAC00307	cytochrome oxidase subunit i									0.142	0.333	0.314	-0.033
EAC00950	dimethyladenosine transferase									0.188	0.230	0.226	1.133
EAC00212	dna replication licensing factor mcm7									0.512	0.614	0.261	-0.090
EAC00484	aspartate aminotransferase 1									1.169	1.015	0.866	0.780
EAC00903	glyceraldehyde-3-phosphate dehydrogenase									0.491	0.168	0.448	0.467
EAC00316	heat shock cognate 71 kda									0.177	0.248	0.359	0.229
EAC00181	intermediate filament protein									0.789	0.747	0.816	0.828
EAC00363	intermediate filament protein									0.658	0.648	0.784	0.713
EAC00999	intermediate filament protein									0.848	0.750	0.687	0.429

EAC00822	isoform a									-0.659	-0.476	-0.177	-1.079
EAC00290	lysozyme									0.590	0.720	0.279	1.209
EAC01098	mads flc-like protein 2									0.236	0.421	0.480	1.512
EAC00433	methyltransferase mb3374									-0.561	-0.728	-0.454	0.324
EAC00732	monosaccharide-transporting atpase									0.152	0.384	0.009	0.654
EAC00378	myosin alkali light chain 1									-0.309	-1.199	-0.265	-0.843
EAC00108	myosin heavy chain									0.563	0.650	0.552	0.247
EAC01023	myosin light chain 2									0.349	0.468	0.541	0.348
EAC00513	ovulatory protein-2 precursor									0.495	0.622	0.659	1.483
EAC00256	peptidase inhibitor 16									0.062	0.049	0.207	-2.214
EAC00471	PREDICTED: predicted protein [Hydra magnipapillata]									0.316	0.599	0.372	0.290
EAC00214	protein									1.097	0.975	0.634	0.792
EAC00476	ribosomal protein l15									0.590	0.800	0.578	0.517
EAC00520	ribosomal protein l3									0.350	0.432	0.477	0.270
EAC00482	ribosomal protein s15									0.132	0.220	0.280	0.149
EAC00200	ribosomal protein s16									0.041	0.037	-0.038	-0.135
EAC00143	ribosomal protein s7									0.165	0.152	0.233	0.111
EAC01078	ribosomal protein s7									0.364	0.316	0.284	0.219
EAC00663	ribosomal protein s7									-0.444	-0.479	-0.441	-0.461
EAC00309	s enesis abnormal family member (syg-2)									0.239	0.384	0.507	0.680
EAC00045	scbp3 protein									0.627	0.556	0.486	0.353
EAC00408	scbp3 protein									0.391	0.518	0.625	0.163
EAC01045	scbp3 protein									0.602	0.558	0.569	0.407
EAC01022	short chain dehydrogenase									-0.060	-0.044	0.188	0.931
EAC01050	tektin 2									0.676	0.626	0.344	0.374
EAC00545	tropomyosin									0.630	0.611	0.752	0.457
EAC01032	tropomyosin									0.595	0.494	0.641	0.245
EAC00175	tubulin alpha chain									0.578	0.758	0.510	1.058
EAC00657	ubiquinol-cytochrome c subunit									-0.506	-0.613	0.122	0.166
EAC00306	ubiquitin c									0.066	-0.096	0.095	0.607
EAC00550	venom c-type lectin mannose binding isoform 2									-0.372	-0.873	-0.267	-1.460
EAC00431	vesicle-associated protein (vap-1)									-0.235	-0.363	0.493	-0.245
Genes affected by dimethoate and atrazine													
EAC00009	betaine-homocysteine methyltransferase	-0.324	0.187	-0.138	0.509	-0.630	-0.385	0.123	-0.174				
EAC00233	chaperonin containing subunit 7	0.581	0.494	0.098	0.230	0.079	0.326	-0.080	-0.130				
EAC00463	protein	0.029	-0.018	-0.717	-0.148	-0.257	-0.015	0.349	-0.666				
EAC00387	retinol dehydrogenase 3	-0.115	0.415	-0.353	0.303	0.007	0.689	0.398	0.797				
EAC00479	ribosomal protein l10ae	0.273	0.253	-0.294	-0.228	-0.161	-0.266	-0.195	-0.659				
EAC00716	sec14-like 2 (cerevisiae) isoform cra_b	-0.454	-0.159	-0.157	-0.850	0.281	-0.142	-0.732	-0.130				
EAC00994	translation elongation factor 1-alpha	-0.638	-0.174	-0.511	0.088	-0.951	-0.202	0.224	0.363				
EAC00831	u6 snrna-associated sm-like protein lsm3	-0.135	0.112	0.050	0.261	0.109	0.512	0.302	0.322				
Genes affected by dimethoate and carbendazim													
EAC00457	26s protease regulatory subunit 8	-0.192	-0.177	-0.022	0.499					0.404	0.332	0.103	0.705
EAC00281	actin	-0.361	-0.302	0.325	0.228					0.679	0.601	0.709	0.546
EAC00579	adp-ribosylation factor-like protein 2-binding protein	-0.020	0.134	-0.349	-0.523					-0.117	0.100	-0.339	-0.585
EAC00867	aquaporin 1	-0.545	-0.242	0.604	0.499					-0.444	-0.442	-0.133	1.151
EAC00240	atp synthase lipid-binding mitochondrial precursor	0.494	0.633	-0.073	-0.227					-0.056	-0.054	-0.131	-0.311
EAC00421	b chain structural basis for giant hemoglobin	-0.010	-0.070	0.544	0.857					-0.112	-0.374	-0.141	0.501
EAC00377	beta tubulin	-0.284	-0.095	0.466	0.537					0.405	0.191	0.499	0.948
EAC00697	calcium-dependent protein kinase	0.269	0.562	-0.681	-1.056					0.026	0.120	0.117	-0.841
EAC00222	cathepsin b	0.250	-0.026	0.494	0.436					-0.065	0.070	0.117	0.814
EAC00311	cysteine-rich protein 1	0.227	0.760	0.093	0.420					1.270	1.018	1.448	1.725
EAC00407	cytoplasmic 2-like isoform 1	-0.204	-0.501	0.318	-0.079					-0.447	-0.487	-0.549	-0.280
EAC00371	cytoplasmic intermediate filament protein	-1.016	-0.589	0.066	0.789					0.718	0.694	0.656	1.023
EAC00816	endonuclease-reverse transcriptase	-0.221	-0.508	-0.877	-0.620					-0.471	-1.045	-0.379	-0.791
EAC00354	gl12416-like	-0.402	-0.252	0.182	0.371					0.231	0.282	0.472	0.648
EAC00710	hypothetical protein TGME49_026570	-0.106	-0.239	-0.951	0.220					-0.097	-0.617	-0.471	0.236
EAC00558	light chain regulatory	0.432	0.285	0.359	0.670					-0.557	-0.661	-0.744	0.230
EAC01006	mads flc-like protein 2	0.383	0.355	-0.132	-0.261					-0.255	-0.404	-1.092	-1.260
EAC01039	mads flc-like protein 2	-0.185	0.292	-0.638	-0.440					0.851	0.983	1.000	-0.141
EAC01049	mads flc-like protein 2	2.076	1.417	5.112	5.081					-0.909	-2.326	-1.874	1.676
EAC01066	mads flc-like protein 2	2.021	1.365	4.991	4.914					-0.716	-2.232	-1.791	1.800
EAC01116	mads flc-like protein 2	2.202	1.432	4.964	5.152					-0.905	-2.054	-1.750	1.663
EAC00204	mitochondrial nadh:ubiquinone oxidoreductase	0.161	0.162	-0.364	-0.075					0.734	0.817	0.549	0.449
EAC00002	myosin heavy chain	-0.208	-0.586	0.284	0.273					0.597	0.592	0.745	0.662
EAC00616	nadh dehydrogenase	0.333	0.366	-0.029	-0.209					-0.106	-0.085	-0.332	-0.274
EAC00504	NADH dehydrogenase, putative	0.360	0.515	-0.290	-0.129					0.288	0.338	0.286	-0.023
EAC00179	plastin 3 (t isoform)	0.186	0.479	0.379	0.929					-0.236	-0.394	0.073	0.597
EAC00809	protein	0.411	0.218	-0.654	0.071					-0.180	-0.491	-0.520	-0.101
EAC00208	ribosomal protein l3	-0.459	-0.538	-0.197	-0.053					0.503	0.617	0.607	0.430
EAC00517	ribosomal protein l36a	0.171	0.149	-0.402	-0.111					0.358	0.485	0.485	0.378
EAC00518	ribosomal protein l36a	0.161	0.122	-0.488	-0.183					0.431	0.494	0.447	0.382
EAC00006	sea urchin arp2	0.121	-0.042	0.320	0.769					-0.103	-0.103	-0.113	0.677
EAC00554	short-chain dehydrogenase	-0.196	-0.617	0.346	-0.183					0.056	0.110	0.130	0.902
EAC00992	stathmin 1 oncoprotein 18 variant 8	0.352	0.394	-0.119	-0.272					-0.356	-0.380	-1.060	-1.239
EAC00473	t-complex protein 1 subunit beta	0.102	0.194	0.614	0.481					-0.524	-0.792	-0.145	-0.270

EAC00207	transketolase	-0.334	-0.224	0.220	1.098					0.932	0.644	0.671	1.163
EAC00340	troponin t	-0.435	-0.443	-0.261	-0.300					0.491	0.440	0.577	0.401
EAC00485	tubulin beta-2b chain-like	-0.585	-0.495	-0.188	-0.064					0.600	0.605	0.494	0.408
EAC00390	type alpha isoform cra_b	-0.293	-0.519	0.626	0.618					0.750	0.351	0.881	1.017
EAC00880	yippee-like 5	0.249	0.131	-0.673	0.035					-0.452	-0.705	-0.678	0.064
Genes affected by atrazine and carbendazim													
EAC00910	adenosine kinase isoform 2t					-0.127	-0.079	0.369	-0.715	-0.313	-0.701	-0.455	0.107
EAC00099	alpha 1					0.072	0.310	0.754	0.410	0.451	0.397	0.670	0.392
EAC00356	cytochrome c oxidase subunit via polypeptide 1					-0.121	0.375	0.893	0.692	0.682	0.636	0.786	0.754
EAC00106	cytochrome oxidase subunit i					-0.658	0.065	0.589	0.592	0.203	0.433	0.449	-0.048
EAC00455	cytoplasmic actin					-0.067	-0.229	-0.427	-0.438	-0.511	-0.502	-0.569	-0.290
EAC00261	extracellular hemoglobin linker l4 subunit precursor					-0.187	-1.662	-1.967	-0.360	-1.082	-1.346	-0.460	0.138
EAC00265	glucan endo- -beta-glucosidase					-0.210	0.157	0.690	0.557	0.173	0.334	0.236	0.514
EAC00481	heat shock protein 60					-0.291	-0.184	0.241	-0.719	-0.282	-0.535	-0.284	-0.074
EAC01019	larval visceral protein d					0.019	0.316	0.514	0.752	0.149	0.113	0.286	0.261
EAC01087	mads flc-like protein 2					-0.063	1.126	1.238	1.160	1.380	1.369	1.484	2.191
EAC00103	mitochondrial atp synthase gamma subunit					-0.053	0.510	0.807	0.611	0.729	0.675	0.623	0.452
EAC00388	mitochondrial nadh:ubiquinone oxidoreductase esss					-0.077	-0.229	-0.356	-0.265	-0.321	-0.319	-0.464	-0.425
EAC00332	muscle actin					-0.612	-0.732	-0.520	-0.810	-0.835	-0.904	-1.100	-0.710
EAC00541	myosin alkali light chain 1					-0.037	0.204	0.434	0.410	0.521	0.686	0.529	0.432
EAC00632	proteasome isoform a					0.429	0.696	0.167	0.341	0.288	0.323	-0.021	0.826
EAC00067	sarcoplasmic calcium-binding protein short=scp					-0.535	-0.008	0.625	0.313	0.432	0.551	0.583	0.311
EAC00675	tripartite motif-containing 59					0.271	0.596	0.463	0.455	0.416	0.626	0.596	0.508
EAC00533	tropomyosin					-0.026	-0.013	-0.141	-0.243	-0.131	-0.248	-0.185	-0.347
EAC01110	tropomyosin					0.213	0.294	0.219	0.256	0.266	0.230	0.357	0.309
Genes affected by all 3 pesticides													
EAC00415	cysteine-rich repeat-containing protein crim1	0.255	-0.231	2.209	1.421	-0.206	-1.057	-1.591	-0.452	-0.706	-1.815	-2.018	0.165
EAC00826	eukaryotic peptide chain release factor subunit 1	-0.363	-0.047	0.723	1.233	0.671	0.448	0.016	0.710	0.368	0.106	0.245	1.293
EAC00488	heat shock protein 90	-0.630	-0.733	0.530	0.752	0.198	0.627	0.855	0.992	0.844	0.910	0.681	1.160
EAC00664	ILKAP	0.443	0.227	-0.115	-0.402	0.555	0.292	-0.316	-0.151	-0.078	-0.122	-0.351	-0.228
EAC00397	keratinocyte associated protein 2	0.192	0.154	0.531	0.933	-0.182	0.362	0.470	0.562	0.540	0.248	0.720	0.848
EAC00260	lombricine kinase	-0.396	-0.247	0.186	0.410	-0.142	0.176	0.467	0.406	0.386	0.463	0.514	0.692
EAC00167	myosin heavy chain	-0.325	-0.678	0.165	-0.019	0.174	0.091	0.134	0.158	0.000	0.219	0.184	0.258
EAC00636	neutral and basic amino acid transport protein rbat	-0.171	-0.246	0.324	0.046	0.485	0.243	-0.005	0.369	0.441	0.225	0.013	0.142
EAC00549	replication factor a protein 3 domain-containing protein	0.139	0.107	-0.724	0.679	-0.407	0.342	-0.015	-0.846	-0.284	-0.792	-0.720	-0.214
EAC00298	ribosomal protein s16	-0.301	-0.389	0.073	-0.262	0.024	-0.426	-0.620	-0.619	-0.572	-0.485	-0.577	-0.599
EAC00628	type alpha partial	0.452	0.628	0.171	-0.243	0.089	-0.137	-0.468	-0.687	-0.444	-0.761	-0.521	-0.793

Table S3: All significant differential expressed genes of the significant (p<0.05) GO terms.

GO ID	Term	Library Annotated	# Sig. Annotated	Significant genes
Genes uniquely affected by dimethoate				
GO:0010035	*response to inorganic substance*	3	3	EAC00182, EAC00183, EAC00577
GO:0045214	sarcomere organization	4	3	EAC00182, EAC00496, EAC01081
GO:0050896	response to stimulus	23	8	EAC00182, EAC00183, EAC00276, EAC00372, EAC01038, EAC00559, EAC00577, EAC00835
GO:0032989	cellular component morphogenesis	11	5	EAC00182, EAC00259, EAC00496, EAC01038, EAC01081
GO:0051592	response to calcium ion	2	2	EAC00182, EAC00183
GO:0007163	establishment or maintenance of cell polarity	2	2	EAC00259, EAC01038
GO:0016070	RNA metabolic process	20	7	EAC00491, EAC01038, EAC01052, EAC00583, EAC00677, EAC00787, EAC00854
Genes uniquely affected by atrazine				
GO:0051641	cellular localization	8	2	EAC00350, EAC00221
GO:0031532	actin cytoskeleton reorganization	1	1	EAC00350
GO:0007155	cell adhesion	1	1	EAC00627
GO:0032507	maintenance of protein location in cell	1	1	EAC00350
GO:0070972	protein localization in endoplasmic reticulum	1	1	EAC00221
GO:0008360	regulation of cell shape	1	1	EAC00627
GO:0006616	SRP-dependent cotranslational protein targeting to membrane	1	1	EAC00221
Genes uniquely affected by carbendazim				
GO:0051716	cellular response to stimulus	4	3	EAC00181, EAC00363, EAC00484
GO:0048870	cell motility	4	3	EAC00904, EAC01033, EAC01050
GO:0006950	response to stress	13	5	EAC00178, EAC00181, EAC00290, EAC00316, EAC00363
GO:0006066	alcohol metabolic process	5	3	EAC00178, EAC00484, EAC00903
GO:0006259	DNA metabolic process	5	3	EAC00181, EAC00212, EAC00363
GO:0051103	DNA ligation involved in DNA repair	2	2	EAC00181, EAC00363
GO:0016477	cell migration	2	2	EAC00904, EAC01033
GO:0007243	intracellular protein kinase cascade	2	2	EAC00904, EAC01033
GO:0043408	regulation of MAPKKK cascade	2	2	EAC00904, EAC01033
GO:0023014	signal transmission via phosphorylation event	2	2	EAC00904, EAC01033
GO:0006412	Translation	36	9	EAC00200, EAC00226, EAC00408, EAC00476, EAC00482, EAC00520, EAC01014, EAC01078, EAC00663
GO:0044267	cellular protein metabolic process	56	12	EAC00200, EAC00214, EAC00226, EAC00306, EAC00316, EAC00408, EAC00476, EAC00482, EAC00520, EAC01014, EAC01078, EAC00663
GO:0040011	Locomotion	6	3	EAC00904, EAC01033, EAC01050
GO:0048511	rhythmic process	3	2	EAC00178, EAC00408
GO:0042274	ribosomal small subunit biogenesis	3	2	EAC00663, EAC00200
GO:0006281	DNA repair	3	2	EAC00181, EAC00363
GO:0006974	response to DNA damage stimulus	3	2	EAC00181, EAC00363
GO:0010033	response to organic substance	7	3	EAC00178, EAC00316, EAC00484
GO:0006414	translational elongation	12	4	EAC00200, EAC00226, EAC00476, EAC00663
Genes affected by dimethoate and atrazine				
GO:0008202	steroid metabolic process	2	2	EAC00387, EAC00716
GO:0006629	lipid metabolic process	6	2	EAC00387, EAC00716
GO:0016070	RNA metabolic process	20	3	EAC00479, EAC00716, EAC00831
GO:0008203	cholesterol metabolic process	1	1	EAC00716
GO:0016101	diterpenoid metabolic process	1	1	EAC00387
GO:0006775	fat-soluble vitamin metabolic process	1	1	EAC00387

GO:0001523	retinoid metabolic process	1	1	EAC00387
GO:0006721	terpenoid metabolic process	1	1	EAC00387
GO:0006396	RNA processing	12	2	EAC00479, EAC00831
GO:0006412	Translation	36	3	EAC00233, EAC00479, EAC00994
GO:0010467	gene expression	99	5	EAC00233, EAC00479, EAC00994, EAC00716, EAC00831
Genes affected by dimethoate and carbendazim				
GO:0022607	cellular component assembly	20	5	EAC00340, EAC00354, EAC00377, EAC00485, EAC00616
GO:0007018	microtubule-based movement	4	3	EAC00354, EAC00377, EAC00485
GO:0043623	cellular protein complex assembly	7	4	EAC00354, EAC00377, EAC00485, EAC00616
GO:0023052	Signaling	15	4	EAC00992, EAC01006, EAC00579, EAC00616
GO:0023034	intracellular signaling pathway	6	3	EAC00992, EAC01006, EAC00616
Genes affected by atrazine and carbendazim				
GO:0007040	lysosome organization	1	1	EAC00265
GO:0006643	membrane lipid metabolic process	1	1	EAC00265
GO:0006665	sphingolipid metabolic process	1	1	EAC00265
GO:0007033	vacuole organization	1	1	EAC00265
GO:0005975	carbohydrate metabolic process	12	2	EAC00265, EAC01019
Genes affected by all 3 pesticides				
GO:0044267	cellular protein metabolic process	56	5	EAC00298. EAC00397, EAC00488, EAC00664, EAC00826
GO:0006486	protein amino acid glycosylation	1	1	EAC00397
GO:0046209	nitric oxide metabolic process	1	1	EAC00488
GO:0042026	protein refolding	1	1	EAC00488
GO:0006470	protein amino acid dephosphorylation	1	1	EAC00664
GO:0007090	regulation of S phase of mitotic cell cycle	1	1	EAC00664
GO:0006464	protein modification process	12	2	EAC00397, EAC00664
GO:0034621	cellular macromolecular complex subunit organization	15	2	EAC00488, EAC00826
GO:0006986	response to unfolded protein	2	1	EAC00488
GO:0006449	regulation of translational termination	2	1	EAC00826

Chapter VIII

General Discussion and Concluding Remarks

VIII – GENERAL DISCUSSION AND CONCLUDING REMARKS

The aim in this thesis was to understand the overall effects, and gain insight on the mechanisms behind the responses, to metal and pesticide contamination in *Enchytraeus albidus*, studying different levels of biological organization. To pursue this goal, several experiments were conducted which aimed at determining effects on various endpoints under similar experimental conditions: survival, reproduction, avoidance behaviour, antioxidant defences, energetic status and gene expression alterations. The chemical compounds tested were the essential and non-essential metals zinc and cadmium respectively, and the insecticide dimethoate, the herbicide atrazine and the fungicide carbendazim (for the assessment of traditional endpoints in chapter II, two pesticides from each class were tested).

Traditional ecotoxicological bioassays [chapters II and III] allowed the determination of effect concentrations on survival and reproduction. Results indicated that pesticides were more toxic than metals, affecting survival, reproduction and avoidance behaviour at lower chemical concentrations. Comparisons between these three endpoints indicated that avoidance behaviour was less sensitive than reproduction. This particular result further indicated that, if the organisms in the field are not able to avoid concentrations which affect their reproduction or even survival, effects are likely to occur on that population. Avoidance tests were not effect predictive, hence should not be used for risk assessment but from an ecological point of view, are extremely valuable and relevant. Additionally, the fact that soil type is an important factor was confirmed. Toxicity of both metal and pesticides were higher in the natural LUFA 2.2 soil than in the artificial OECD soil, which has a higher organic matter content. Again, it indicates the risk of underestimation if only data from studies with OECD soil are used.

To understand the mechanisms of toxicity which result in e.g. decrease of the reproductive output, effects were assessed at the subcellular and molecular levels using concentrations with known reproduction effects.

The present thesis novelty relied on the genomic information to provide mechanistic insight in how enchytraeids respond to toxicity. One of the core aspects was the

development of genomic data, limited at the start date. To increase the potentiality of the existing microarray, and assess transcriptional responses, the existing cDNA library was enriched with transcripts differentially expressed in the conditions of interest [chapter IV]. After the considerable increase in the number of gene sequences, a new custom oligonucleotide Agilent microarray was developed. This platform, in comparison with spotted cDNA arrays, has the advantage of higher specificity and reproducibility of results, also between laboratories. A new web freely available database was implemented, gathering all the existing genomic data for *E. albidus*: EST sequences, BLAST homologies, GO terms, KEGG pathways and efficiently designed primers. EnchyBASE will also be a depository of all gene expression data generated and it is expected to be complemented, in the near future, with genomic information on other relevant enchytraeids species. The database developed in the course of this thesis provided the scientific community with information that has the potential to be used in functional genomics and molecular ecology to answer relevant ecological questions in the terrestrial environment.

Having this new tool in place, it was used to evaluate transcription responses to metal [Chapter V] and pesticide [Chapter VII] contamination. The combination of SSH-PCR methodology and microarray technology proved to be effective in the identification of known, as well as non-described biological pathways affected by metal and pesticide stress. Moreover, with these tools, most interesting and novel information on the mechanisms of chemical toxicity was obtained, with the identification of common and specific key pathways affected by each compound.

In the microarray study of Cd and Zn, results showed commonly affected biological processes, associated with regulation of gene expression, calcium homeostasis, cellular respiration and metabolisms of carbohydrates and proteins. The transcription responses indicated a tendency for a reduction on the available energy reserves, along with increased cellular respiration for both metal exposures. This tendency was further confirmed [Chapter VI] where the actual content of lipids, carbohydrates and proteins was reduced and an increased activity of the electron transport system was observed, suggesting an additional energy requirement to handle metal toxicity. Processes related with detoxification or antioxidant defense were not depicted by the microarray analysis but the experiment performed in Chapter III demonstrated that these same concentrations of Cd

induced significant effects on several antioxidant enzyme activities and substrate levels, confirming that in fact an energy costly response is undergoing to cope with this stress. From the oxidative stress study it is important to highlight that glutathione seems to play an important role in the antioxidant defense against metal stress and that the levels of GSH and GSSG are potentially good biomarkers for this type of exposure in *E. albidus*. Oxidative damage on membrane cells was also observed for metal stress and, although only lipid peroxidation levels were measured, damage on other macromolecules is expected, e.g. DNA damage. Evidences of this DNA damage induced by Cd are also shown in the transcription study by the inhibition of expression of genes involved in DNA repair.

The transcriptomic study of pesticides [Chapter VII] represents the first gene expression results of dimethoate and carbendazim exposures in invertebrates. Although some information is available on the expression of specific genes in mammals, this is the first microarray study of an animal exposed to these pesticides. From the analysis of results it was possible to identify biological processes affected by all three pesticides, effects that were shared by only two of them or even key processes exclusively affected by one of the pesticides. For this gene expression analysis of pesticide contamination, a large range of concentrations were tested at the reproduction effect level - EC_{10} EC_{20}, EC_{50}, EC_{90}. Results showed an increase in the number of differentially expressed genes with increasing concentrations. The EC_{10} also induced significant gene expression alterations and, in some cases, opposite of what was observed for the higher concentrations. This gene expression response in a dose-related manner, if confirmed in further studies, can potentially be useful in risk assessment. The present results showed the importance of testing a range of concentrations to adequately understand the extent of the molecular events and obtain the maximum mechanistic information.

For pesticides, no clear relation between gene expression responses and Cellular Energy Allocation (CEA) was observed, probably due to the short period of exposure (2 days). For future experiments, it is recommended an extent in exposure duration till 4 or 8 days as it has been shown for Cd and Zn that changes in the energy budget were only detectable after 2 days.

Influence of time of exposure was also addressed in the studies with metals for oxidative stress biomarkers and transcriptional responses. Responses were influenced by time, as often verified in various studies, and allowed to see the evolution of events.

Biochemical and molecular responses were observed within short periods of exposure (2 to 8 days), hence an advantage in comparison to the traditional and more time-consuming tests. Additionally, information on the mechanisms of toxicity is added. Interestingly, some of the proposed mechanisms of action for *E. albidus* were comparable to the ones known for mammals, suggesting across species conserved modes of action and underlining the usefulness of this soil invertebrate as a model species within the 3R concept, and potential use to read across species, including human.

Over the past few years, the application of transcriptomics in ecotoxicology has proven to successfully enhance the scientific knowledge on the effects of environmental stressors, as also confirmed in the present results. Recent advances in sequencing techniques (next-generation sequencing) are very promising and hopefully in the near future it will be possible to have the full *E. albidus* genome sequenced to understand the gene responses to their full extent.

The molecular studies performed in this thesis focused only on gene expression (transcriptomics). For future research it would be interesting to go slightly further at higher levels to study protein (proteomics) and metabolite expressions (metabolomics). In fact, mRNA abundance does not always correlate with protein and further metabolite abundances due to translational and post-translational regulatory events. The application of all these “omics” technologies in a complementary context, integrated with effects on higher and ecologically relevant effects, is the ideal approach to understand the complete picture of stress events and represents a challenge in ecotoxicology (systems biology approach).